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Cultivation and Utilization

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BIOMASS NOW – CULTIVATION AND UTILIZATION

Edited by **Miodrag Darko Matovic**

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Notice

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Meet the editor

Dr. Miodrag Darko Matovic is professor at Queen's University in Kingston, Canada, Department of Mechanical and Materials Engineering. He traded his long experience with fossil fuel combustion with work on renewable energy resources, especially biomass. He looks at biomass as the sustainable source of energy, soil amendments, raw material for plastics and other organic materials and as means to capture atmospheric carbon and fix it away from the atmosphere. His current research includes various aspects of biomass for energy use, including storage safety, novel uses of ash and potentials of biochar use in soil remediation.

Contents

Preface XIII

Section 1 Biomass Cultivation 1

Chapter 1 **Crop Rotation Biomass and Effects on Sugarcane Yield in Brazil 3**
Edmilson José Ambrosano, Heitor Cantarella, Gláucia Maria Bovi Ambrosano, Eliana Aparecida Schammas, Fábio Luis Ferreira Dias, Fabrício Rossi, Paulo Cesar Ocheuze Trivelin, Takashi Muraoka, Raquel Castellucci Caruso Sachs, Rozario Azcón and Juliana Rolim Salomé Teramoto

Chapter 2 **Generation and Utilization of Microbial Biomass Hydrolysates in Recovery and Production of Poly(3-hydroxybutyrate) 33**
Jian Yu, Michael Porter and Matt Jaremko

Chapter 3 **Considerations for Sustainable Biomass Production in *Quercus*-Dominated Forest Ecosystems 49**
Viktor J. Bruckman, Shuai Yan, Eduard Hochbichler and Gerhard Glatzel

Chapter 4 **Biomass Production in Northern Great Plains of USA – Agronomic Perspective 75**
Qingwu Xue, Guojie Wang and Paul E. Nyren

Chapter 5 **Design of a Cascade Observer for a Model of Bacterial Batch Culture with Nutrient Recycling 97**
Miled El Hajji and Alain Rapaport

Chapter 6 **Harvest Systems and Analysis for Herbaceous Biomass 113**
Jude Liu, Robert Grisso and John Cundiff

Section 2 Bio-Reactors 151

Chapter 7 **Biological Activated Carbon Treatment Process for Advanced Water and Wastewater Treatment 153**
Pengkang Jin, Xin Jin, Xianbao Wang, Yongning Feng and Xiaochang C. Wang

Chapter 8 **Biomass Digestion to Produce Organic Fertilizers: A Case-Study on Digested Livestock Manure 193**
Alessandra Trinchera, Carlos Mario Rivera, Andrea Marcucci and Elvira Rea

Chapter 9 **Removal of Carbon and Nitrogen Compounds in Hybrid Bioreactors 213**
Małgorzata Makowska, Marcin Spychała and Robert Mazur

Chapter 10 **Animal Manures: Recycling and Management Technologies 237**
María Gómez-Brandón, Marina Fernández-Delgado Juárez, Jorge Domínguez and Heribert Insam

Chapter 11 **Methods and Applications of Deoxygenation for the Conversion of Biomass to Petrochemical Products 273**
Duminda A. Gunawardena and Sandun D. Fernando

Section 3 Aquatic Biomass 299

Chapter 12 **Biofloc Technology (BFT): A Review for Aquaculture Application and Animal Food Industry 301**
Maurício Emerenciano, Gabriela Gaxiola and Gerard Cuzon

Chapter 13 **Phytoplankton Biomass Impact on the Lake Water Quality 329**
Ozden Fakioglu

Section 4 Novel Biomass Utilization 345

Chapter 14 **Towards the Production of Second Generation Ethanol from Sugarcane Bagasse in Brazil 347**
T.P. Basso, T.O. Basso, C.R. Gallo and L.C. Basso

Chapter 15 **Sugarcane Biomass Production and Renewable Energy 355**
Moses Isabirye, D.V.N Raju, M. Kitutu, V. Yemeline, J. Deckers and J. Poesen

Chapter 16 ***Paenibacillus curdlanolyticus* Strain B-6 Multienzyme Complex: A Novel System for Biomass Utilization 369**
Khanok Ratanakhanokchai, Rattiya Waeonukul, Patthra Pason, Chakrit Tachaapaikoon, Khin Lay Kyu, Kazuo Sakka, Akihiko Kosugi and Yutaka Mori

Chapter 17 **Methanogenic System of a Small Lowland Stream Sitka, Czech Republic 395**
Martin Rulík, Adam Bednařík, Václav Mach, Lenka Brablcová, Iva Buriánková, Pavlína Badurová and Kristýna Gratzová

Chapter 18 **How Soil Nutrient Availability Influences Plant Biomass and How Biomass Stimulation Alleviates Heavy Metal Toxicity in Soils: The Cases of Nutrient Use Efficient Genotypes and Phytoremediators, Respectively 427**
Theocharis Chatzistathis and Ioannis Therios

Preface

The increase in biomass related research and applications is driven by overall higher interest in sustainable energy and food sources, by increased awareness of potentials and pitfalls of using biomass for energy, by the concerns for food supply and by multitude of potential biomass uses as a source material in organic chemistry, bringing in the concept of bio-refinery. The present, two volume, Biomass book reflects that trend in broadening of biomass related research. Its total of 40 chapters spans over diverse areas of biomass research, grouped into 9 themes.

The first volume starts with the Biomass Sustainability and Biomass Systems sections, dealing with broader issues of biomass availability, methods for biomass assessment and potentials for its sustainable use. The increased tendency to take a second look at how much biomass is really and sustainably available is reflected in these sections, mainly applied to biomass for energy use. Similarly, Biomass for Energy section specifically groups chapters that deal with the application of biomass in the energy field. Notably, the chapters in this section are focused to those applications that deal with waste and second generation biofuels, minimizing the conflict between biomass as feedstock and biomass for energy. Next is the Biomass Processing section which covers various aspects of the second-generation bio-fuel generation, focusing on more sustainable processing practices. The section on Biomass Production covers short-rotation (terrestrial) energy crops and aquatic feedstock crops.

The second volume continues the theme of production with the Biomass Cultivation section, further expanding on cultivation methods for energy, the feedstock crops and microbial biomass production. It is followed by the Bio-reactors section dealing with various aspects of bio-digestion and overall bio-reactor processes. Two more chapters dealing with aquatic microbial and phytoplankton growth technologies are grouped into the Aquatic Biomass section, followed by the Novel Biomass Utilization section which concludes the second volume.

I sincerely hope that the wide variety of topics covered in this two-volume edition will readily find the audience among researchers, students, policy makers and all others with interest in biomass as a renewable and (if we are careful) sustainable source of organic material for ever wider spectrum of its potential uses. I also hope that further

exploration of second-generation energy sources from biomass will help in resolving the conflict of biomass for food and biomass for energy.

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Biomass Cultivation

Chapter 1

Crop Rotation Biomass and Effects on Sugarcane Yield in Brazil

Edmilson José Ambrosano, Heitor Cantarella, Gláucia Maria Bovi Ambrosano, Eliana Aparecida Schammas, Fábio Luis Ferreira Dias, Fabrício Rossi, Paulo Cesar Ocheuze Trivelin, Takashi Muraoka, Raquel Castellucci Caruso Sachs, Rozario Azcón and Juliana Rolim Salomé Teramoto

Additional information is available at the end of the chapter

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1. Introduction

Healthy soils are vital to a sustainable environment. They store carbon, produce food and timber, filter water and support wildlife and the urban and rural landscapes. They also preserve records of ecological and cultural past. However, there are increasing signs that the condition of soils has been neglected and that soil loss and damage may not be recoverable [1]. Soil is a vital and largely non-renewable resource increasingly under pressure. The importance of soil protection is recognized internationally.

In order to perform its many functions, it is necessary to maintain soil condition. However, there is evidence that soil may be increasingly threatened by a range of human activities, which may degrade it. The final phase of the degradation process is land desertification when soil loses its capacity to carry out its functions. Among the threats to soil are erosion, a decline in organic matter, local and diffuse contamination, sealing, compaction, a decline in bio-diversity and salinisation.

The authors in [2] made the interesting observation when study of nine great groups of New Zealand soils, that although many soil quality indicators will be different between soil types differing in clay and organic mater contents, land use had an overriding effect on soil quality: agricultural systems could be clearly differentiated from managed and natural forests, and grass land and arable land were also clearly separated.

Regular additions of organic matter improve soil structure, enhance water and nutrient holding capacity, protect soil from erosion and compaction, and support a healthy community of soil organisms. Practices that increase organic matter include: leaving crop

residues in the field, choosing crop rotations that include high residue plants, using optimal nutrient and water management practices to grow healthy plants with large amounts of roots and residue, applying manure or compost, using low or no tillage systems, using sod-based rotations, growing perennial forage crops, mulching, and growing cover crops as green manure [3].

Addition to being a frequent addition of organic matter should be diversified. Diversity cropping systems are beneficial for several reasons. Each plant contributes a unique root structure and type of residue to the soil. A diversity of soil organisms can help control pest populations, and a diversity of cultural practices can reduce weed and disease pressures. Diversity across the landscape can be increased by using buffer strips, small fields, or contour strip cropping. Diversity over time can be increased by using long crop rotations. Changing vegetation across the landscape or over time not only increases plant diversity, but also the types of insects, microorganisms, and wildlife that live on your farm [3]. In addition a dedicated management approach is needed to maintain or increase the soil organic matter content.

The incorporation of plant materials to soils, with the objective of maintaining or improving fertility for the subsequent crop is known as green manuring. The inclusion of a legume fallow within a sugarcane cropping cycle is practiced to reduce populations of detrimental soil organisms [5, 6], provide nitrogen (N) through biological fixation [7,8] and for weed suppression [9, 10].

Interest in the use of green manure's biomass has revived because of their role in improving soil quality and their beneficial N and non-N rotation effects [11]. Because of its nitrogen fixation potential, legumes represent an alternative for supplying nutrients, substituting or complementing mineral fertilization in cropping systems involving green manuring. This practice causes changes in soil physical, chemical and biological characteristics, bringing benefits to the subsequent crop both in small-scale cropping systems and in larger commercial areas such as those grown with sugarcane [12].

The area cropped with sugarcane (*Saccharum spp*.) in Brazil shows rapid expansion, with most of the increase for ethanol production. The area cultivated with sugarcane is now 9.6 Mha, with an increase of 5 Mha from 2000 and over 8.6 Mha of fresh sugarcane harvested per year [13]. Sugarcane crops in Brazil are replanted every five to ten years. In southeastern Brazil, the interval between the last sugarcane harvest and the new plantings occurs during the spring-summer season, under high temperature and heavy rainfall (almost 1,000 mm in six months) [14].

Green manure fertilization of the soil with legumes has been recommended before a sugarcane field is replanted. This practice does not imply on losing the cropping season, does not interfere with sugarcane germination, and provides increases in sugarcane and sugar yield, at least during two consecutive cuts [12]. Additionally, it protects the soil against erosion, prevents weed spreading and reduces nematode populations [15, 16]. Legumes usually accumulate large quantities of N and K, the nutrients which are taken up in the highest amounts by the sugarcane plants.

Residue incorporation studies of legumes using ^{15}N label indicate that 10 to 34% of the legume N can be recovered in the subsequent rye or wheat crop, 42% in rice, 24% recovery from Velvet bean by corn crop, around 15% of N recovery from sunn hemp by corn plants in no-till system, 30% by maize [51],and 5% of N recovery from sunn hemp by sugarcane [12], and ranged from 19 to 21% when the recovery was observed from sunn hemp by two sugarcane harvest [17].

The authors suggested that legume residue decomposition provided long-term supply of N for the subsequent crops, by not supplying the nutrient as an immediate source.

There are many benefits to the sugarcane crop of leguminous plants rown in rotation in sugarcane renovation areas; these include the recycling of nutrients taken up from deep soil layers by the rotational crop, which may prevent or decrease leaching losses, and the addition of N from biological fixation. Leguminous plants can accumulate over 5 t ha^{-1} of dry mass during a short period of time during the summer and take up large amounts of N and K. Most of this N comes from the association of legumes with rhizobia. In this way crop rotation with legumes can replace partially or totally N mineral fertilization of sugarcane, at least for the first ratoon [18, 12].

Another important microbial association is that of mycorrhizal fungi and plant roots. These fungi are present in over 80% of plant species [19]. In contrast with the large diversity of plants, which includes sugarcane, that have their roots colonized by mycorrhizas, only 150 fungi species are responsible for that colonization [19]. A crop whose roots are colonized by mycorrhizal fungi can raise the soil mycorrhizal potential which can benefit plants which are responsive to this fungi association and that are cultivated in sequence. This could be particularly useful for the nutritional management of crops in low nutrient, low input–output systems of production [20].

This study evaluated biomass production, N content, characterizing the biomass and the natural colonization of arbuscular mycorrhizal fungi (AMF) of leguminous green manure and sunflower (*Helianthus annuus L.*) in rotation with sugarcane and their effect in the soil. Their effect on stalk and sugar yield and nematode that occur on sugarcane cv. IAC 87-3396 grown subsequently was also studied. The economic balance considered the costs of production and revenues of the rotational crops as well as three or five harvests of sugarcane was too evaluated.

2. Development

Three long term assays were developed during December 4, 1999 to October 10, 2005 to complete this study as following:

The experiments were carried out in Piracicaba, state of São Paulo, Brazil (22°42′ S, 47°38′ W, and 560 m a.s.l). The soil, classified as an Arenic Hapludult, and a Typic Paleudult, was chemically characterized at different depths after cutting the green manure crop, before the sugarcane first planting. The soil was acidic and had low amounts of nutrients (Table 1), typical of many sugarcane growing areas.

Total monthly rainfall and local temperature were measured at the meteorological station near the experimental site (Figure 1).

Soil characteristics*	Arenic Hapludult		Typic Paleudult	
	0.0-0.2	0.2-0.4	0.0-0.2	0.2-0.4
	Soil depth, m			
pH (0.01mol l^{-1})	4.1	4.0	5.5	5.5
O.M. (g dm^{-3})	26	22	20	19
P (mg dm^{-3})	3	14	13	10
K (mmolc dm^{-3})	0.7	0.5	0.6	0.4
Ca (mmolc dm^{-3})	7	6	33	29
Mg (mmolc dm^{-3})	6	5	21	19
H + Al (mmolc dm^{-3})	50	68	23	25
CEC (mmolc dm^{-3})	64	80	79	74
V %	22	14	68	64

*Adapted from [12 and 16]

Table 1. Soil chemical characteristics before sugarcane planting, in plots without green manure, at depths of 0.0-0.2 and 0.2-0.4 m.

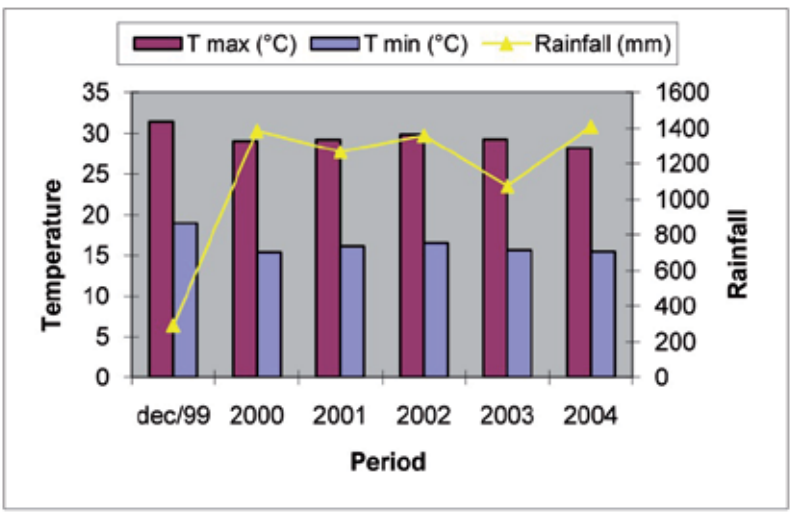

Figure 1. Climatological data of maximum and minimum annual average temperature, and annual average rainfall from December 1999 to December 2004 (experiment 1) adapted from [14].

2.1. Effect of seven rotational crops in sugarcane yield

The experiment 1 consisted to evaluate seven rotational crops plus control (fallow) grown before sugarcane was planted.

The soil is as a Typic Paleudult and was chemically characterized at different depths with samples taken after the green manures were cut but before sugarcane was planted (Table 1).

The experimental design was a randomized block with eight treatments and five replications.

The rotational crops were peanut (*Arachis hypogaea* L.) cv. IAC-Tatu, peanut cv. IAC-Caiapo, sunn hemp cv. IAC-1 (*Crotalaria juncea* L.), velvet bean (*Mucuna aterrima* Piper and Tracy), soybean (*Glycine max* L. Merrill) cv. IAC-17, sunflower (*Helianthus annuus* L.) cv. IAC-Uruguai, and mung bean (*Vigna radiata* L. Wilczek).

The green manures were sowed always in December on 7 m × 10 m size plots, with rows 0.50 m apart. The experimental area was weeded 30 d after sowing, and the weed residues were left on the soil surface.

During seed filling, the plants used as green manure were manually cut and spread on the soil covering the entire plot surface in pieces less than 0.25 m and left there for six months. Peanut, soybean, sunflower and mung bean were harvested after physiological maturation for the grain yield, and the remaining plant parts were cut and spread on the soil. Biomass production of the rotational crops was evaluated in 1 m^2 of the plot area.

At the harvest stage, the roots of each rotational crop were sampled in order to evaluate the natural colonization level of arbuscular mycorrhizal fungi (AMF). The colonization percentage was estimated using the root coloration technique according to [21]. The percentage of colonization by AMF was estimated by counting the roots' stained portions using a reticular plate under a microscope following the procedures described by [22].

To evaluate sugarcane stalk yield 2-m sections of each of the three central rows were cut and weighed.

Ten successive stems were separated from each plot for the technological evaluation of the Brix, pol, and total recovered sugar [23]. Sugar yield, expressed in terms of tons of pol per hectare (TPH), was estimated with the stem yield and technological analysis data.

The economic balance considered the costs of production and revenues of the rotational crops as well as three harvests of sugarcane. The basic costs of production of sugarcane (including land preparation, seed stalk, fertilizer, herbicides feedstock and application, and harvesting) were the average of the 2004, 2005, and 2006 prices, based on an average stalk yield of 70 t ha^{-1}. For the control treatment, which did not include the crop rotations, the cost of production of sugarcane was estimated as U$ 3,111 ha^{-1}. The costs of production of the green manures crotalaria and velvet beans, U$ 100 ha^{-1}, include seeds, planting, and cutting. For the grain crops, the costs of grain harvesting and of chemicals needed for phytosanitary control were added: sunflower (U$ 422 ha^{-1}), peanut cv. IAC-Tatu (U$ 1,289 ha^{-1}), Peanut cv. IAC-Caiapó (U$ 1,480 ha^{-1}), mung bean (U$ 2,007 ha^{-1}) and soybean (U$ 513 ha^{-1}). The sales prices of grain and cane stalks for the period between 2004 and 2006 (according to a database of the Institute of Agricultural Economics of the São Paulo State Secretary of Agriculture) were: sugarcane stalks, U$ 17.56 t^{-1}; sunflower, U$ 178 t^{-1}; peanut cv. IAC-Tatu, U$ 260 t^{-1}; peanut cv. IAC-Caiapó, U$ 260 t^{-1}; soybean, U$ 197 t^{-1}; and mung bean, U$ 2,222 t^{-1}. Mung bean is not sold as a commodity but as a specialty crop; its prices are highly variable, and the market for it is relatively small; therefore, the data on the economical return for mung bean must be taken with care.

2.2. Recovery of nitrogen in sugarcane fertilized with sunn hemp and ammonium sulfate

The utilization of nitrogen by sugarcane (*Saccharum spp*.) fertilized with sunn hemp (SH)(*Crotalaria juncea* L.) and ammonium sulfate (AS) was evaluated using the ^{15}N tracer technique in the experiment 2, that consisted of four treatments with four replications in a randomized block design as fallow: a) control with no N fertilizer or green manure; b) ammonium sulfate (AS) at a rate of 70 kg ha^{-1} N; c) sunn hemp (SH) green manure; d) and sunn hemp plus ammonium sulfate (SH + AS). Microplots consisting of three rows of sugarcane 2-m long were set up in plots c and d with the ^{15}N-labeled sunn hemp.

N was added at the rate of 196 and 70 kg ha^{-1} as ^{15}N labeled sunn hemp green manure (SH) and as ammonium sulfate (AS), respectively. Treatments were: (i) Control; (ii) $AS^{15}N$; (iii) $SH^{15}N$ + AS; (iv) $SH^{15}N$; and (v) $AS^{15}N$ + SH. Sugarcane was cultivated for five years and was harvested three times. ^{15}N recovery was evaluated in the two first harvests.

Sunn hemp (*Crotalaria juncea* L, cv IAC-1) was sown at the rate of 25 seeds per meter on the 4 Dec 2000 and emerged in nine days. Microplots, consisting of 6 rows, 2- m long and spaced by 0.5 m within the sunn hemp plots were used for ^{15}N enrichment as described by Ambrosano [24]. After 79 days the sunn hemp was cut, and the fresh material was laid down on the soil surface. Total dry mass of sunn hemp was equivalent to 9.15 Mg ha^{-1}, containing 21.4 g kg^{-1} N, corresponding to 195.8 kg ha^{-1} N with an ^{15}N enrichment of 2.412 atoms % excess.

Microplots with AS-labeled fertilizer (3.01 ± 0.01 atoms % ^{15}N), with two contiguous rows 1-m long, were set up in plots b and also in plots d; therefore, these plots had microplots for both sunn hemp and AS-labeled materials.

Ammonium sulfate was sidedressed to sugarcane 90 days after planting in both main plots and microplots. N rate (70 kg ha^{-1}) is within the range (30 to 90 kg ha^{-1} N) recommended for the plant cane cycle in Brazil [25]. A basal fertilization containing 100 kg ha^{-1} P_2O_5 as triple superphosphate and 100 kg ha^{-1} K_2O as potassium chloride was applied to all treatments to ensure a full sugarcane development. Cane yield was determined outside the microplots by weighing the stalks of three rows of sugarcane, 2-m long.

Stalks yields were measured after 18 months (plant-cane cycle, on 24 Aug 2002), 31 months (1st ratoon crop, on 8 Oct 2003), and 43 months after planting (2nd ratoon crop, on 20 Sep 2004). Samples consisting of ten stalks were used for the determination of apparent sucrose content (Pol) in the cane juice, according to [23]. The expressed cane juice was analyzed for Pol (apparent sucrose) by a saccharimeter. Just before harvesting of the plant cane (24 Aug 2002) and of the first ratoon (8 Oct 2003) whole plants were collected from 1-m row of plants in the center of the microplots. Leaves and stalks were analyzed separately for determination of ^{15}N abundance and N content in a mass spectrometer coupled to an N analyzer, following the methods described in [26].

The fraction and amount of nitrogen in the plant derived from the labeled source (Ndff) and the fraction of N recovery of the labeled source (R%) were calculated based on the isotopic results (atoms %), according to Trivelin [26], Equations 1 to 3:

$$Ndff = \left(a / b\right) 100 \quad (1)$$

$$QNdff = \left[Ndff / 100\right] TN \quad (2)$$

$$R\% = \left[Ndff / NF\right] 100 \quad (3)$$

where: Ndff (%) is the fraction of nitrogen in the plant derived from the labeled source, a and b are ^{15}N abundance values (atoms % excess) in the plant and in the labeled source (AS or SH), respectively; QNdff (kg ha^{-1}) is the amount of nitrogen in the plant derived from the labeled source, TN (kg ha^{-1}) is total cumulative nitrogen in the sugarcane plant (kg ha^{-1}); R% is the fraction of N recovery of the labeled Sugarcane cultivar IAC- 87-3396 was planted on Mar or April on plots with ten sugarcane rows, 10-m long and spaced at 1.4 m.

The biological nitrogen fixation (BNF) by leguminous plants was determined by natural abundance of ^{15}N technique ($\delta^{15}N$) [27], and sunflower was the non-N fixing specie. The chemical analysis of plants to determine macro and micronutrient contents were performed according to the methods proposed by [28].

2.3. Effect of four rotational crops in sugarcane yield

The experiment 3 consisted to evaluate and characterize the biomass of leguminous residues, the natural arbuscular mycorrhizal (AM) fungus occurrence and the effect of leguminous on the nematodes (*Pratylenchus* spp.) in sugarcane crop. The experiment was carried out in Piracicaba, São Paulo State, Brazil. The soil was classified as Typic Paleudult and the sugarcane (*Saccharum* spp.) cultivar was IAC87-3396. The effects of previous cultivation of legumes were evaluated for five consecutive harvests. The treatments consisted of previous cultivation of legumes: peanut (*Arachis hypogaea* L.) cultivars IAC-Tatu and IAC-Caiapó, sunn hemp IAC 1 (*Crotalaria juncea* L.) and velvet-bean [*Mucuna aterrima* (Piper & Tracy) Holland], and a control treatment. We adopted the randomized block design with five replications.

The chronology of the events on the experimental field in experiment 3 is the same that experiment 1.

3. To evaluate seven rotational crops

The changes in soil properties were relatively small as should be expected with only one rotation. The rotational crops can contribute with organic residues, but, in general, the amounts of organic C added to the soil are usually not enough to cause significant changes in soil organic matter in the short term (Table 2).The letters in the tables represent statistical comparisons. Means followed by at least one equal letter do not differ statistically. Means followed by all the different letters differ significantly. The rotational crops also affected some soil attributes (Table 2). The organic matter content increased in the soil upper layer (0-0.2 m) with the cultivation of peanut cv. IAC-Tatu and velvet bean, and in the 0.2-0.4 m layer, with mung bean, sunflower IAC-Uruguai, and peanut cv. IAC-Tatu. The increase of

soil exchangeable magnesium was also observed for peanut cv. IAC-Tatu and velvet bean, although the original Mg content was already high.

Rotational crops	Organic matter			Mg		
	0-0.2 m	0.2-0.4 m	Average	0-0.2 m	0.2-0.4 m	Average
	---------- g kg^{-1} ----------			-------- mmolc dm^{-3} ------		
Control	20 Ab	19 Ab	19	21	19	20 b
Mung bean cv. M146	19 Ab	20 Aa	20	19	18	19 b
Peanut cv. IAC-Caiapó	21 Ab	19 Bb	20	24	15	20 b
Peanut cv. IAC-Tatu	23 Aa	21 Aa	22	29	23	26 a
Soybean cv. IAC-17	19 Ab	17 Bb	18	20	17	18 b
Sunflower cv. IAC-Uruguai	20 Ab	20 Aa	20	20	19	19 b
Sunn hemp IAC 1	19 Ab	18 Ab	18	19	17	18 b
Velvet bean	23 Aa	18 Bb	21	28	18	23 a
Average	21 A	19 B	20	22 A	18 B	20
[1]C.V.(%)	8.1	8.1		18.4	22.6	

Means followed by the same lower-case letter in the columns and capital letter in the rows are not different (Comparisons among means were made according to Tukey-Kramer test, $p > 0.1$).
[1]Coefficient of variation. Adapted from [14].

Table 2. Organic matter and exchangeable magnesium in soil sampled after rotational crops.

Sunflower accumulated more above-ground dry matter of total biomass and soybean more grain yield than the other crops (Table 3). Soybean, sunn hemp, velvet bean, and sunflower extracted the greatest amounts of N and P (Table 4). Sunflower also recycled more of K, Ca, Mg, and Zn than the other rotational crops, probably as a consequence of the higher biomass yield (Tables 3 and 4).

Soybean presented the highest N content, and sunflower the lowest. No differences were observed between peanuts and velvet bean and between sunn hemp and mung bean (Table 5). Among the macronutrients, N had the highest and P the lowest accumulation in the rotational crops. On the average Fe was recycled in the highest amounts in the above-ground parts of the rotational crops and Zn in the lowest (Table 4). The same results were observed by [29]) who evaluated pigeon pea (*Cajanus cajan*) and stylo plants (*Stylosanthes guianensis* var. vulgaris cv. Mineirão).

The high AMF infection rate, which helps the uptake of micronutrients (Table 3), may explain the high amounts of Zn returned to the soil when sunflower was grown before sugarcane. There is an increasing utilization of sunflower as a crop rotation with sugarcane in Brazil, due to its use for silage, seed oil production, and to its potential as a feedstock for biodiesel [30].

The amounts of N in the above-ground parts of sunn hemp (Table 4) were relatively low compared to those of [31], who reported the extraction of up to 230 kg ha^{-1} of N, and to those of [12], who found 196 kg ha^{-1} of N. However, the amounts of N returned to the soil

are directly related to the nutrient concentration in the plant, which varies with the local potential for biological nitrogen fixation (BNF) and with the growth stage of the crop at the time of cutting, and with the biomass yield, which is affected by the weather, soil, and crop growing conditions.

Rotational crop	Above ground dry matter[2]	Grain yield[2]	Natural infection of AMF
	----------kg ha^{-1}----------		%
Control	-	-	-
Mung bean cv. M146	2,225 d	798 d	51 b
Peanut cv. IAC-Caiapó	1,905 d	1,096 c	74 a
Peanut cv. IAC-Tatu	1,783 d	1,349 c	57 b
Soybean cv. IAC-17	3,669 c	2,970 a	56 b
Sunflower cv. IAC-Uruguai	15,229 a	1,805 b	73 a
Sunn hemp IAC 2	6,230 b	-	49 c
Velvet bean	5,049 b	-	65 a
[1]C.V. %	5.1	22.1	15.9

Means followed by the same letter in each column are not different (Comparisons among means were made according to Scott-Knott test, p = 0.05).
[1]Coefficient of variation.
[2] Analysis of variance were made after data transformation to log (×).Adapted from [14].

Table 3. Dry mass and grain yields of the rotational crops and percentage of infection of natural arbuscular mycorrhizal fungus (AMF) in roots of rotational crops.

Rotational crops	N	P	K[2]	Ca[2]	Mg[2]	Fe[2]	Mn[2]	Zn[2]
	---------- kg ha^{-1} ----------					---------- g ha^{-1} ----------		
Control	-	-	-	-	-	-	-	-
Mung bean cv. M146	27 b	2.4 b	17 d	17 d	12 d	2,073 a	258 a	43 c
Peanut cv. IAC-Caiapo	39 b	2.7 b	35 c	19 d	13 c	3,279 a	222 a	47 c
Peanut cv. IAC-Tatu	34 b	3.8 b	27 c	18 d	11 c	1,679 a	81 b	37 c
Soybean cv. IAC-17	122 a	8.7 a	14 d	46 c	28 b	1,424 a	186 a	56 c
Sunflower IAC-Uruguai	71 a	7.7 a	120 a	171 a	98 a	2,736 a	324 a	259 a
Sunn hemp IAC 2	97 a	5.8 a	33 c	34 c	21 b	1,313 b	178 a	84 b
Velvet bean	109 a	8.9 a	50 b	61 b	17 b	792 a	159 a	90 b
[1]C.V. %	10.9	42.5	13.3	9.7	13.0	9.5	10.1	9.9

Means followed by the same letter in each column are not different (Comparisons among means were made according to Scott-Knott test, p = 0.05).
[1]Coefficient of variation.
[2] Analysis of variance were made after data transformation to log (×).Adapted from [14].

Table 4. Nutrient content of above ground biomass of the rotational crops, excluding the grains.

Perin [32] found substantial amounts of N derived from BNF present in the above ground parts of sunn hemp (57.0%) grown isolated and 61.1% when intercropped with millet (50% seeded with each crop). The sunn hemp+ millet treatment grown before a maize crop resulted in higher grain yield than when sunn hemp alone was the preceding rotation. This effect was not observed when N-fertilizer (90 kg N ha^{-1}) was added. Intercropping legume and cereals is a promising biological strategy to increase and keep N into the production system under tropical conditions [32]. A large proportion of the N present in soybeans usually comes from BNF. Guimarães [33] found that 96% of the N present in above ground parts of soybeans were derived from BNF, values which are in agreement with those obtained by [32] for sunn hemp. However, in the present study, only about 27% of the N present in the soybean residues were from BNF (Table 5), probably because of poor specific population of fixing bacteria for soybeans in the experimental site, which have been grown with sugarcane for long time. No inoculation of soybean with Bradyrhizobium was done. The contribution of BNF for the peanut varieties was significantly different: it reached 70% of the N in the cv. IAC-Caiapó but only 37.7% in the cv. IAC-Tatu (Table 5). Usually the natural population of rhyzobia is high enough to guarantee root colonization for peanuts but probably the bacteria population in the soil of the experimental site was not efficient for peanuts cv. IAC-Tatu.

Rotational crop	C content	N content	C : N	N-BNF
	---------- g kg^{-1} ----------			%
Mung bean cv. M146	426 a	12.5 c	34.1 b	89 a
Peanut cv. IAC-Caiapó	424 a	20.9 b	20.3 b	70 b
Peanut cv. IAC-Tatu	440 a	19.2 b	23.0 b	38 c
Soybean cv. IAC-17	426 a	31.9 a	13.3 b	27 c
Sunflower IAC-Uruguai	429 a	4.6 d	92.4 a	-
Sunn hemp IAC 2	449 a	17.2 c	26.1 b	69 b
Velvet bean	446 a	21.6 b	20.7 b	62 b
[1]C. V. %	2.8	19.1	19.6	13.7

Means followed by the same letter in each column are not different (Comparisons among means were made according to Scott-Knott test, p = 0.05).
[1]Coefficient of variation. Adapted from [14].

Table 5. Carbon and nitrogen concentration, carbon to nitrogen ratio, and N derived from biological N_2 fixation (BNF) in the aboveground parts of the rotational crops at harvesting.

The rate of natural colonization with AMF was relatively high in all crops (Table 3). Peanut cv. IAC-Caiapo and sunflower cv. IAC-Uruguai, followed by velvet bean, had at least 64% of root infection with AMF. At the same time, sunflower produced the greatest amount of above-ground biomass, followed by C. juncea and velvet bean. Soybean had the highest grain yield (Table 3) and also presented a considerable percentage of root infection with AMF: 56% (Table 3). Besides the symbiotic association with rhizobia, roots of the legumes can be colonized by fungi of the family Endogonaceae that form vesicular-arbuscular (VA) endomycorrhizas, which help enhance the uptake of phosphorus and other nutrients [34].

Results of a nursery study on the effect of a short season pre-cropping with different mycotrophic herbaceous crops on growth of arbuscular mycorrhiza-dependent mandarin orange plants at an early stage after transplantation were presented by [20]. Mandarin orange seedling plants 180 days after transplantation showed variation in shoot growth in response to single season pre-cropping with seven different crops—maize, Paspalum millet, soybean, onion, tomato, mustard, and ginger, and two non-cropped fallow treatments—non-weeded and weeded fallows. Net growth benefit to the orange plants due to the different pre-crops and the non-weeded fallow treatment over the weeded fallow treatment plants showed a highly positive correlation with mycorrhizal root mass of the orange plants as it varied with the pre-crop treatments. Increase in citrus growth varied between 0 and 50% depending upon the mycorrhizal root mass of the pre-crops and weeds, AMF spore number, and infective inoculum density of the pre-cropped soils. These pre-crop variables individually and cumulatively contributed to the highly significant positive correlation between the AMF potential of the pre-cropped soils and growth of mandarin orange plants through their effect on mycorrhizal root mass development (i.e. extent of mycorrhization) of the mandarin orange plants. The choice of a pre-crop from the available options, grown even for a short season, can substantially alter the inherent AMF potential of soils to a significant influence on the performance of the mycorrhiza-dependent orange plant. The relationship between soil mycorrhizal potential left by a pre-crop and mycorrhizal benefit drawn by the succeeding AMF responsive plant can be of advantage for the exploitation of native AMF potential of soils for growth and nutrition management of crops in low nutrient, low input–output systems of production [20].

Sugarcane yield increased more than 30%, in average, due to the rotational crops as compared with the control treatment; those benefits lasted up to the third harvest (Table 6). In the first cutting, sunflower was the rotational crop that induced the greater yield increase, followed by peanut cv. IAC-Caiapó, and soybean cv. IAC 17. [35] observed that sunn hemp residues increased the sugarcane yield; in the first harvest after the green manure, the effect of the legume crop was better than that of chemical fertilization with nitrogen. Similar results were reported later by [36], with a yield rise of 15.4 tons ha^{-1} of sugarcane stalks, which represented about 24% increase in relation to the control. Positive effects on stalk yields were also found by [31] when sugarcane was grown after *Crotalaria spectabilis*, and by [36], who cultivated sugarcane after sunn hemp and velvet bean.

Sunflower was the best rotational treatment, causing an yield increased of around 46% in the first harvest after the rotational crops (Table 6). Meanwhile, in the average of three cuttings, peanut showed an yield increase of around 22% whereas sunflower presented a 10% yield increase; these results are in agreement with those of [31, 36].

The sugar content of sugarcane stalks is important because the raw material remuneration takes into account this parameter. Some crops that preceded sugarcane had a high effect on sugar yield (Table 7); this was observed mainly in the first harvest in areas where sunflower, peanuts and C. juncea were previously cultivated (Table 2). The 3-year average data showed a sugar yield increase, in the best treatment, of 3 t ha^{-1} in relation to the control. These results were already observed by [35, 31] who found an average increase of 2.98 ton^{-1} ha due to green manure crops grown before sugarcane.

Rotational crops	Stem yield			
	First cut	Second cut	Third cut	Average
	---------- ton ha^{-1} ----------			
Control	47.6 Bc	111.2 Aa	50.7 Ba	69.8
Mung bean cv. M146	61.6 Bb	131.9 Aa	54.7 Ba	82.7
Peanut cv. IAC-Caiapó	67.6 Ba	130.6 Aa	58.0 Ba	85.4
Peanut cv. IAC-Tatu	60.6 Bb	114.9 Aa	66.8 Ba	80.8
Soybean cv. IAC-17	67.5 Ba	124.9 Aa	56.7 Ca	83.1
Sunflower cv. IAC-Uruguai	69.5 Ba	105.2 Aa	55.3 Ca	76.7
Sunn hemp IAC 1	65.9 Bab	125.8 Aa	51.1 Ca	80.9
Velvet bean	61.3 Bb	116.3 Aa	61.2 Ba	79.6
Average	62.7	120.1	56.8	
SEM[1]	0.85	3.80	1.65	

Means followed by the same lower-case letter in the columns and capital letter in the rows are not different (Comparisons among means were made according to Tukey-Kramer test, $p > 0.1$).
[1]Standard error of the mean. SEM for comparison of rotational crops is 4.22. Adapted from [14].

Table 6. Yield of millable stems of sugarcane grown after rotational crops planted before the first sugarcane cycle.

Rotational crop	Sugar yield[1]				
	First cut	Second cut	Third cut	Average	SEM[2]
	---------- ton ha^{-1} ----------				
Control	6.9 Bb	18.1 Aa	7.5 Ba	10.3	1.4
Mung bean cv. M146	9.3 Ba	19.6 Aa	8.3 Ba	12.4	1.7
Peanut cv. IAC-Caiapó	9.9 Ba	21.2 Aa	8.9 Ba	13.3	1.6
Peanut cv. IAC-Tatu	8.8 Cab	18.5 Aa	10.5 Ba	12.6	1.3
Soybean cv. IAC-17	10.0 Ba	17.7 Aa	8.8 Ba	12.2	1.3
Sunflower cv. IAC-Uruguai	10.3 Ba	15.5 Aa	8.1 Ca	11.3	1.0
Sunn hemp IAC 2	9.3 Ba	19.2 Aa	7.5 Ca	12.0	1.5
Velvet bean	9.2 Ba	18.5 Aa	9.5 Ba	12.4	1.3
Average	9.2	18.6	8.6		
SEM[2]	0.2	0.6	0.3		

Means followed by the same lower-case letter in the columns and capital letter in the rows are not different (Comparisons among means were made according to Tukey-Kramer test, $p > 0.1$).
[1] Apparent sucrose content in the cane juice.
[2]Standard error of the mean. Adapted from [14].

Table 7. Sugar yields of three consecutive cuttings of sugarcane grown after rotational crops.

Studying crop rotation with legume plants in comparison with a control with and without a mineral N addition, [35] observed that, after a crop rotation, the sugarcane yield was higher after C. juncea and velvet bean, with 3.0 and 3.2 stalk tons ha^{-1} increase, respectively. The treatments with an addition of N fertilizer but no-rotation with green manure resulted in only 1.1 tons ha^{-1} of a sugar yield increase, in the average of three years, suggesting that the

beneficial influence of leguminous plants is not restricted to the N left by the leguminous plants after harvest.

Farmers must combine the resources of land, labor, management, and capital in order to derive the most profit. Since resources are usually scarce, maximizing returns on each one is important. Crop rotations provide income diversification. If profitability of one crop is reduced because of price variation or some unpredicted reason, income is not as likely to be adversely affected as if the whole farm was planted to this crop, provided that a profit potential exists for each crop in a rotation. This is especially important to the farmer with limited capital.

Some of the general purposes of rotations are to improve or maintain soil fertility, reduce the erosion, reduce the build-up of pests and diseases, best distribute the work load, reduce the risk of weather damage, reduce the reliance on agricultural chemicals, and increase the net profits. Crop rotations have fallen somewhat into disfavor because they require additional planning and management skills, increasing the complexity of farming operations.

Crop rotation can positively affect yield and increase profit (Table 8). Except for peanuts, all other rotational crops contributed to raise the net income. This was true both for the green manures (crotalaria juncea and velvet bean), as for the grain crops (soybean, sunflower and mung bean). Peanuts caused an increase in the sugarcane stalk yields relative to the control, especially in the first harvest (Table 8), but the high cost of production of this grain somewhat cancelled out the benefit of this rotation. However, in many sugarcane regions in São Paulo State peanuts are extensively grown in rotation with sugarcane, probably because in those sites yields are higher and the cost of production, lower. Mung beans are a niche crop. Although it provided a relatively high net return in the present study (Table 8), the risks may be high due to the market restrictions and price fluctuations.

Rotational crop	Gross revenue	Cost of production	Net income
	---------- U$ ha^{-1} ----------		
Control	3,710	3,111	599 b
Mung bean cv. M146	6,131	5,118	1,012 a
Peanut cv. IAC-Caiapó	4,784	4,591	193 b
Peanut cv. IAC-Tatu	4,606	4,401	205 b
Soybean cv. IAC-17	4,961	3,624	1,337 a
Sunflower cv. IAC-Uruguai	4,431	3,584	847 a
Sunn hemp IAC 2	4,263	3,195	1,068 a
Velvet bean	4,193	3,212	981 a
C.V.(%)	-	-	24.1

Means followed by the same letter in the column are not different (Comparisons among means were made according to Scott-Knott test, $p > 0.05$).

[1]Gross revenue includes sales of the three harvests of sugarcane plus grains of rotational crops. Cost of production includes land and crop management, chemicals, feedstock, and harvesting costs of all sugarcane and rotational crops, but excludes land rental. Adapted from [14].

Table 8. Economic balance[1] of sugarcane production including revenues and costs of crop rotation.

4. To evaluated the recovery of nitrogen by sugarcane when applied green manure crop and mineral N

To evaluate the utilization of nitrogen by sugarcane (*Saccharum spp*.) fertilized with sunn hemp (SH) (*Crotalaria juncea* L.) and ammonium sulfate (AS):

The presence of a green manure crop and mineral N applied together caused some soil alterations that could be detected in samples collected in the sugarcane planting and harvesting seasons (Table 9). There was an increase in calcium and magnesium availability, and consequently in base saturation and pH, in relation to the AS-^{15}N treatment, at planting. Similar results were obtained by [38], who worked with four velvet bean cultivars, velvet bean, Georgia velvet bean, cow itch, and cratylia. The presence of green manure caused a significant sum of bases increase, due to increases in calcium and magnesium; consequently, treatments involving velvet bean showed higher CEC values. The presence of organic acids in the plant mass could be the reason for this change.

During sugarcane harvest, increases in Mg concentration, pH, and base saturation (V%) were observed in the treatments containing SH-^{15}N+ AS in relation to the treatment containing AS-^{15}N alone. Also, a significant reduction in potential acidity was observed in treatments containing SH-^{15}N+ AS in relation to the treatment containing AS-^{15}N alone (Table 9).

	Soil sampling at sugarcane planting					
Treatment	pH (CaCl2)	Ca	Mg	H+Al	SB	V
	0.01mol l^{-1}	----------mmolc dm^{-3}----------				----- % -----
Control	5.1 ab	20.5 ab	14.5 ab	37.8 a	35.4 ab	48.2 a
AS-^{15}N[2]	4.7 b	15.8 b	9.8 b	47.0 a	25.9 b	36.0 a
SH + AS -^{15}N	5.3 a	24.8 a	17.8 a	32.0 a	42.8 a	55.8 a
SH-^{15}N	5.0 ab	18.0 ab	13.0 ab	39.0 a	31.4 ab	44.5 a
Mean	5.0	19.8	13.8	39.0	33.9	46.1
C.V.%	5.12	7.55	10.76	20.77	6.81	22.52
	Soil sampling at sugarcane harvest					
Control	5.0 ab	17.8 a	14.0 ab	39.8 ab	32.2 a	44.5 ab
AS-^{15}N	4.7 b	15.3 a	9.8 b	46.5 a	25.4 a	35.8 b
SH + AS -^{15}N	5.6 a	24.7 a	26.8 a	25.5 b	44.4 a	67.5 a
SH-^{15}N	5.0 ab	19.0 a	15.3 ab	36.3 ab	34.5 a	48.5 ab
Mean	5.0	18.0	16.4	37.0	33.5	49.1
C.V.%	8.00	30.88	15.18	25.87	32.45	29.57

Means followed by different letters in columns in each sampling season are different (Comparisons among means were made according to Tukey test $P < 0.05$).

[2] Treatments were: Control (no N fertilizer applied), AS-^{15}N (^{15}N-labeled ammonium sulfate), SH + AS -^{15}N (Sunn hemp + ^{15}N-labeled ammonium sulfate), SH-^{15}N (^{15}N-labeled Sunn hemp). Adapted from [12].

Table 9. Chemical characterization of the soil (0.0-0.2 m depth) in the sugarcane planting and harvesting seasons.

The presence of organic acids in decomposing plant residues can help Mg movement in the soil [39]. Crops with high C:N ratio may release N more slowly and cause an increase in N uptake by succeeding crop In addition, rotational plants that were grown before sugarcane could recycle nutrients that would otherwise be leached contribute with N derived from BNF and keep some elements in plant available forms, which could be transformed into more recalcitrant forms if the soil lie fallow for some time.

There was no variation in nutrient contents for macronutrients N and P, and for micronutrients B and Zn in sugarcane stalks at harvest time (Table 10). However, there were differences in Ca and K contents; the latter showed higher values in treatments involving fertilizer application, either mineral or organic, while Ca showed a higher value in the treatment with green manure and mineral N, indicating better nutrition with this element in the treatment containing higher nitrogen supply.

Nitrogen and potassium absorption is greatly influenced by moisture; this relation has been known for a long time [40], and the fact that treatments involving green manure crops maintained environments with higher moisture due to soil mulching with plant mass could have favored better potassium nutrition. With regard to calcium, nitrogen seems to favor absorption [41].

Treatment	N	K	P	Ca	Zn	B
	Contents determined in sugarcane stalks at harvest					
	----------g kg^{-1}----------				--mg kg^{-1}--	
Control	7.2 a	3.3 b	0.8 a	1.6 b	10.9 a	12.1 a
AS-^{15}N[2]	8.1 a	6.7 a	0.9 a	1.7 b	15.3 a	14.9 a
SH-^{15}N	7.7 a	7.1 a	0.9 a	1.8 b	13.3 a	14.8 a
SH + AS -^{15}N	8.8 a	8.5 a	1.0 a	2.4 a	13.7 a	15.4 a
Mean	8.0	6.4	0.9	1.88	13.3	14.3
C.V.%	11.52	27.89	15.61	8.14	19.80	18.00

Means followed by different letters in columns are different (Comparisons among means were made according to Tukey test $P < 0.05$).

[2] Treatments were: Control (no N fertilizer applied), AS-^{15}N (^{15}N-labeled ammonium sulfate), SH-^{15}N (^{15}N-labeled Sunn hemp), SH + AS -^{15}N (Sunn hemp + ^{15}N-labeled ammonium sulfate). Adapted from [12].

Table 10. N, K, P, Ca, Zn, and B contents in sugarcane stalks at harvest time.

When sugarcane was cultivated for five years and was harvested three times. ^{15}N recovery was evaluated in the two first harvests. In the sum of the three harvests, the highest stalk yields were obtained with a combination of green manure and inorganic N fertilizer; however, in the second cutting the yields were higher where sunn hemp (SH) was used than in plots with ammonium sulfate (AS) (Table 11).

Millable stalk yields of the first cycle (plant cane, harvested 18 months after planting) were higher than those of the second and the third cycle (Table 11). The yield decline with time is common, especially in the cases such as the present experiment when only the first cycle crop was fertilized in order to evaluate the residual effect of N application in the mineral or green manure forms. In the first year the stalk yield was numerically higher in plots

fertilized with a combination of green manure and AS; however, in the second year the plots that received SH produced more cane than those fertilized only with AS or the control treatment, indicating that the green manure applied before planting still affected plant growth and yield after 34 months. In the third cycle, there were no differences among the treatments, showing that the residual effect of both N sources had disappeared (Table 11). In the sum of three cuttings, the combination of AS and green manure resulted in highest yields.

Treatments[2]	Harvests			Total of three cuttings	Mean ± SEM[3]
	24 Aug 2002	08 Oct 2003	20 Sep 2004		
	---------- Stalk yield, Mg ha^{-1} ----------				
Control	86.0 Ba	61.1 Bab	47.1 Ab	$_{b}$194.2	64.7 ± 4.6
AS^{15}N	106.2 ABa	64.7 Bb	42.3 Ab	$_{ab}$213.2	71.1 ± 4.6
SH + AS^{15}N	128.7 Aa	84.5Ab	45.0 Ac	$_{a}$258.2	86.1 ± 4.6
SH^{15}N	92.4 ABa	83.8 Aa	41.2 Ab	$_{ab}$217.3	72.4 ± 4.6
Mean ± SEM	103.3 ± 3.8 a	73.5 ± 3.8 b	43.9 ± 3.8 c	215.4 ± 18.9	
	----------POL, Mg ha^{-1} ----------				
Control	11.9	10.4	17.9	$_{b}$40.2	13.5 ± 0.7 B
AS^{15}N	14.9	11.1	17.5	$_{ab}$43.5	14.5 ± 0.6 AB
SH + AS^{15}N	17.0	14.1	18.4	$_{a}$49.5	16.5 ± 0.6 A
SH^{15}N	12.9	14.2	18.1	$_{ab}$45.2	15.1 ± 0.6 AB
Mean ± SEM	14.2 ± 0.9 b	12.4 ± 0.9 b	18.0 ± 0.9 a	43.8 ± 2.4	

Means followed by a different letter lower-case letter, in the rows, and upper-case letter, in the columns, are different (Comparisons among means were made according to Tukey-Kramer $p \leq 0.1$). Means followed by superscript letters differ vertically (Comparisons among means were made according to Tukey-Kramer $p \leq 0.1$).

[1] Cane was planted on 01 Mar 2001. POL =apparent sucrose content in the cane juice.

[2] Treatments were: Control (no N fertilizer applied), AS^{15}N (^{15}N-labeled ammonium sulfate); SH + AS^{15}N (Sunn hemp + ^{15}N-labeled ammonium sulfate); SH^{15}N (^{15}N-labeled Sunn hemp).

[3] Standard error of the mean. Adapted from [17].

Table 11. Millable stalk yield and POL[1] of sugarcane plants in three consecutive harvests as a function of N applied at planting as ammonium sulfate (AS) or Sunn hemp (SH) green manure[1]

[36] showed evidence of the positive effect of green manure fertilization with sunn hemp in sugarcane, with greater sugarcane yield increase than with the application of 40 kg ha^{-1} mineral N to the soil. [43], studying lupine in maize, and [44], studying velvet bean and sunn hemp in rice, could not find response to mineral N applied after green manure, and no N fertilizer was needed when vetch (*Vicia spp*.) was grown after wheat, and when cotton followed faba beans [42].

The effect of fertilizer source on sugar concentration was less evident. In the average of three cuttings, the value of pol in plots, treated with both AS + SH was higher than in that observed in plots that received no N (Table 11). Pol in cane juice was higher in the third cutting than in the two previous ones. Variations in pol measurements among cropping seasons are usually more affected by environmental conditions (temperature and drought)

that determine cane maturation than by nutrition. However, high N tends to decrease sugar content and delay maturation [45]; therefore, after two years with no N fertilization, sugar content in cane plants was more likely to be high.

The recovery of N by the first two consecutive harvests accounted for 19 to 21% of the N applied as leguminous green manure and 46 to 49% of the N applied as AS.

Nitrogen derived from AS and SH in the leaves and top parts of the sugarcane plant, excluding the stalks, varied from 6.9 to 12.3 % of the total N at the end of the first cycle (plant cane) and was not affected by N source (Table 12). But the amounts of N from both sources accumulated in the leaves and tops were in the range of only 4.5 to 6.0 kg ha^{-1}, which represent a recovery of 6.4 to 8.1% of the N applied as AS and 2.7 and 3.1% of the N from the green manure (Table 12). The recovery of ^{15}N in the second cycle decreased when the N source was the inorganic fertilizer. In the second year the percentage of N derived from sunn hemp was greater than that from the AS, indicating a slightly higher residual effect of the green manure (Table 12).

Sampling dates	Treatments[2]				Mean ± SEM[3]
	$AS^{15}N$	SH-^{15}N + AS	$SH^{15}N$	$AS^{15}N$+ SH	
	---------- Ndff ,% ----------				
24 Aug 2002	12.3 Aa	11.1 Aa	10.9 Aa	6.9 Aa	10.3 ± 1.1
08 Oct 2003	1.7 Bb	5.5 Aa	4.1 Aab	1.7 Bb	3.2 ± 1.1
Mean ± SEM	7.0 ± 1.6	8.3 ± 1.6	7.5 ± 1.6	4.3 ± 1.6	
	---------- QNdff, kg ha^{-1} ----------				
24 Aug 2002	5.7	6.0	5.2	4.5	5.4 ± 0.6 A
08 Oct 2003	1.8	6.8	4.6	2.9	4.0 ± 0.6 A
Mean ± SEM	3.7 ± 1.0 a	6.4 ± 1.0 a	4.9 ± 1.0 a	3.7 ± 1.0 a	
	---------- R, % ----------				
24 Aug 2002	8.1 Aa	3.1 Aa	2.7 Aa	6.4 Aa	5.1 ± 0.6
08 Oct 2003	2.6 Ba	3.5 Aa	2.3 Aa	4.1 Aa	3.1 ± 0.6
Mean ± SEM	5.3 ± 0.9	3.3 ± 0.9	2.5 ± 0.9	5.3 ± 0.9	

Means followed by a different letter lower-case letter, in the rows, and capital letter, in the columns, are different [Comparisons among means were made according to Tukey-Kramer and F′ tests ($p \leq 0.1$), respectively].

[1] Cane was planted on 01 Mar 2001.

[2] Treatments were: Control (no N fertilizer applied), $AS^{15}N$ (^{15}N-labeled ammonium sulfate); SH + $AS^{15}N$ (Sunn hemp ^{15}N-labeled ammonium sulfate); $SH^{15}N$ (^{15}N-labeled Sunn hemp).

[3] Standard error of the mean. Adapted from [12].

Table 12. Percentage (Ndff) and quantity (QNdff) of nitrogen in leaves derived from the labeled fertilizer source and nitrogen recovery (R) in samples taken in the first and second harvests[1].

The percentage of N derived from the AS or SH accumulated in the stalks harvested in the first cycle were similar and ranged from 7.0 to 10.5% of the total N content. In the plant cane cycle the amounts of N in the stalks that had been applied as inorganic or organic fertilizers were higher than those measured in the leaves and tops and varied from 27.3 to 24.1 kg N ha^{-1} (Table 13). The recovery of N derived from inorganic fertilizer - 30.1 to 34.4% - was higher than that of the sunn hemp - 8.8 to 9.8%. However, in the second harvest the N the green manure supplied more N to the cane stalk than AS (Table 13). The difference in the amounts of N in the sugarcane plants derived from green manure and mineral fertilizer in the ratoon crop was around 1to 2 kg ha^{-1} N in leaves and tops (Table 12) and 4 to 7 kg N ha^{-1} in the stalks (Table 13), which were relatively small compared to the amounts of N accumulated in the ratoon plants (179 kg N ha^{-1} in plants supplied with AS and 243 kg ha^{-1} N in the SH treatments, or a 64 kg N ha^{-1}difference - Table 13). These results suggest that the effect of green manure on the yield of the second ratoon crop (Table 11) may not be only due to the extra N supply, but rather to other beneficial role of green manure on soil physical-chemical or biological activity properties.

Adding up the amounts of N taken up by the sugarcane plant and contained in the above-ground parts of the plant (leaves, tops and stalks), AS supplied 32.4 to 34.2 kg N ha^{-1}or about 46 to 49% of N recovery; the N taken up by sugarcane from sunn hemp varied from 37.4 to 40.0 kg ha^{-1}, which represented 19.1 to 20.8% N recovery (Tables 12 and 13).

The recovery of N from fertilizers by sugarcane is usually lower than that of grain crops: the latter varies from 50 to 70% [46] whereas for sugarcane the figures vary from 20 to 40% [47-49, 25]. Results of several studies show that the utilization of N from green manure by subsequent crops rarely exceeds 20% [43, 50, 12, 51] and most of the N remains in the soil, incorporated in the organic matter fraction. In the present study the application of AS along with SH increased N utilization by sugarcane plants. This result is in line with that of [44] who used an organic source isolated or combined with an inorganic fertilizer in rice crops and concluded that the green manures improved the mineral N utilization, resulting in N use efficiency of up to 79%.

In a study in pots [51] observed that maize plants took up more N from sunn hemp incorporated to a sandy soil (Paleudalf) than to a clayey soils (Eutrudox) and that the N derived from the roots was more recalcitrant than that of the shoots. Between 50 and 68% of the ^{15}N of the sunn hemp shoots remained in the soil whereas the figures for roots varied from 65 to 80%. Unaccounted for ^{15}N, probably lost in gaseous forms, varied from 5 to 15% of the sunn hemp N [51].

In a detailed account of the first year of the present experiment, [12] showed that 8 months after planting, the recovery by sugarcane plants (above ground parts) of the N derived from AS or from sunn hemp was similar: 3 to 6% of the added N. However, 12 and 15-month-old sugarcane plants recovered between 20 and 35% of the AS but only 6 to 8% of the sunn hemp-derived N.

The percentage of recovery of the inorganic fertilizer N contained in the stalk when the sugarcane plants were harvested after 18 months of planting varied from 30 to 34%; the corresponding figures for the N derived from sunn hemp were significantly lower: around 9

to 10% (Table 13). The residual effect of the N from both sources in the second harvest of the sugarcane plant was similar: between 4 and 6% of the N supplied at planting as AS or SH was recovered in the stalks of the sugarcane plant 31 months after planting (Table 12).

Sampling dates	Treatments[2]				Mean ± SEM[3]
	$AS^{15}N$	SH ^{15}N + AS	$SH^{15}N$	$AS^{15}N$ + SH	
	---------- Ndff, % ----------				
24 Aug 2002	10.5 Aa	7.0 Aa	8.2 Aa	10.3 Aa	9.0 ± 1.2
08 Oct 2003	1.4 Bb	3.8 Aa	3.7 Aa	1.7 Bb	2.6 ± 0.1
Mean ± SEM	6.0 ± 1.2	5.4 ± 1.2	5.9 ± 1.2	6.0 ± 1.2	
	---------- QNdff, kg ha^{-1} ----------				
24 Aug 2002	24.1 Aa	19.3 Aa	17.3 Aa	21.1 Aa	20.4 ± 2.78
08 Oct 2003	2.7 Bb	8.6 Aa	10.3 Aa	3.9 Bb	6.4 ± 0.8
Média ± SEM	13.4 ± 2.6	14.0 ± 2.6	13.8 ± 2.6	12.5 ± 2.6	
	---------- R, % ----------				
24 Aug 2002	34.4 Aa	9.9 Abc	8.8 Ac	30.1 Aab	20.8 ± 1.9
08 Oct 2003	3.9 Ba	4.4 Aa	5.3 Aa	5.6 Ba	4.8 ± 1.9
Mean ± SEM	19.1 ± 3.2	7.1 ± 3.2	7.0 ± 3.2	17.8 ± 3.2	
	---------- Cumulative N kg ha^{-1} ----------				
24 Aug 2002	177.4	235.6	257.0	220.4	222.6 ± 9.2 A
08 Oct 2003	181.0	190.8	228.0	270.8	217.6 ± 9.2 A
Mean ± SEM	179.2 ± 12.0 a	213.2 ± 12.0 a	139.9 ± 12.0 a	245.6 ± 12.0 a	

Means followed by a different letter lower-case letter, in the rows, and capital letter, in the columns, are different [Comparisons among means were made according to Tukey-Kramer and F´ tests ($p \leq 0.1$), respectively].
[1] Cane was planted on 01 Mar 2001
[2] Treatments were: Control (no N fertilizer applied), $AS^{15}N$ (^{15}N-labeled ammonium sulfate); SH + $AS^{15}N$ (Sunn hemp + ^{15}N-labeled ammonium sulfate); $SH^{15}N$ (^{15}N-labeled Sunn hemp).
[3] Standard error of the mean. Adapted from [12].

Table 13. Percentage (Ndff) and quantity (QNdff) of nitrogen derived from the labeled fertilizer source, nitrogen recovery (R) in sugarcane stalks and nitrogen accumulated in samplings carried out in the first and second harvestings[1].

In the present study, about 69% of the N present in the sunn hemp residues were from BNF. The data obtained in the present study are also in agreement with those obtained by [18] for green manure produced in the field, in the inter-rows of the ratoon crop.

Perin [32] found substantial amounts of N derived from BNF present in the above ground parts of sunn hemp (57.0%) grown isolated and 61.1% when intercropped with millet (Pennisetum glaucum, (L.) R. Brown) (50% seeded with each crop). The sunn hemp+millet treatment grown before a maize crop resulted in higher grain yield than when sunn hemp alone was the preceding rotation. This effect was not observed when N-fertilizer (90 kg N ha^{-1}) was added; Perin [32] concluded that intercropping legume and cereals is a promising biological strategy to increase and keep N into production system under tropical conditions.

No difference was observed in relation to the cumulative N listed in Table 10. The cumulative N results are similar to those found by [47], who obtained, during plant cane harvesting, mean values of 252.3 kg ha^{-1} cumulative nitrogen, with high nitrogen and plant material

accumulation during the last three months, as also observed by [49]. The nitrogen contents found in the above-ground part of sugarcane, Table 10, are in agreement with results of [47].

As the amounts of N applied as AS or SH to sugarcane in the first cycle were different (70 kg N ha^{-1}as AS and 196 kg N ha^{-1} as SH), the quantities of N derived from the green manure in the second harvest were larger than those from the inorganic fertilizer, although the percentage of N recovery was similar (Table 12).

Because less N derived from the green manure was recovered by the sugarcane plant in the first cycle it would be expected that a higher proportion of that N would be taken up in the second cycle (first ratoon), but this did not happen. It seems that the residual N that is incorporated to the soil organic matter has a somewhat long turnover. Other authors have reported low recovery (about 3.5% of the N) by the second crop after sunn hemp cover crop [52] or hairy vetch (*Vicia villosa* Roth) plowed into the soil [53]. Low recovery of residual N has also been observed for inorganic fertilizer sources: less than 3% of the N derived from fertilizers was taken up by soybeans (*Glicine max* (L) Merril) [54], maize (*Zea Mays* L.) [52], [53] or sugarcane (*Saccharum spp*) [55], results similar to those obtained in the present study (Table 12 and 13).

The amounts of inorganic N, derived from both N sources, present in the 0-0.4 m layer of soil in the first season after N application and were below 1 kg ha^{-1}.

The average concentration of inorganic nitrogen in the 0-40 cm layer of soil was relatively low in most samples taken after 8, 12, 15, and 18 months of planting (Table 14). Samples taken in February, in the middle of the rainy and hot season, presented somewhat higher values of ($NH4^+$ + $NO3^-$)-N probably reflecting higher mineralization of soil organic N (Figure 2). Later in the growing season (samples of May and Aug 2002) soil inorganic N content decreased again. This coincides with beginning of the dry season with mild temperatures, when the sugarcane plant reached maturity and probably had already depleted the soil for most of the available N.

Treatments[1]	Sampling dates				
	29 Oct 2001	20 Feb 2002	28 May 2002	24 Aug 2002	Mean ± SEM[2]
----------mg kg^{-1}----------					
Control	2.7 Ab	7.3 ABa	2.3 Ab	2.8 Ab	3.8 ± 0.22
$AS^{15}N$	2.6 Ab	9.1 Aa	2.2 Ab	3.2 Ab	4.3 ± 0.22
$SH^{15}N$ + AS	2.9 Ab	7.0 ABa	2.7 Ab	3.1 Ab	3.9 ± 0.22
$SH^{15}N$	2.8 Ab	5.8 Ba	1.5 Bc	2.8 Ab	3.2 ± 0.22
SH + $AS^{15}N$	2.7 Ab	7.2 ABa	3.1 Ab	2.6 Ab	3.9 ± 0.22
Mean ± SEM	2.7 ± 0.19	7.3 ± 0.19	2.4 ± 0.19	2.9 ± 0.19	

Means followed by a different letter lower-case letter, in the rows, and capital letter, in the columns, are different (Comparisons among means were made according to Tukey-Kramer test $p \leq 0.1$).
[1] Treatments were: Control (no N fertilizer applied); $AS^{15}N$ (^{15}N-labeled ammonium sulfate); SH + $AS^{15}N$ (Sunn hemp + ^{15}N-labeled ammonium sulfate); $SH^{15}N$ (^{15}N-labeled Sunn hemp).
[2] Standard error of the mean. Adapted from [12].

Table 14. Soil mineral N ($NH4^+$ + $NO3^-$) determined in four sampling dates during the plant cane cycle. Data are average of samplings of the 0-0.2 and 0.2-0.4 m soil layers.

The percentage of the inorganic N derived from AS or SH present in the soil from the 8th to the 18th month after sugarcane planting represented only 1 to 9% of total inorganic N (Table 15). The proportion of N that was originated from AS decreased with time whereas that from the green manure increased, indicating that the mineralization of this organic source could supply more N at the end of the season (Table 15). Indeed, [12] showed that sugarcane stalks sampled in 15-month old plants had significantly higher percentage of N derived from AS than from SH; in the 18th month that difference had disappeared. Nonetheless, throughout the season, the amounts of inorganic N in the soil derived from either AS or SH were of very little significance for the nutrition of the sugarcane plant – less than 1 kg ha^{-1} of inorganic N in a 40 cm soil layer (Table 15), indicating that little residual N is expected in soils grown with this crop. Although the rate of N applied as SH was almost 200 kg N ha^{-1}, little nitrate leaching losses are expected under the conditions of this experiment.

Treatments[1]	Sampling dates				
	29 Oct 2001	20 Feb 2002	28 May 2002	24 Aug 2002	Mean ± SEM[2]
	---------- Ndff, % ----------				
AS^{15}N	5.9 Aa	0.7 Aa	3.2 Aa	1.0 Ba	2.7 ± 0.57
SH^{15}N + AS	2.6 Ab	3.2 Aab	9.0 Aa	5.7 ABab	5.1 ± 0.62
SH^{15}N	2.9 Aa	4.3 Aa	7.0 Aa	5.9 Aa	5.0 ± 0.58
SH + AS^{15}N	2.9 Aa	0.3 Aa	4.0 Aa	1.3 ABa	2.1 ± 0.62
Mean ± SEM	3.6 ± 0.58	2.1 ± 0.56	5.8 ± 056	3.5 ± 0.55	
		----------QNdff, kg ha-1 ----------			
AS^{15}N					
	0.3 Aa	0.3 Aa	0.4 Aa	0.5 Aa	0.40 ± 0.16
SH^{15}N + AS	0.1 Aa	0.2 Aa	0.2 Aa	0.2 Aa	0.18 ± 0.18
SH^{15}N	0.1 Aa	0.2 Aa	0.1 Aa	0.2 Aa	0.15 ± 0.16
SH + AS^{15}N	0.1 Aa	0.1 Aa	0.1 Aa	0.0 Aa	0.07 ± 0.16
Mean ± SEM	0.15 ± 0.09	0.21 ± 0.09	0.24 ± 0.09	0.22 ± 0.09	

For Ndff: means followed by a different letter lower-case letter, in the rows, and capital letter, in the columns, are different (Comparisons among means were made according to Tukey-Kramer and F tests $p \leq 0.1$), respectively.
For Qndff: means followed by a different letter lower-case letter, in the rows, and capital letter, in the columns, are different (Comparisons among means were made according to Tukey-Kramer test $p \leq 0.1$).
[1] Treatments were: Control (no N fertilizer applied); AS^{15}N (^{15}N-labeled ammonium sulfate); SH + AS^{15}N (Sunn hemp + ^{15}N-labeled ammonium sulfate); SH^{15}N (^{15}N-labeled Sunn hemp).
[2] Standard error of the mean. Adapted from [12].

Table 15. Percent (Ndff) and amount (QNdff) of soil mineral N ($NH4^+ + NO3^-$) derived from the labeled fertilizer source (Ndff). Data are average of samplings of the 0-0.2 and 0.2-0.4 m soil layers.

Soil N is often the most limiting element for plant growth and quality. Therefore, green manure may be useful for increasing soil fertility and crop production. With regard to

fertilization, organic matter such as a green manure can be potentially important sources of N for crop production [56].

Sugarcane is a fast growing plant that produces high amounts of dry matter. Therefore, it tends to rapidly deplete the soil of inorganic N, especially in soils fertilized with small rates of soluble N as in the case of this study. Cantarella [25] reviewed several Brazilian studies showing little nitrate leaching losses in sugarcane. More recently, [57] showed that only 0.2 kg ha^{-1} $NO3^-$-N derived from 120 kg ha^{-1} of N as urea enriched to 5.04 ^{15}N At% applied to the planting furrow leached below 0.9 m in a sugarcane field, although the total N loss reached 18 kg ha^{-1} N, mostly derived from soil organic matter mineralization or residual N already present in the soil. As in the present study, the data of [57] refer to N applied at the end of the rainy season when excess water percolating through the soil profile is limited (Figure 2).

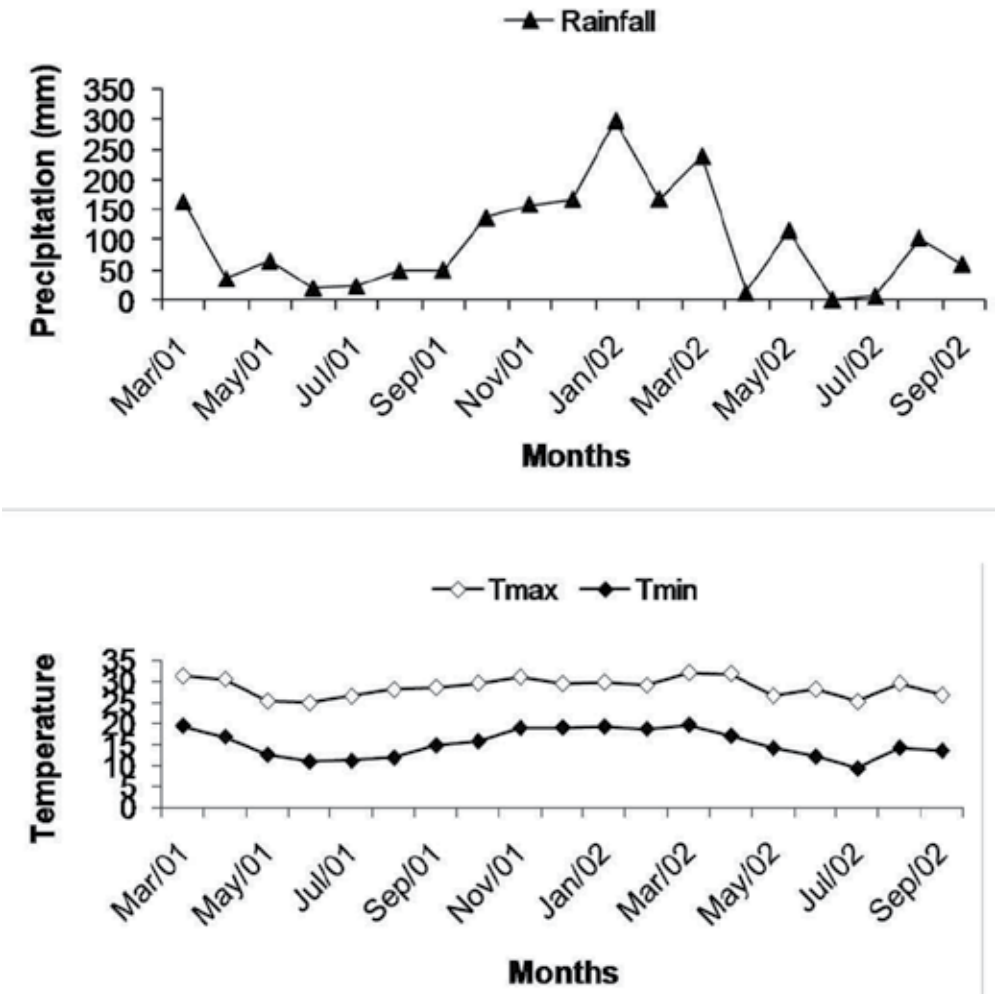

Figure 2. Climatic data for maximum and minimum temperature and rainfall during the first sugarcane growing season (plant cane cycle experiment 2), adapted from [12].

5. To evaluate the effect of biomass on the occurrence of nematodes (*Pratylenchus spp*.) and sugarcane yield after five cuts

The legume most productive was sunn hemp crotalaria juncea IAC-1 with 10,264 kg ha^{-1}, followed by velvet-bean with 4,391 kg ha^{-1} and peanuts IAC-Caiapó and IAC-Tatu with 3,177 kg ha^{-1} and 1,965 kg ha^{-1}, respectively.

There was an increase of Stalk yield of sugarcane in the average of the five cuts, compared to control treatment (Table 16). It can be seen that the effect of planting green manure in the fields of sugarcane promoted reform of benefits in terms of increased productivity of sugarcane, and this is lasting reaching in this case until the fifth cut, and the only treatment that stood out was the rotation of the witness with sunn hemp. Notably, the sunn hemp had higher dry matter production, and this may be a positive influence on growth of sugarcane. After five harvests, sunn hemp crotalaria was the leguminous crop that induced the greatest sugarcane yield, with 30% increase in cane yield and 35% in sugar yield.

Rotational crop	Harvests					
	25 Oct 2001	9 Sep 2002	1Aug 2003	7Nov 2004	6 Oct 2005	Mean
	---------- Stalk yield, Mg ha^{-1} ----------					
Sunn hemp IAC 1	145.36	122.30	79.70	51.86	39.30	87.70 A
Velvet bean	141.2	121.88	75.72	51.78	28.12	85.56 AB
Peanut cv. IAC-Tatu	149.92	108.79	74.58	52.16	29.64	83.02 AB
Peanut cv. IAC-Caiapó	122.74	122.30	67.42	49.44	36.78	79.74 AB
Control	129.90	85.31	55.38	46.40	36.15	67.51 B
Mean	138.39 a	113.23 b	71.00 c	50.43 d	34.16 e	

C.V.% (plot) = 7.57, CV% (subplots) = 4.20. Means followed by same lower-case letter in rows and capital letters in columns do not differ (Comparisons among means were made according to Tukey test p> 0.05). For statistical analysis the data were transformed into log (x). Adapted from [16].

Table 16. Millable stalk yield of sugarcane plants in five consecutive harvests as a function of treatments.

Crop rotation with non-host species of nematodes, when well planned, can be an efficient method for integrated control of nematodes. It is common in some areas, the practice of cultivation of Fabaceae in the period between the destruction of ratoon sugarcane field and planting the new. There are several plants used in systems of crop rotation with sugarcane, the most common are crotalarias, velvet beans, soybeans and peanuts. However, depending on nematode species occurring in the area, some of these cultures may significantly increase the population of these parasites. Thus, the sugarcane crop, can be greatly impaired by increasing the inoculum potential of the nematodes [58, 59].

The peanut IAC-Caiapó and velvet bean were the leguminous crops that resulted in the greater percentage of AM fungus. The lowest population of *Pratylenchus spp*. was found in the treatments with peanut IAC-Tatu and IAC-Caiapó (Figure 3).

Thet peanut IAC-Caiapó showed a minimum of 10 nematodes per 10 g of roots and a maximum of 470, while on the control this variation was from 80 to 2,510, indicating the smaller presence of the nematode in treatments with peanut IAC-Caiapó (Figure 3).

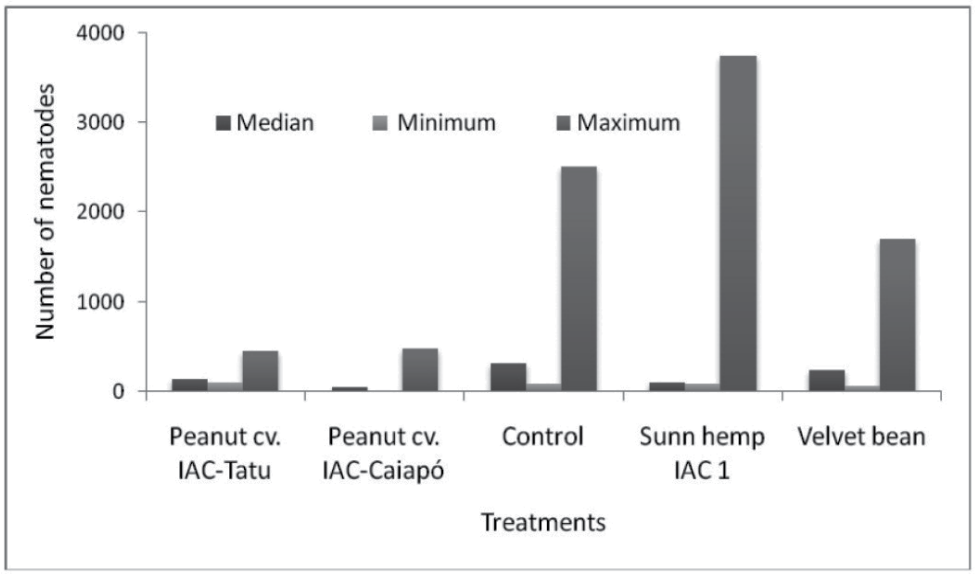

Figure 3. Number of nematodes of the genus *Pratylenchus spp*. by 10 grams of roots of sugar sugarcane cultivation influenced by the previous legume species. Adapted from [16].

6. Conclusions

Crop rotation can positively affect yield and increase profit, contributed to raise the net income. This was true both for the green manures (sunn hemp and velvet bean), as for the grain crops (soybean, sunflower and mung bean). Peanuts caused an increase in the sugarcane stalk yields relative to the control, especially in the first harvest, but the high cost of production of this grain somewhat cancelled out the benefit of this rotation.

However, in many sugarcane regions in São Paulo State (Brazil) peanuts are extensively grown in rotation with sugarcane, probably because in those sites yields are higher and the cost of production, lower. Mung beans are a niche crop. Although it provided a relatively high net return in the present study, the risks may be high due to the market restrictions and price fluctuations.

The biomass of green manure induced a complete N substitution in sugarcane and can cause positively affect yield and increase Ca and Mg contents, sum of bases, pH, and base saturation, and decreasing potential acidity and increase profit.

The combination of inorganic fertilizer and green manure resulted in higher sugarcane yields than either N source separately. The recovery of N from ammonium sulfate was higher in the first year whereas in the green manure presented a longer residual effect and

resulted in higher yields of cane in the second cycle. The recovery of ^{15}N- labeled fertilizers by two successive sugarcane crops summed up 19 to 21% of the N applied as sunn hemp and 46 to 49% of the N applied as ammonium sulfate. Very little inorganic N was present in the 0-40 cm soil layer with both N sources.

The sugar content of sugarcane stalks is important because the raw material remuneration takes into account this parameter in Brazil. Some crops that preceded sugarcane had a high effect on sugar yield; this was observed mainly in the first harvest in areas where sunflower, peanuts, velvet beans and sunn hemp were previously cultivated. The 3-year average data showed a sugar yield increase, in the best treatment, of 3 t ha^{-1} in relation to the control.

The peanut IAC-Caiapó, sunflower and velvet bean were the leguminous crops that resulted in the greater percentage of AM fungus. The lowest population of *Pratylenchus* spp. was found in the treatments with peanut IAC-Tatu and IAC-Caiapó.

After five harvests, sunn hemp crotalaria was the leguminous crop that induced the greatest sugarcane yield, with 30% increase in cane yield and 35% in sugar yield.

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Chapter 2

Generation and Utilization of Microbial Biomass Hydrolysates in Recovery and Production of Poly(3-hydroxybutyrate)

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Additional information is available at the end of the chapter

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1. Introduction

In moving towards sustainable manufacturing with reduced carbon footprint, bio-based fuels, chemicals and materials produced from renewable resources have attracted great interest. Microbial cells, in working with other chemical and enzymatic catalysts, are often used in the conversion of feedstocks to desired products, involving different species of bacteria, yeast, filamentous fungi, and microalgae. A substantial amount of microbial biomass is generated in the industrial fermentations and often discarded as a waste. Because of a high cost associated with growth and disposal of the cell mass, reusing the microbial biomass may be an attractive alternative to waste disposal. In contrast to the biomass as energy storage (e.g. starch and oil) or plant structure (e.g. cellulose and hemi-cellulose), microbial biomass is biologically active, consisting primarily of proteins (10-60 wt%), nucleic acid (1-30 wt%), and lipids (1-15%) [1]. Few cases of reusing microbial biomass exist in industrial processes.

Poly(3-hydroxybutyrate) (PHB) is a representative polyhydroxyalkanoate (PHA) that is formed by many bacterial species as carbon and energy reserve [2,3]. Although the biopolyesters made from renewable feedstocks have the potential of replacing petroleum-based thermoplastics in many environmentally friendly applications, they are not widely accepted in the markets because of the high production cost [4]. Extensive research has been conducted to use cheap feedstocks [5,6], develop high cell density fermentation technology for high PHA productivity [7,8], and improve microbial strains that exhibit good performance under high osmotic pressure and environmental stress [9-11]. One major cost factor of PHA production is the recovery and purification of biopolyester for desired purity and material properties [4, 12]. Depending on strains and culture conditions, biopolyester may account for 50-80 wt% of cell mass [13]. They are stored in microbial cells as tiny

amorphous granules [0.2-0.5 μm in diameter] and need a sophisticated treatment to separate them from the residual cell mass [14-16]. Two technologies, based-on solvent extraction or biomass dissolution, are usually adopted in PHA recovery. With solvent extraction, the PHA granules are dissolved in appropriate organic solvents, leaving cells or residual biomass intact [17,18]. With cell mass dissolution, the PHA granules are left intact while the non-PHA cell mass is decomposed and dissolved in aqueous solutions with help of biological and/or chemical agents [19,20]. Following either treatment, PHA and non-PHA cell mass can be separated with conventional solid/liquid separations. Separating biopolyester from the cells would generate a substantial amount of residual microbial biomass, 0.25 to 1 kg dry mass per kg of PHA resin, depending on the initial PHA content. As a mixture of proteins, nucleic acids, lipids, and wall fragments, the residual biomass has no market value and is discarded at an extra disposal cost.

According to the microbial structure and cellular composition [1], the residual microbial biomass is actually consisting of true biological compounds formed during cell growth while PHA is just a carbon storage material. In a conventional PHA fermentation, sufficient substrates and nutrients (C, N, P, minerals and some organic growth factors) are supplied to grow enough cell mass that in turn or simultaneously synthesize biopolyester from carbon substrates. A large portion of organic carbons and nutrients are therefore consumed to generate new cell mass that is going to be discarded as a solid waste after polymer recovery. Ideally, the residual biomass should be reused by microbial cells to generate new cell mass and/or PHA polymers. This would not only reduce the cost of waste treatment and disposal, but also save the cost of nutrients in PHA fermentation. The bacterial cells, however, cannot assimilate their cell mass because they lack appropriate enzymes to break down various biological macromolecules and their complex structure such as cellular walls and membranes [19]. If the cells or mutants from genetic engineering could easily assimilate their structural components, they might not be suitable to industrial PHA production because the cells would undergo autolysis under high environmental stress. It is highly possible, however, to make the residual biomass reusable during PHA recovery in which the non-PHA cell mass is decomposed and hydrolyzed in aqueous solutions [20]. Integration of PHA recovery with reusing of residual biomass in microbial PHA fermentation is a novel and challenging technology. This work shows the generation of biomass hydrolysates in a downstream PHB recovery and the beneficial utilization of the hydrolysates in cell growth and PHB formation.

2. Materials and methods

2.1. Microbial cultures

A laboratory strain of *Ralstonia eutropha* maintained on agar slant was used in this work. The agar medium contained (per liter): 5 g yeast extract, 5 g peptone, 2.5 g meat extract and 15 g agar. A 16S RNA analysis indicates that the strain has 100% alignment with *Ralstonia eutropha* H16. The cells were cultivated in 200 mL mineral medium containing: 160 mL glucose mineral solution, 8 mL inoculum, 5-20 mL solutions of residual microbial biomass

hydrolysates, and the rest pre-sterilized water. The glucose mineral solution contained (per liter): 12 g glucose, 2 g NaH_2PO_4, 2.8 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, 1 g $(NH_4)_2SO_4$ and 1 mL trace solution [21]. The culture solution was shaken in a 500 mL baffled flask at 30 ^oC and 200 rpm in a rotary incubator for 24 or 48 hours. Cell mass was harvested from 50 mL medium with centrifugation at 5,000 g for 10 min and freeze dried for measurement of cell mass concentration and PHB content.

A large amount of PHB-containing cell mass for biopolyester recovery was produced with a fed-batch culture in a 3L bench-top bioreactor (BioFlo 110, New Brunswick Scientific Co. NJ). The temperature, pH and dissolved oxygen were controlled at 30 ^oC, 6.8, and 10% air situation, respectively. A feed solution prepared with a sugar manufacturing byproduct containing about 50% sucrose was introduced into the bioreactor till the cell density reached 130 g/L and PHB concentration 94 g/L (72% PHB of cell mass). The cell mass was harvested with centrifugation and re-suspended in an acidic water (0.2M H_2SO_4) to make a cell slurry of 278 g dry mass/L. The slurry was reserved for later use.

2.2. PHB recovery

The biopolyester was recovered and purified by dissolving the non-PHA cell mass (28% w/w) in sequential treatments consisting of acid pretreatment, base treatment, hypochlorite whitening, washing and drying [22].

Acid pretreatment: The cell mass in acidic solution was heated to boil and maintained for one hour under ambient conditions. The pretreated cellular solids were cooled to room temperature and separated from acid solution with centrifugation at 5,000 g for 10 min. The dissolved microbial biomass in the supernatant solution is referred to acid hydrolysates. The insoluble wet pellets were subjected to next base treatment.

Base treatment: The insoluble solids from the acid pretreatment were re-suspended in an equivalent volume of water to form slurry of about 200 g DM/L. The solution pH was raised to 10 to 11 with 5 M NaOH solution and stirred under ambient conditions for 30 to 60 min. A small amount of surfactants such as sodium dodecylsulfate (SDS, $CH_3(CH_2)_{11}OSO_3Na$) might be added to a concentration of 5 to 10 g/L. The slurry was then heated to boiling and maintained for 10 min under ambient conditions. After centrifugation, the dissolved biomass in the supernatant solution is referred to base hydrolysates. The sequential acid and base treatments above could also be performed without separation of the acid hydrolysates. In this case, sodium hydroxide was directly added into the acidic cell slurry and the pH was raised to 10-11 for base treatment. After centrifugation, the supernatant solution contained the hydrolysates generated from both acid and base treatments and is refereed to acid-base hydrolysates.

Whitening and washing: The insoluble wet pellets from base treatment were re-suspended in a commercially available bleaching solution containing 6% w/w of hypochlorite. The slurry was stirred for 1 to 2 hours under ambient conditions. The white PHB pellets were recovered with centrifugation. A small amount of biomass was dissolved and mineralized in

the bleaching solution because of chemical oxidation, which is not considered for reuse in this work. The wet PHA pellets were washed two times with water and dried in oven. The final PHB product is a white powder.

2.3. Chemical and material analysis

The dissolution of non-PHA cell mass was monitored by measuring the characteristic absorption of amino acid residues at 280 nm with a UV/VIS spectrophotometer (Beckman Coulter DU530, Fullerton, CA). The concentration of proteins that can be stained in Bradford assay was measured with the spectrophotometer after protein-dye binding [23]. The content of PHB in original cell mass, in sequential treatments, and final product were determined via methanolysis of the biopolyester in methanol (3 wt% sulfuric acid) at 100 ^{o}C for 8-10 hours [24]. The 3-hydroxybutyric methyl ester was hydrolyzed into 3-hydroxybutyric acid when the solution pH was raised to 11 with a 10N NaOH solution. The liquid samples were analyzed using an HPLC equipped with a UV detector (Shimadzu, Japan) and an organic acid column (OA-1000, Alltech, Deerfield, IL). The column was maintained at 65 ^{o}C and eluted with a water-sulfuric acid solution (pH 2) at 0.8 mL/min. The monomeric acid and crotonic acid, a trace byproduct formed in methanolysis, were detected at 210 nm. For data quality control, the biopolyester was also extracted from the freeze-dried cell mass in hot chloroform followed by precipitation with methanol [21]. The PHB content was calculated from the purified PHB and compared with the results of HPLC analysis.

The purified PHB and non-PHB cell mass were examined with a Nicolet Avatar 370 FTIR spectrometer (Thermo Electron Co., Madison, WI). The solids were pressed on a germanium crystal window of micro-horizontal attenuated total reflectance (ATR) for measurement of single-reflection and absorption of infrared radiation by the specimens. The thermal properties of PHB powder were examined with a differential scanning calorimeter (DSC). A Modulated 2920 instrument (TA Instruments, New Castle, DE) equipped with a refrigerated cooling system was run in heat-cool-heat mode at a rate of 5 ^{o}C/min under nitrogen. The selected temperature range was 30^{o}C – 210 ^{o}C with sample weights of 4.5 – 5.5 mgs. Images of cell and PHB granules were obtained with an energy-filtering transmission electron microscopy (120 kV LEO 912, Carl Zeiss SMT Inc. MA). The instrument has an in-column electron energy loss spectrometer, allowing analysis of light element in thin sections.

3. Results and discussion

3.1. Sequential treatment for PHB recovery

Figure 1 elucidates the sequential treatment of PHB-containing cells in a process of PHB recovery and purification. Starting with 100 dry cell mass, the cells in a slurry of 278 g DM/L were first treated in an acidic solution (0.2M H_2SO_4). A substantial amount of microbial proteins was released from the damaged cells, depending on temperature and time as shown in Figure 2.

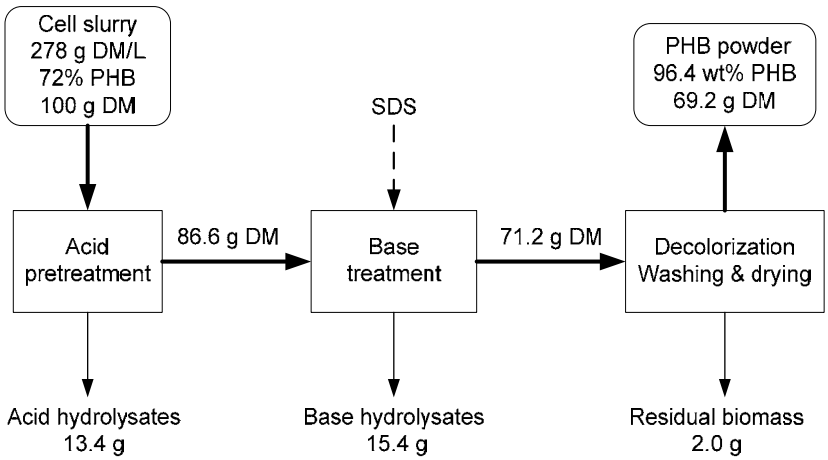

Figure 1. Sequential treatment of PHB-containing biomass (100 g dry mass) and generation of biomass hydrolysates. Surfactant SDS is optional for high PHB purity.

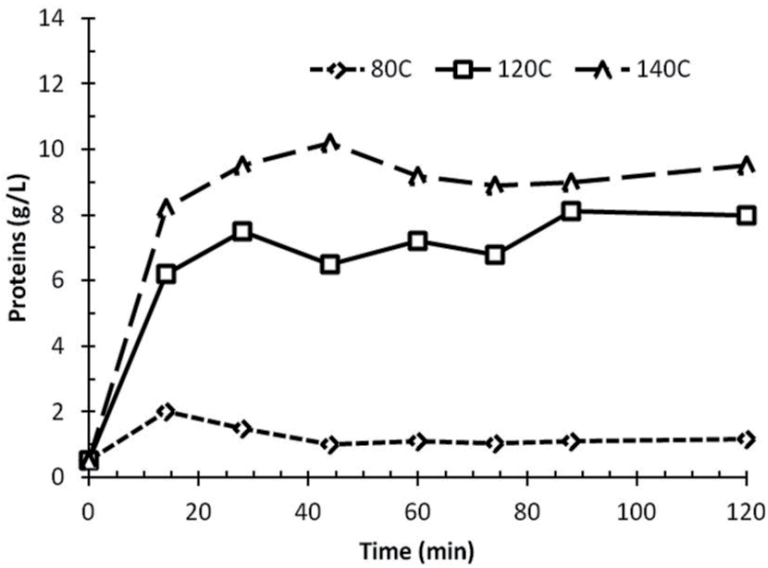

Figure 2. Effect of temperature and time on release of proteins from microbial cells in acidic solution.

During the acid pretreatment, the original amorphous PHB granules became partially crystallized (data not shown here), which improved the granule's resistance to abiotic degradation in the following treatments [20]. The biomass hydrolysates (13.4 g dry mass) dissolved in supernatant solution was discharged as acid hydrolysates. The residual PHB-containing biomass was further subjected to a base treatment by raising the slurry pH to 10.5 with a 10M NaOH solution. About 15.4 g dry mass was dissolved in the supernatant solution and discharged as base hydrolysates. After a small amount of residual biomass (2 g dry mass) was removed via oxidation with hypochlorite, the final PHB powder (69.2 g dry mass) contained 96.4 wt% PHB. The overall PHB recovery yield was 92.6%, or 7-8% PHB

was lost in repeated hydrolysis and solid/liquid separations. When a small amount of surfactant such as sodium dodecylsulfate (SDS) was added in the base treatment, the PHB purity of the final biopolyester resin could be increased to 99.4%.

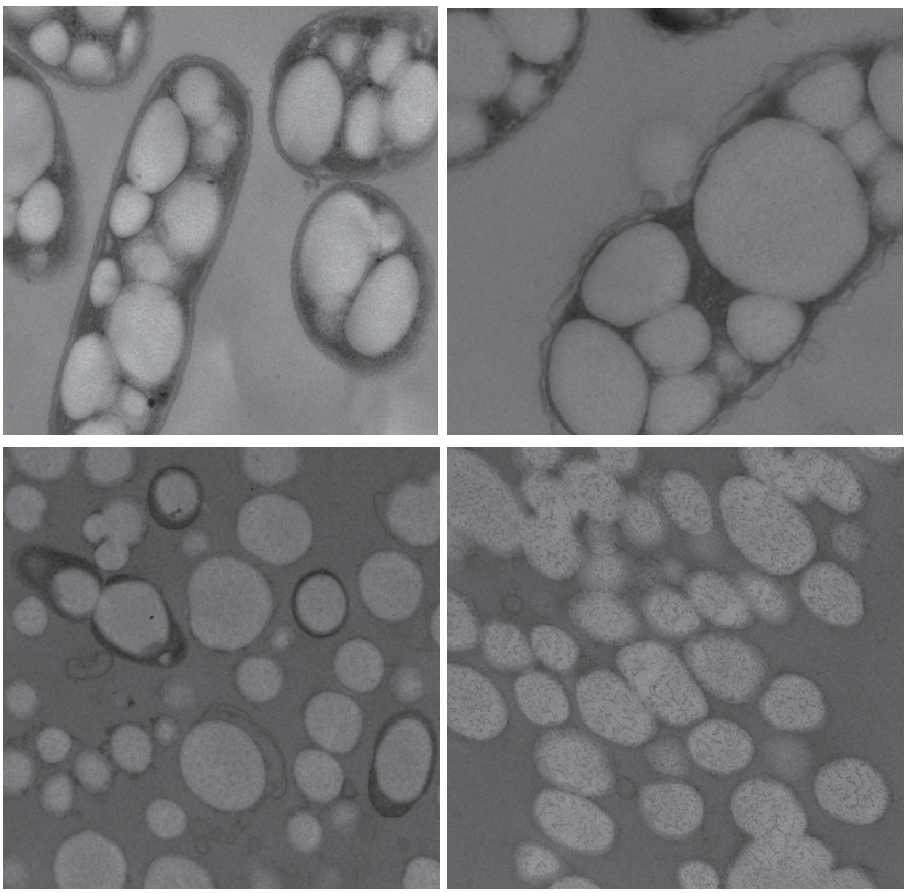

Figure 3. Transmission electron microscope images: microbial cells containing native PHB granules (top left), cells with damaged walls in acid pretreatment (top right), PHB granules with attached residual cell mass (bottom left), and purified PHB granules (bottom right)

Figure 3 is the electronic microscopy images of the original cells with PHB inclusion bodies, the cells with damaged porous cell walls in acid pretreatment, the PHB granules with residual cellular mass in base treatment, and the purified PHB granules after whitening and washing. It is interesting to see that the cell walls became porous in the acid pretreatment, which allowed release of proteins and other biological components in cytoplasm. The original cell structure, however, was maintained to keep the PHB granules within the damaged cells. After base treatment, the cell walls were almost completely decomposed, and a small amount of residual cell mass, probably some hydrophobic cellular components, was attached to PHB granules. After whitening and washing, the non-PHA cell mass was removed to give purified PHB granules.

3.2. Purified PHB versus original cell mass

Figure 4 compares the FTIR spectra of the purified PHB granules and the original oven-dried PHB-containing cell mass. The peaks of amide I band at ~1650 cm^{-1} and amide II band at ~1540 cm^{-1} (N-H bend) are characteristic infrared radiation absorption of the proteins in cell mass [25,26]. They disappeared in the spectrum of purified PHB granules. This was confirmed with a pure PHB prepared with solvent extraction (the spectrum not shown here). It was also noticed that the native amorphous PHB granules became crystallized during the process of purification, which will be further discussed. This structural change in PHB matrix was also reflected in the absorption of infrared radiation at wave numbers of 1180, 1210, and 1280 nm^{-1} [27].

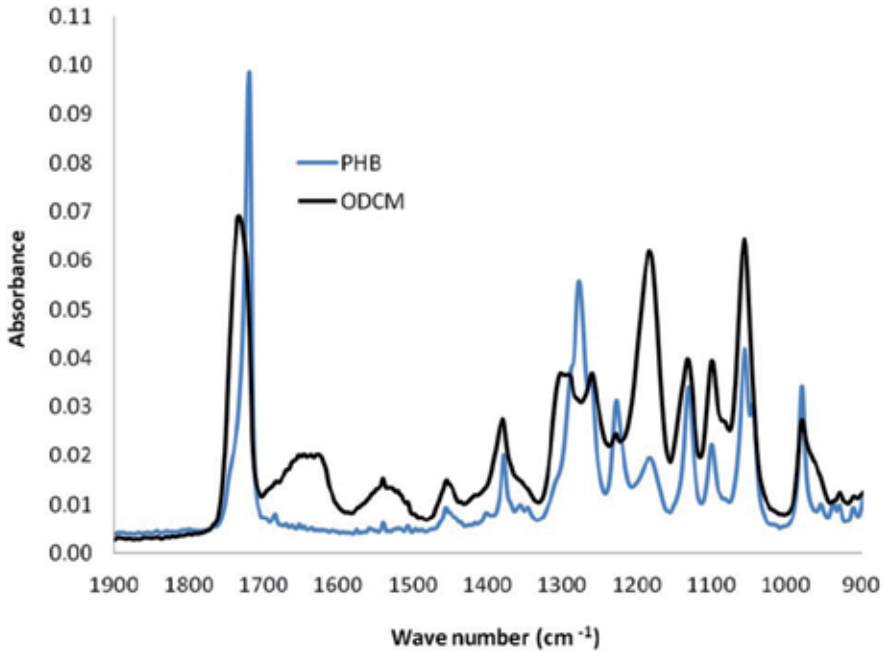

Figure 4. FTIR spectra of purified PHB granules and PHB-containing oven-dried cell mass (ODCM).

The purified PHB granules were subjected to repeated heating and cooling in a differential scanning calorimeter (DSC), and the results are presented in Figure 5. In the first heating (solid blue line), two melting peaks were observed, around 156 °C and 167 °C, respectively, indicating that the PHB powder had two types of crystalline structures. The melted polymer was re-crystallized again during the first cooling (dotted blue line), starting at 100 °C and ending at 80 °C. When the crystallized PHB was subjected to the second heating (solid black line), it was noticed that the relatively small melting peak at 156 °C (or crystalline structure) observed in the first heating disappeared. Only one melting peak (crystalline structure) was observed, starting at 160 °C and ending at 181 °C with a peak around 174 °C. This phenomenon indicates that the first melting peak in the first heating represents a type of crystalline structure that could not be formed at a cooling rate of 5 °C/min. The whole endothermic event in the second heating absorbed 87J/g PHB. Based on a theoretical melting

enthalpy of 100% crystalline PHB (146 J/g) [28], it can be estimated that about 60% of PHB matrix was crystallized during the first cooling (Eq. 1).

$$X_C = \frac{\Delta H_m}{\Delta H_t} = \frac{87}{146} \approx 60\% \quad (1)$$

Where X_c is the PHB crystallinity, ΔH_m and ΔH_t are the melting enthalpies of PHB powder and a theoretical PHB crystal [28].

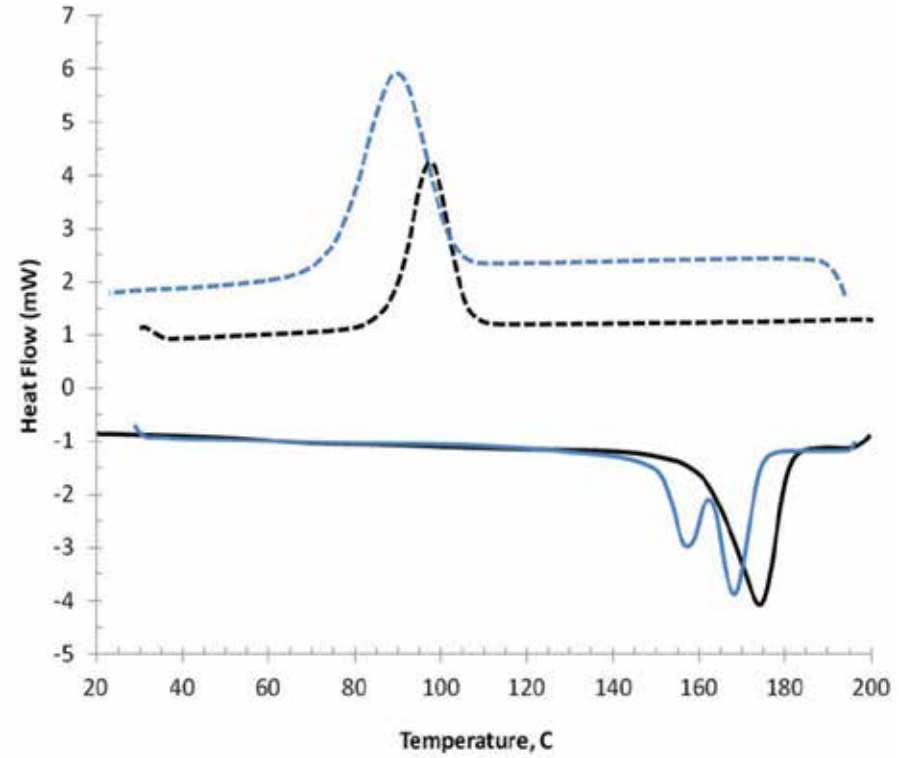

Figure 5. Differential scanning calorimetry (DSC) measurement of purified PHB granules in repeated heating and cooling: the first heating (solid blue line) followed by the first cooling (dotted blue line) and the second heating (solid black line) following by the second cooling (dotted black line).

In contrast to the thermal plastic behavior of PHB powders, the oven-dried cell mass containing 73% PHB could not be melted till carbonization. This fact reveals a complicated interaction between the biopolyester and the residual biomass. It also shows that a composite of 73% PHB and 27% cellular mass is not a thermoplastic material, but a rigid composite. The role of cellular mass in the PHB composite is not clear yet.

3.3. Cell debris solutions

As shown in Figure 1, about 94 wt% of residual microbial biomass was decomposed and hydrolyzed in the acid pretreatment (~44% biomass) and base treatment (~50% biomass). The remaining small amount (~6 wt %) of residual cell mass is most likely mineralized via

oxidation with hypochlorite, a strong oxidation agent. The acid hydrolysates are primarily the cytoplasm proteins released from the damaged cells (Figures 2 and 3). The released biological macromolecules were subjected to further hydrolysis in the thermal acidic solution. The acid hydrolysates solution had a clean brownish color and contained 30-45 g/ of soluble biomass, depending on the density of cell slurry and treatment conditions. The base hydrolysates solution with a dark color contained the hydrolysis products of hydrophobic cell components including lipids and membrane proteins. After centrifugation, the concentration of soluble biomass in the supernatant solution was 30 to 50 g/L. The sequential treatments disrupted and dissolved the structural components so that they could be removed from PHB granules. Equally important, the biomass and biological macromolecules were decomposed into small molecule hydrolysates such as amino acids and organic acids. These hydrolysates could become appropriate substrates that can be assimilated by microbial cells in microbial PHB production.

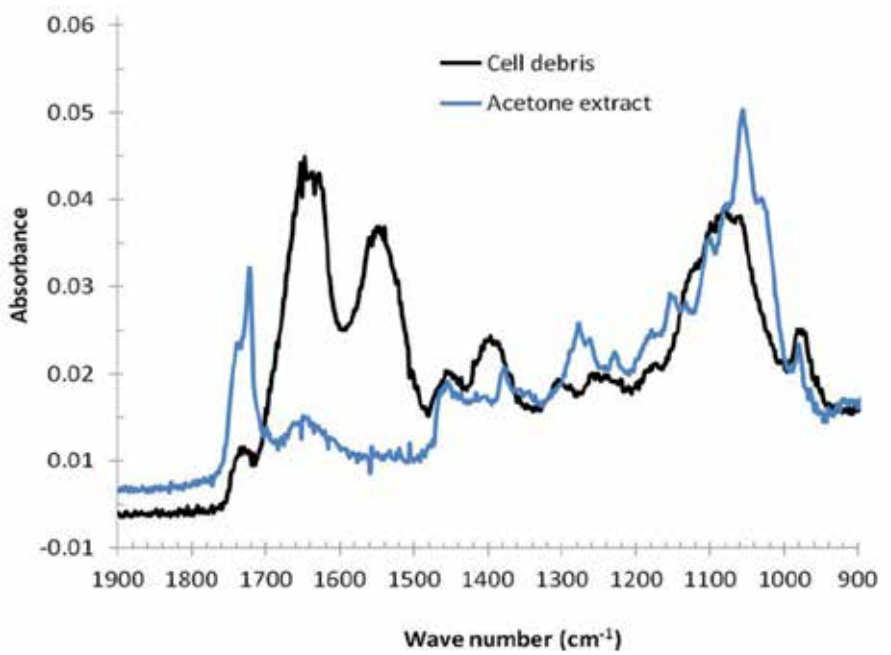

Figure 6. FTIR spectra of acid-base biomass hydrolysates (black line, cell debris) and cellular components extracted with acetone (blue line)

In addition to the two types of biomass hydrolysates described above, a mixed hydrolysates of residual biomass was generated when the acid pretreatment and base treatment were performed sequentially without solid/liquid separation. It eliminated one operation of solid/liquid separation, but the acid hydrolysates (primarily proteins) were subjected to additional hydrolysis in the base solution. Figure 6 shows the FTIR spectrum of an acid-base hydrolysates. As observed in the IR spectrum of the original cell mass in Figure 4, a major component of the hydrolysates was the amino acids or proteins with infrared radiation absorbance at 1500 to 1700 cm^{-1} [25]. Another major component in the acid/base hydrolysates had the IR absorbance at 1000 to 1200 cm^{-1}, which was attributed to cell lipids

and/or similar compounds. This was confirmed with the spectrum of cell mass extract in acetone. Acetone is a common solvent used to remove hydrophobic lipids, steroids and pigments from PHB-containing cell mass [17]. It does not dissolve and extract PHB and proteins. Based on the observations above, it was concluded that the major cellular components in the acid hydrolysates were derived from cytoplasm proteins and in the base hydrolysates from cell walls, lipids and membrane proteins. In the acid-base hydrolysates, the products were derived from both groups, i.e. amino acids or peptides derived from proteins, and lipids derived from cell walls and membranes. It should be pointed out that the composition of acid-base hydrolysates is not a simple mixture of acid- and base-hydrolysates because the acid hydrolysates were further hydrolyzed in base treatment.

Because of the hydrophobic properties of PHB granules, the residual hydrophobic impurities of cell mass might be attached to the granules and difficult to remove by washing with water. A surfactant such as SDS in the base treatment can remove most of the impurities to a high PHB purity (>99 % w/w). A large portion of the surfactant, however, may be left in the hydrolysates solution and may have an adverse effect on the reuse of the hydrolysates in PHB production.

3.4. Utilization of acid hydrolysates in PHB biosynthesis

An acid hydrolysates solution containing 38 g/L of soluble solids was added into a glucose medium to give a predetermined percentage of residual biomass to glucose at 0, 10, 20 and 25% of sugar, respectively. The initial glucose concentration was controlled at a constant level of 9.6 g/L. The flask cultures of no biomass hydrolysates were run in parallel as controls. As shown in Figure 7, the acid biomass hydrolysates were beneficial to both cell growth and PHA formation. Because the residual biomass might also contain some insoluble solids and PHB granules lost in PHB recovery, both cell density and PHB concentration were compared at 24 hours and 48 hours to show the net gains. The benefits of biomass hydrolysates were statistically significant based on the deviations of duplicates.

The acid hydrolysates might have two positive effects on microbial PHA formation. First, the hydrolysates promoted cell activity on glucose utilization, giving higher cell densities than the controls in the first 24 hours. This nutritional effect was similar to those of organic nutrients such as yeast extract and peptone, which are widely used in microbial cultures to provide nutrients and growth factors to the cells. A fast cell growth can reduce the cultivation time, resulting in a high PHB productivity. Second, the biomass hydrolysates might also be used as an extra carbon source to generate more cell mass than the controls in 48 hours. This carbon source effect, however, might play a minor role because the cell density did not increase with cell debris load. In fact, too much acid hydrolysates deteriorated the gains as shown in Figure 7. The reason is not clear yet. A load of acid biomass hydrolysates to glucose from 10 to 20 wt% seems appropriate for both cell growth and PHB formation.

3.5. Utilization of acid-base biomass hydrolysates

An acid-base biomass hydrolysates solution containing 48.5 g/L of soluble solids was added into a glucose medium at predetermined percentage of cell debris to glucose from 0 to 40%. The glucose medium without biomass hydrolysates was run in parallel as controls. As shown

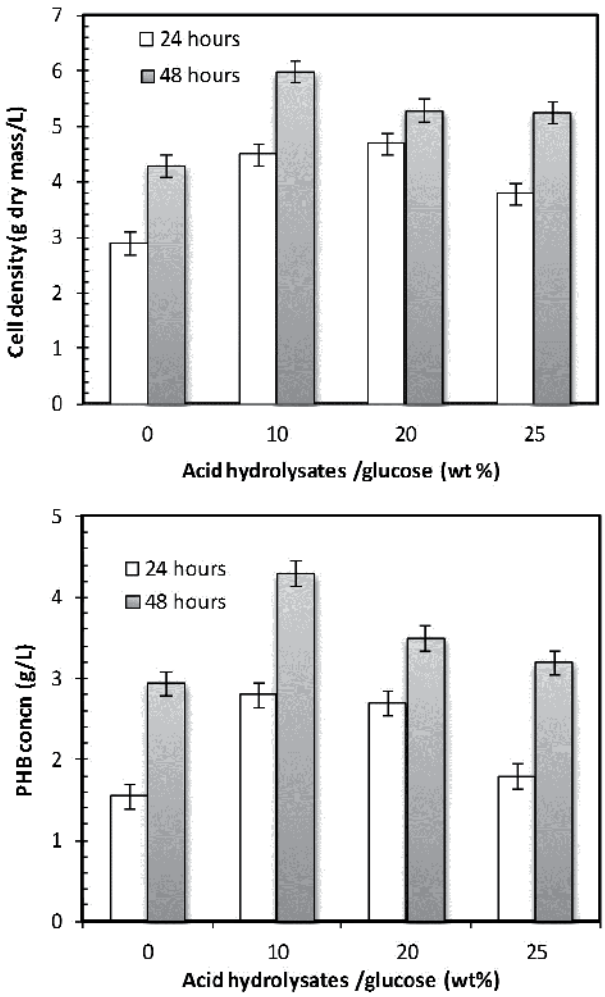

Figure 7. Effect of acid hydrolysates to glucose loading ratio on cell growth (top) and PHB formation (bottom) in reuse of the residual biomass for PHB production.

in Table 1, the concentrations of both cell mass and PHA content, after 48 hours cultivation, were substantially higher than those of the control. The overall cell growth yield (Yx/s) and PHA formation yield (Yp/s) are calculated from the amounts of cell mass and PHA formed in 48 hours based on the initial concentration of glucose. The relative yields (Yx' and Yp') based on the controls were increased by 100 – 300%. More interestingly, the inhibitory effect of acid biomass hydrolysates was not observed in the use of acid-base hydrolysates, and the load of cell debris to glucose can be increased to about 39 wt%. Most likely, the unknown inhibitors in acid hydrolysates were further decomposed into less inhibitory hydrolysates in the base treatment. Since the acid biomass hydrolysates contained primarily the cytoplasm proteins released from the damaged cells, the soluble proteins might adversely affect cell growth at high concentrations. After being hydrolyzed in base solution into peptides and amino acids, the small molecule hydrolysates become less inhibitory and more usable to the cells.

Biomass hydrolysates (g/L)	Biomass /Glucose (wt%)	Cell density (g/L)	PHB (wt%)	Yx/s (g/g)	Yp/s (g/g)	Yx′	Yp′
0.0	0	2.1 ± 0.5	45 ± 1.2	0.18	0.081	1.0	1.0
1.15	9.4	4.0 ± 0.2	55 ± 1.6	0.33	0.18	1.8	2.24
2.30	18.8	4.5 ± 0.3	60 ± 1.7	0.38	0.23	2.1	2.84
4.60	38.8	5.6 ± 0.4	61± 1.5	0.47	0.29	2.6	3.58

Note: the flask cultures were maintained at 30 °C and 200 rpm in a rotary incubator for 48 hours. The yields of cell mass (Yx/s) and PHB (Yp/s) were based on the initial glucose concentration. The relative yields (Yx′ and Yp′) are the ratios of yields with hydrolysates to the control without hydrolsyates.

Table 1. Effect of acid-base biomass hydrolysates on cell growth and PHB formation

3.6. Comparison of three types of cell debris

In the process of PHB recovery (Figure 1), three types of biomass hydrolysates may be generated, depending on operations: acid, base, and acid-base biomass hydrolysates. They may have different nutrient values or inhibitory effects on cell growth and PHB synthesis. Solutions of three types of biomass hydrolysates were added into a glucose medium for pre-determined concentrations of cell debris. Controls without hydrolysates were run in parallel. The ratios of cell densities (g/L) to the controls were compared after 48 hours cultivation as shown in Figure 8. The nutritional value of acid hydrolysates in cell growth is similar to that of base hydrolysates. The nutrient value of acid-base hydrolysates, however, is significantly higher than those of hydrolysates from individual treatment.

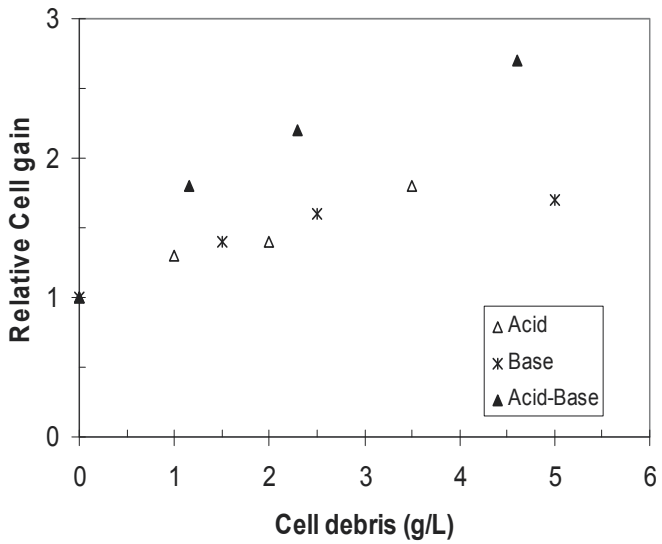

Figure 8. Comparison of three types of biomass hydrolysates (acid, base, and acid-base) on cell growth in a glucose mineral medium. The relative cell gain is the ratio of cell density to the controls.

Based on an average cell yield (Yx/s = 0.45) of PHB fermentation on glucose [29], 45 kg of cell mass containing 70 wt% of PHB is generated from 100 kg of glucose consumed. A

downstream recovery and purification as shown in Figure 1 can generate 31 kg PHB resin and 13 kg acid-base hydrolysis of residual microbial biomass. It is assumed that the acid hydrolysates are not separated, but hydrolyzed sequentially in the base treatment and discharged with the base hydrolysates together. If this amount of residual biomass is reused in next PHB fermentation, the percentage of biomass hydrolysates to glucose is 13% at maximum (13 kg for 100 kg glucose), a moderate load of biomass hydrolysates (Table 1). In real fermentations, more glucose is often added because of the residual glucose in the spent medium. This quick calculation indicates that most of residual biomass discharged from downstream separations can be reused in the next PHA fermentation. In addition to the elimination of a waste stream, the productivity and yields of PHA fermentation can also be significantly improved.

3.7. Effect of SDS in biomass hydrolysates solution

SDS is a popular surfactant used in PHA recovery to disrupt the cells or remove a small amount of hydrophobic impurities from PHB granules [30, 31]. The purity of PHB granules can be increased from 96.4% to above 99% when a small amount of SDS was added in the base treatment. The surfactant left in the base solution, however, may have an adverse effect when the cell debris is reused in microbial PHB fermentation. An acid-base biomass hydrolysates solution containing 48.5 g/L of soluble solids and SDS were added into a glucose medium. The cell debris concentration was kept at 1.94 g/L, and SDS concentration was increased from 0.2 to 0.8 g/L with SDS. Controls without biomass hydrolysates and SDS were run in parallel. Table 2 gives the results of cell growth and PHA formation at different surfactant levels at 24 and 48 hours, respectively.

Biomass hydrolysates (g/L)	SDS (g/L)	24 hours		48 hours	
		Cell mass (g/L)	PHB (wt%)	Cell mass (g/L)	PHB (wt%)
0	0	2.08 ± 0.02	36.8 ± 1.1	2.7 ± 0.02	44.4 ± 0.6
1.94	0.2	3.69 ± 0.06	49.7 ± 2.1	4.99 ± 0.04	60.6 ± 1.1
1.94	0.4	3.27 ± 0.05	39.6 ± 1.2	4.58 ± 0.04	53.8 ± 0.8
1.94	0.6	2.70 ± 0.04	33.9 ± 1.5	3.51 ± 0.03	50.3 ± 0.7
1.94	0.8	2.59 ± 0.04	17.8 ± 1.3	3.54 ± 0.03	25.8 ± 1.2

Note: flask cultures were maintained at 30 °C and 200 rpm in a rotary incubator.

Table 2. Effect of surfactant SDS and biomass hydrolysates on cell growth and PHB formation

Compared with the controls of no biomass hydrolysates and surfactant, all the cultures containing the acid-base hydrolysates exhibited better cell growth. Particularly, the increase of cell concentration from 24 hours to 48 hours was the new cell mass formed in 24 hours from glucose and cell debris in the presence of SDS. The formation of PHB, however, was deteriorated at high SDS concentrations (0.6-0.8 g/L). At a low or moderate SDS concentrations (0.2-0.4 g/L), the positive effect from biomass hydrolysates was much higher than the negative effect of surfactant. The PHB concentrations, after 48 hours cultivation,

were 2.4 to 3 g/l, in comparison with 1.2 g/L of the control. The results in Table 2 indicate that the dosage of SDS in PHA recovery should be controlled according to the amount of residual microbial biomass generated. The mass ratio of SDS to biomass hydrolysates should be less than 20% w/w or better at 10% w/w. In a typical PHB recovery process as shown in Figure 1, the amount of SDS used should be less than 2.9 g for 10% of acid-base cell debris or 5.8 g for 20% of acid-base cell debris. The consumption of SDS is therefore 4-8 % of PHB resin produced. It is much lower than the SDS dosages used in the conventional separations [30, 31].

4. Conclusion

Residual microbial biomass is an inevitable waste generated in downstream recovery of polyhydroxyalkanoates from microbial cells. With a separation technology based on sequential dissolution of no-PHB cell mass in aqueous solutions, the cell mass separated from the PHB-granules is decomposed and hydrolyzed into small molecule hydrolysates that can be assimilated by microbial cells as nutrients and/or carbon source. A type of biomass hydrolysates generated from continuing treatment in acid and base solutions exhibits the best nutrient value for cell growth and PHA formation. The acid-base hydrolysates contains two major water-soluble components derived from the cell proteins and lipids, respectively. When PHB-producing cells are fed with the hydrolysates in a glucose mineral solution, the cells grow faster and form more biopolyester in comparison with the controls that do not contain the hydrolysates. The glucose-based yields of cell mass and PHA bioplastics are significantly improved. SDS is an efficient surfactant to remove the small amount of hydrophobic residues for high PHB purity, but also a potential inhibitor to microbial PHA formation. When the amount of surfactant is less than 20% of an acid-base biomass hydrolysates, its negative effect is overwhelmed by the nutritional value of hydrolysates. Under these conditions, it is highly possible to reuse most of the residual biomass discharged from PHB recovery in the next microbial PHB fermentation. It therefore eliminates a waste stream from bioplastics production and saves the nutrients with improved PHA productivity and yield.

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Chapter 3

Considerations for Sustainable Biomass Production in *Quercus*-Dominated Forest Ecosystems

Viktor J. Bruckman, Shuai Yan, Eduard Hochbichler and Gerhard Glatzel

Additional information is available at the end of the chapter

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1. Introduction

Our current energy system is mainly based on carbon (C) intensive metabolisms, resulting in great effects on the earth's biosphere. The majority of the energy sources are fossil (crude oil, coal, natural gas) and release CO_2 in the combustion (oxidation) process which takes place during utilization of the energy. C released to the atmosphere was once sequestered by biomass over a time span of millions of years and is now being released back into the atmosphere within a period of just decades. Fossil energy is relatively cheap and has been fuelling the world economy since the industrial revolution. To date, fossil fuel emissions are still increasing despite a slight decrease in 2009 as a consequence of the world's economic crisis. Recently, the increase is driven by emerging economies, from the production and international trade of goods and services [1]."If we don´t change direction soon, we´ll end up where we´re heading" is the headline of the first paragraph in the executive summary of the World Energy Outlook 2011 [2]. It unfortunately represents systematic failure in combating climate change and the emphatic introduction of a "green society", leaving the fossil age behind. Certainly such far reaching transformations would take time, but recovery of the world economy since 2009, although uneven, again resulted in rising global primary energy demands [2]. It seems that more or less ambitious goals for climate change prevention are only resolved in phases of a relatively stable economy. Atmospheric carbon dioxide (CO_2) is the second most important greenhouse warming agent after water vapour, corresponding to 26% and 60% of radiative forcing, respectively [3]. Together with other greenhouse gases (GHG's) (e.g. methane (CH_4), nitrous oxide (N_2O) or ozone (O_3)) they contribute to anthropogenic global warming. The industrialization has been driven by fossil sources of energy, emerging in the 17th and 18th century in England as a historical singularity, but soon spreading globally.

Today, our economies still rely on relatively cheap sources of fossil energy, mainly crude oil and natural gas, and consequently emitting as much as 10 PG C per year in 2010 [4]. The Mauna Loa Observatory in Hawaii carries out the most comprehensive and longest continuous monitoring of atmospheric CO_2 concentration. It publishes the well-known Keeling Curve, representing the dynamic change since 1958. Observing the Keeling Curve, one can easily recognize the seasonal variability which is directly triggered by CO_2 uptake of vegetation (biomass) in the northern hemisphere during the vegetation period and secondly, which is even more important in terms of global change, a steady increase of CO_2 concentration from 315 ppmv in 1958 to 394 ppmv in March 2012 [5]. Earlier concentrations could still be derived from air occluded in ice cores. Neftel et al. [6] presents accurate gas concentration measurements for the past two centuries. However, the theoretical knowledge of the warming potential of CO_2 in the atmosphere evolved in the late 19th century when a theory of climate change was proposed by Plass [7], pointing out the "influence of man's activities on climate" as well as the CO_2 exchange between oceans and atmosphere and subsequent acidification. He highlighted the radiative flux controlled by CO_2 in the 12 to 18 micron frequency interval, agreeing with a number of studies published in the forthcoming decades, e.g. Kiehl and Trenberth [3]. In order to understand the fate of anthropogenic CO_2 emissions, research soon focussed on estimating sources and sinks as well as their stability, since it was obvious that the atmospheric concentrations did not rise at the same magnitude as emissions. Available numbers on current fluxes are principally based on the work of Canadell et al., [8] and Le Quéré et al., [1]. In their studies, it is emphasized that the efficiency of the sinks of anthropogenic C is expected to decrease. Sink regions (of ocean and land) could have weakened, source regions could have intensified or sink regions could have transitioned to sources [8]. Another explanation might be the fact that the atmospheric CO_2 concentration is increasing at a higher rate than the sequestration rate of sinks [1]. Moreover, CO_2 fertilization on land is limited as the positive effect levels off and the carbonate concentration which buffers CO_2 in the ocean steadily decreases according to Denman, K.L. et al. [1]. Fossil fuel combustion and land use change (LUC) are the major sources for anthropogenic C emissions (Figure 1). Land use change is usually associated with agricultural practices and intensified agriculture triggers deforestation in developing countries [9] and consequently causes additional emissions.

Another consideration is the availability of fossil fuel, which is limited by the fact that it is a non-renewable and therefore finite resource. Since the fossil energy system is based on globally traded sources with centralized structures, the vulnerability to disturbance is high. Recent examples of price fluctuations caused by political crises or other conflicts in producing countries or along transportation lines demonstrate potential risks. Moreover, a shift towards alternative energy sources and a decentralization of the energy system may contribute to system resilience and create domestic jobs. It prevents capital outflow to unstable political regimes and it helps to protect the environment not only by reducing GHG emissions, but also by reducing impacts of questionable methods of extracting fossil sources of energy (e.g. tar sands exploitation, fracking etc.).

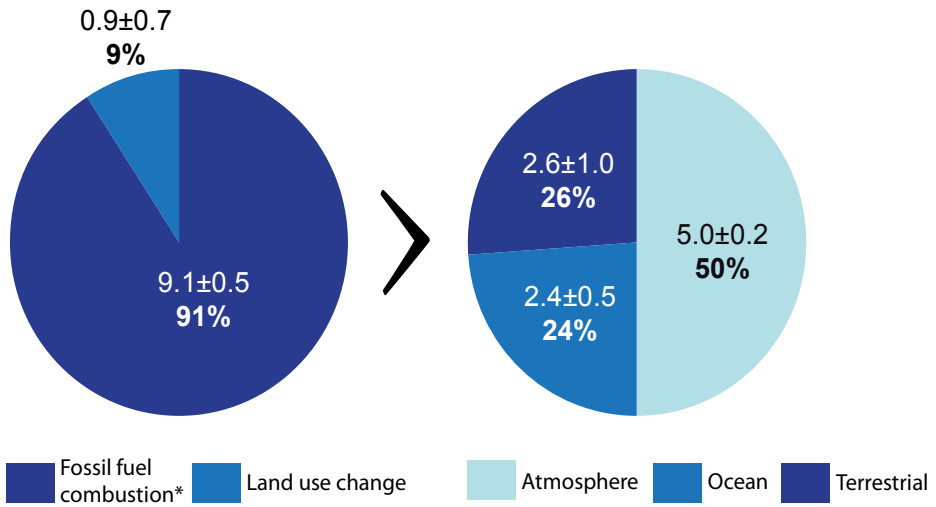

Figure 1. The fate of anthropogenic CO_2 emissions in 2010, showing sources (left) and sinks (right). Presented numbers are Pg C yr^{-1}. The values for 2010 were presented at the Planet under Pressure 2012 conference in London [4].
*includes cement production and flaring.

Biomass could play a significant role in the renewable energy mix. It is a feedstock for bioenergy production and currently thermal utilization (combustion) is by far the most important conversion process, but research activities are focussed on a range of different processes. This includes, for instance, the Fischer-Tropsch synthesis where any kind of biomass may be used as feedstock to produce liquid biofuels. This process is known as biomass – to – liquid (BtL). Research is pushed by national and international regulations (e.g. the EU's 2020 bioenergy target) and commitments, as a climate change mitigation strategy.

This chapter focuses on aspects of sustainable woody biomass production in *Quercus* dominated forest ecosystems with emphasis on different silvicultural management systems. Short rotation woody crops (SRWC), coppice with standards (CS), high forest (HF) and Satoyama are characterized according to their biomass potential and sustainability considerations. CS and HF are directly compared based on our own research and links to similar systems (SRWC and Satoyama) are drawn in order to provide a holistic view of the current topic. The chapter aims at providing an interdisciplinary view on biomass production in forest ecosystems, considering impacts on C and nutrient metabolism as well as other effects (e.g. biodiversity, technical, silvicultural and cultural issues). Considerations for sustainable biomass production in *Quercus* dominated forest ecosystems are presented for each management system.

1.1. Biomass production and carbon sequestration

Terrestrial C sequestration accounts for approximately one quarter of the three main sinks as indicated in Figure 2, where forests contribute the largest share. An intact terrestrial sink might be more important in the future in terms of mitigating climate change, since the ocean

sink is expected to decrease. Our current forests are capable of sequestering ~2.4 Pg C yr^{-1} (of 2.6 Pg in total), when excluding tropical land-use change areas [10]. Sequestration of C in forests is controlled by environmental conditions, disturbance and management. However, forests can principally act as a source or sink of C, depending on the balance between photosynthesis and respiration, decomposition, forest fire and harvesting operations. On both European and global scales, forests were estimated to act as sinks on average over the last few decades [11, 12].

The most important process of net primary production is achieved by photosynthesis, which is the chemical transformation of atmospheric CO_2 and water from the soil matrix into more complex carbohydrates and long chain molecules to build up cellulose, which is found in cell walls of woody tissue as well as hemicellulose and lignin. The C remains in the woody compound either until it is degraded by microorganisms, which use the C as source of energy, or until oxidation takes place (e.g. burning biomass, forest fire). In both cases, it becomes part of atmospheric CO_2 again. A certain share, controlled mainly by climatic conditions [13], enters the soil pool as soil organic carbon (SOC). The ratio between aboveground and belowground pools depends on the current stand age, forest management and climate. In temperate managed forests, SOC stocks are typically similar to the aboveground stocks [14], which is confirmed in our own research [13]. SOC (and in particular O-horizon) stocks are typically higher in boreal forests and in high elevation coniferous forests as a consequence of reduced microbial activity and much lower in tropical environments. O-layer C pools are especially sensitive to changes in local climate. A traditional forest management regime in Austrian montane spruce forests is clear-cutting, typically from the top of a hill to the valley to facilitate cable skidding. An abrupt increase of radiative energy and water on the soil surface creates favourable conditions for soil microorganisms and a great amount of C stored in the O-layer will be released to the atmosphere by heterotrophic respiration. Other GHG's, such as N_2O are eventually emitted under moist and reductive conditions as excess nitrogen is removed by lateral water flows. Unfortunately, this effect is likely to happen on a large-scale where massive amounts of C might be released as the global temperature rises and thawing permafrost induces C emissions [15], potentially creating a strong feedback cycle, further accelerating global warming. However, Don et al. [14] found that SOC pools were surprisingly stable after a major disturbance (wind throw event), indicating low short-term vulnerability of forest floor and upper mineral horizons. They explained their findings with herbaceous vegetation and harvest residues, taking over the role of litter C input. The study covers a time-span of 3.5 years which might be too short to observe soil C changes. Likewise, we did not observe significant C stock decrease in our youngest sample plot of the coppice with standards (CS) chronosequence [13]. We expect that the N dynamics might have a profound influence on C retention and the impact of disturbance on SOC pools depends on environmental conditions. Successful long-term sequestration in terms of climate change mitigation is therefore only achieved if C becomes part of the recalcitrant fraction in the subsoil, which is typically between 1 000 and 10 000 years old [16, 17]. The C concentration is lower in the

subsoil, but considerable amounts can still be found if one not only analyses the topsoil layer as recommended by a number of authors, e.g. by Diochon et al. [18]. In contrast, the radiocarbon age of the topsoil may range from less than a few decades [17] to months if considering freshly decomposed organic matter. The reasons for the relatively high age in subsoil horizons are not clear, but unfavourable conditions for soil microbial diversity and strong association of C with mineral surfaces (organo-mineral interactions with clay minerals) might be an explanation [16]. There are, in principal, two pathways for sequestering C in forest soils. Forest litter consists of leaves, needles and woody debris; such as branches, bark and fruit shells which accumulate on the surface (L and F layer). Soil macrofauna degrades it until it becomes part of the organic matter (OM) where it is impossible to recognize its original source (H layer). Parts of it are translocated into deeper horizons by bioturbation (e.g. earthworms) or remain on the surface, to be further degraded by soil microorganisms while organic matter becomes mobile in the form of humic acids and subsequently being mineralized at a range of negatively charged surfaces (humus, clay minerals). The second pathway is through root turnover and rhizodeposition (= excretion of root exudates). Matthews and Grogan [19] and subsequently Grogan and Matthews [20] parameterized their models with values of between 50 and 85% of C from the fine root pool which is lost to the SOC pool on an annual basis, depending on species composition and management. This assumption is consistent with another study where 50% of the living fine roots were assumed to reflect real values [21]. There is evidence that C derived from root biomass [22] and mycorrhizal hyphal turnover [23] might be the most important source for SOC pools rather than from litter decomposition. Since fine root turnover is species specific [24], it could be controlled to some extent by species composition at management unit levels. A further question for a forest owner in terms of carbon sequestration is which management option to choose while still being able to produce products and generate income. Despite the fact that unmanaged forests hold the highest C pools, it was commonly believed that aggrading forests reach a maximum sequestration and it is reduced in old growth forests, where photosynthesis and autotrophic respiration are close to offsetting each other. This is contrasts a number of studies, pointing out that even unmanaged forests at a late successional development stage could still act as significant carbon sinks [25]. The authors of another study claim that advanced forests should not be neglected from the carbon sequestration discussion a priori [26] and they should be left intact since they will lose much of their C when disturbed [27]. Therefore, managing forests implies a trade-off between maximum C sequestration and provision of goods, such as timber and biomass for energetic utilization. While the highest amounts of carbon could be sequestered in unmanaged forests [27, 28], C sequestration through forest management can be a cost-effective way to reduce atmospheric CO_2, despite limited quantities, due to biological limitations and societal constraints [29]. This is in general agreement with the conclusion of Wiseman's [30] dissertation, who argues that there is potential for additional C uptake depending on forest management, but the effect is short-term until a new equilibrium in C stocks is reached and also argues that the effect may be limited.

2. Biomass from forests

There is an on-going debate regarding the potentials of obtaining biomass from forests on multiple scales, from stand to international levels. Biomass is often discussed in the context of a raw material for energetic utilization although it should be emphasized that total biomass figures account for the total harvestable amount of wood, regardless of its utilization or economic value. Especially in the context of energy, it is highlighted that biomass is an entirely CO_2 neutral feedstock since the carbon stored in wood originates from the atmospheric CO_2 pool and it was taken up during plant growth. This is, in principal, true despite biomass from forests not being free of CO_2 emissions per se, since harvesting and further manipulation requires energy, which is currently provided by fossil fuels. However, it is difficult to estimate per-unit of CO_2 emissions since there are many influential variables. Even a single variable could have a profound influence on the per-unit emissions as is shown for the case of chipped fuel [31]. In general, biomass requires a different treatment as compared to fossil sources of hydrocarbons. Chemical transformations over thousands of years under high pressure led to a higher density of yieldable energy per volume unit as compared to biomass, although hydrocarbons are ultimately a form of solar energy. Hence fossil infrastructure does not fit to sources of renewable energy because of intrinsic properties. Centralized structures of energy distribution might work for fossil fuels, but it is questionable if it makes sense to transport woodchips across large distances. The energy invested for (fossil based) transport eventually curbs the benefits of renewable energy resources in terms of C emissions. Biomass from forests to be used for energetic utilization in the context of conventional forestry is often seen as a by-product of silvicultural interventions and subsequent industrial processes. However, there are a number of woodland management systems focussing on woody biomass production for energetic utilization or a combination of traditional forestry and energy wood production. Table 1 compares a number of *Quercus* dominated woodland management systems and highlights the main differences and characteristics. These systems will be further described thereafter.

In conventional forestry (high forest), residues from thinning and subsequent product cycles; e.g. slash and sawdust; are seen as the most important feedstock for energy wood. This opens the floor for controversial discussions and assumptions, based in principal on ecological and economic concerns. While residues of thinning operations are requested by traditional industries (e.g. paper mills), the extraction of slash and other harvest residues eventually leads to nutrient depletion with ecological impacts and ultimately detriment to increments in the long-term perspective. Inherent climate and soil properties control both magnitude and duration of such developments. “Residues” from forestry were traditionally harvested in ancient times. Most of the raw materials extracted from forests served as a source for thermal energy (fuel wood and charcoal) or other feedstock for industrial processes. Moreover, forests in central Europe provided nutrients for agro systems to sustain the human population [32]. Forest pasture, litter raking and lopping (sometimes referred to as pollarding) are some examples. Extraction of nutrients is still a common practice, e.g. litter collection in the Satoyama woodlands of Japan [33]. Since all of these practices tend to extract compartments with a relatively high nutrient content in comparison to wood, soil acidification and nutrient

depletion was a common threat in Central European forest ecosystems. Forests only recovered gradually, mainly because of acidic depositions starting from the beginning of industrialization until the late 1980's, when clear signs of forest dieback caused public awareness and subsequent installation of exhaust filters across Europe.

Woodland management system	**Characteristics**
Coppice	• Management target: traditional method of production of biomass for energetic utilization (fuelwood and charcoal) • Vegetative regeneration • Rotation period ~30 years • Degree of mechanization: low to medium • Extraction of nutrients from the soil: medium to high
Coppice with standards	• Management target: coppice (underwood) e.g. for biomass and an uneven-aged structure of standards (overwood) for timber of high quality. Standards provide shade and act as a back-up if vegetative regeneration is not successful (seed trees). • Vegetative (coppice) and generative regeneration (standards) • Rotation period ~30 years for coppice and 60-120+ years for standards • Degree of mechanization: medium • Extraction of nutrients from the soil: medium to high
Short rotation woody crops (SRWC)	• Management target: biomass for energetic utilization only, maximum increment will be harvested • Vegetative regeneration • Rotation period 2-10+ years (depending on the crop) • Degree of mechanization: high • Extraction of nutrients from the soil: very high
High forest	• Management target: production of high quality stems for trade, biomass for thermal utilization is a by-product ("residues", thinning harvests, slash, sawdust) • Generative regeneration • Rotation period ~120 years • Degree of mechanization: medium • Extraction of nutrients from the soil: low to medium
Satoyama	• Management target: integrative approach, biomass for energetic utilization, fertilizer for agriculture (litter collection) habitat for wildlife, recreation opportunities, small scale • Vegetative regeneration • Rotation period 15-20 years • Degree of mechanization: low • Extraction of nutrients from the soil: high to very high

Table 1. Comparison of *Quercus* woodland management systems and their characteristics with regard to management target, regeneration, rotation period, degree of mechanization and nutrient extraction. *Salix* or *Populus* species are the dominating crop in Central Europe due to higher increment rates in SRWC as compared to *Quercus* species.

Today, forest biomass stocks are increasing in most European countries, due to land use change (abandoned mountain pastures), shifting tree line as a consequence of global warming and elevated CO_2 concentrations as well as atmospheric N deposition. However, this should not lead to short sighted assumptions that biomass can be harvested at levels of growth increment, since a large part of it grows in areas with unsuitable conditions for access. Easily accessible forests at highly productive sites in lowlands are already typically managed at harvesting rates close to increment or even higher, e.g. in cases of natural disasters such as wind throws. In some countries, such as Austria, access to specific land ownership structures might uncover greater potentials of additional harvests.

3. Short rotation woody crops (SRWC) – A model of agriculture in forestry business

The major challenges of shifting forest management goals from traditional forestry to biomass production are sustainability issues, relatively low value of the product in comparison to quality logs and expensive harvesting, being competitive only at a high degree of mechanization in developed nations. As a consequence, rotation periods were shortened and fast growing species are preferred in order to produce woody biomass in an agriculture-like manner. Since the increment is highest at the beginning of stand development and subsequently decreases, only the maximum increment is utilized, ensuring maximum biomass production capacities at a given site. Short-rotation woody crops (SRWC) are hence established, in Europe typically with fast growing willow (*Salix*) or poplar (*Populus*) species. However, fast growing hardwood *Quercus* species are also considered for short rotation [34]. SRWC originated in ancient times, when people coppiced woodlands in order to obtain raw materials, e.g. fuel wood for cooking or heating purposes, but most of the research has been carried out and application of the results has been achieved in the last 50 years [35]. The basic principles of SRWC therefore originate in a coppice land management system, which will be described below. Planting is optimized for maximum biomass production (increment) while minimizing threats of disease and facilitating highly mechanized harvest technologies. Typical rotation periods are between 1 and 15 years [35], and rotations of *Salix* are shorter (< 5 years) than those of *Populus* and *Quercus*. Biomass from short rotations extracts significant amounts of soil nutrients, since a higher share of nutrient rich compartments (bark and thin branches) is extracted from the system. In combination with the short rotation cycles, nutrient extraction rates are larger as compared to conventional forestry. This implies the need of fertilization in most cases and concerns, e.g. about N leaching into groundwater bodies are discussed. However, Aronsson et al.[36] showed that high rates of N fertilization do not necessarily prime leaching, even on sandy soils (Eutric Arenosol) if the demand for N is high. C sequestration in the soil is also primarily controlled by N fertilization and the response of the vegetation [37]. The authors found increasing biomass production and C sequestration in a hybrid poplar plantation following N fertilization. On the other hand, it was argued that short rotations eventually result in the loss of the mineralization phase, thus preventing self-regeneration of the forest ecosystem [38]. Following their argument, a rotation cycle should be long enough to permit

the return of autotrophic respiration and high rates of mineralization. In terms of C sequestration potential, it ultimately depends on the land use prior to SRWC if and to what extent additional C is accumulated. Especially sites that were formerly used for agricultural purposes and where organic carbon was depleted are prone to additional sequestration after land conversion [19, 20]. They pointed out that especially, but not only, non-woody *Miscanthus* plantations, can substantially sequester C with relative high amounts of litter. In a global context, SRWC may interfere with agricultural production if it continues to be a focus and if plantation areas increase since most plantations are not the result of forestland conversion, but rather farmland conversion. One of the reasons for this interference is the varying legal definition of SRWC across nations. While SWRC is considered forest in some countries, it is treated as an agricultural crop in other nations, making comparisons and predictions across borders difficult.

4. Coppice with standards and high forest management in Austria

High forest (HF) and Coppice with standards (CS) are the most common silvicultural management systems for broadleaved forest ecosystems in northeastern Austria. These systems have evolved over a long period of traditional management and they are mainly determined by environmental conditions and economic considerations. However, these systems were locally adapted over time, resulting in a range of intermediate types. Divergent silvicultural structures with diffuse standards are the consequence and are very common in Austria [39].

Quercus dominated high forests aim at producing quality timber with a diameter of at least 30 cm (diameter at breast height (DBH)). Rotation periods are approximately 120 years, followed by shelterwood cuts and natural regeneration. It is one of the most common systems in central Europe and suitable for most species. The rotation period is set according to the dominant or most economically significant species and it is usually shorter for coniferous species (~100 years). The most important difference in comparison to other management systems is the type of regeneration, which in the case of high forest is entirely generative. Generative regeneration may be introduced by shelterwood cuts or similar silvicultural systems, or by planting, while natural regeneration is usually preferred as a consequence of costs and genetic compatibility issues (e.g. climate) and uncertain provenance. Shelterwood cuts promote generative regeneration via seeds when the canopy is opened. Individuals with high quality (i.e. straight stem) may be chosen to initiate regeneration, which implies genetic selection to a certain extent. Thinning is typically performed 4 times, at 30, 50, 70 and 90 years. The main silvicultural goal is to produce straight logs with a minimum number and size of ingrown branches, as a raw material for woodwork, veneer and other similar purposes. Thinning operations and harvesting residues provide biomass for energetic utilization. Individual generative stems might be considered as a source of woody biomass for energetic utilization as well, if they do not meet requirements in terms of quality.

Quercus-Carpinus coppice with standards is a woodland management system to produce biomass for energetic utilization. These forests were once the main source of thermal energy

when producing fuelwood for direct burning or charcoal production. There is evidence of coppice management dating back approximately 400 years in this region of Austria [40]. The management goals shifted during this period, depending on the demands of the landowners. CS is a relatively flexible system regarding supply of different qualities and quantities of wood. Among coppice, some trees are left in four age-classes to grow as larger size timber, called "standards". While standards provide a certain share of higher quality logs for trades, coppice provides fuelwood. Standards typically result from genetic regeneration. This multi-aged traditional system supports sustainable production of timber and non-timber forest products, while enhancing ecosystem diversity and wildlife habitat [41], which is also highlighted in the similar Japanese management system of Satoyama [33]. The rotation period for coppice (understorey) is typically 30 years [39, 42], hence holding a middle position between planted short rotation woody crops (SRWC) [35] and traditional high forests. The system is characterized by cyclic vegetative and generative regeneration [42]. Sprouting occurs rapidly after harvest and standards provide shade and are a source for seeds as backup if sprouting is not successful. Individual standards are managed in four age classes (30-60, 60-90, 90-120, and 120+ years) and harvested depending on certain criteria (e.g. market value, tree health, stand density). However, their importance began to cease with the introduction of fossil sources of energy during the onset industrialization, but significantly after 1960 [39]. Declining fuelwood demands led to a reduced intensity of understorey harvests (coppicing) and a shift towards longer rotation periods. This trend is especially distinct on fertile sites, while coppice was tendentially retained on sites with lower fertility.

The parent material of soils in our study region consists of gravel, sand and silt built up during the Pannonium (between 7.2 and 11.6 Ma before present) resulting from early formation of the Danube River. Consequently, a variety of soils can be found, e.g. Cambisols, Luvisols, Chernozems and even Stagnosols. Younger aeolian deposits of loess (Pleistocene) led to periglacial formation of Chernozems. The soils of our chronosequence series are classified as Eutric Cambisol with a considerable amount of coarse material (≤ 40% volume) in HF and sandy clay loam texture and both Haplic and Vermic Chernozems with loamy texture in CS [43]. Soils with lower fertility are derived from gravel and sand of the Danube River development, while Chernozems are derived from loess. The region receives approximately 500 mm of precipitation annually, with irregular periods of drought during summer. The water holding capacity of soils with a considerable amount of coarse material is lower as compared to loess derived soils, hence vegetative regeneration has the advantage of a fully functional root system at all times, supporting successful regeneration even in periods of drought. Generative regeneration might be obstructed under such conditions as a consequence of drying topsoil horizons. In our case study, we were able to include an outgrown coppice plot (i.e. a coppice with standards system that was not harvested at the theoretical end of the rotation period) aged 50 years to widen the scope for temporal dynamics. Irregular harvesting of standards and rotation periods up to 50 years (outgrown coppice) led to divergent silvicultural structures with diffuse standards [39], as previously mentioned. The plots were established during the summer of 2007 as permanent sample plots for aboveground biomass monitoring and are part of a framework to investigate biomass and carbon pools in this region [44].

4.1. Research methods

Studying temporal aspects of stand development is challenging because of the inherent duration of rotational cycles. Even in the case of CS in our example, it is theoretically 30 years but we included a 50-year-old outgrown stand. In HF, the rotational cycle is twice as long. Pickett [45] recommended a false chronosequence approach where he substitutes time for space. It is therefore crucial to find stands with similar management, species composition and other environmental conditions (microclimate, soil, topography), only differing in stand age. It must be assumed that the stands follow convergent succession trajectories [46], which was ensured in our case by use of inventory data from the past. The chronosequence approach is generally contested since it comes with a set of limitations (e.g. the problem of regional averaging, ignoring major disturbances or site-specific parameters as well as variation between hypothetical stands at the same age). Moreover, it assumes that there are no major disturbances (e.g. windthrows, insect attacks) during the rotational cycle. However, the method allows a researcher to successfully study temporal changes through the judicious use of chronosequences [46] and is often the only possible method to study long-term dynamics. Five plots were chosen for each chronosequence in HF and CS, ranging from 1-50 years in CS and 11-91 years in HF, respectively (Table 1).

A full biomass inventory was performed above- and belowground using allometric functions from Hochbichler [42]. In addition, belowground fine root biomass was determined to a depth of 50 cm by using soil cores. Additionally, soil macronutrient analysis was performed using these samples. Details for plot selection and setup as well as investigation of compartments and subsequent laboratory methods can be derived from Bruckman et al.[13]. The HF forest was chosen for comprehensive soil analysis, including exchangeable cations in soil and nutrient pools of different aboveground compartments, such as foliage, bark, wood and branches as well as regeneration and stems (see Figure 3). Exchangeable cations were determined at different soil horizons by using a $BaCl_2$ extraction and subsequent Inductively Coupled Plasma – Optical Emission Spectroscopy (ICP-OES) analysis. Exchangeable phosphorus pools were estimated using data from the forest soil inventory of adjacent forest sites [47]. Macronutrients N, P and K were determined in biomass compartments (foliage, bark of stems > 8 cm diameter, wood of stems > 8 cm diameter, composite sample of stems <8 cm, branches > 2 cm diameter, branches < 2 cm diameter and regeneration < 1.3 m height) according to inventory data for the most abundant species at each forest site. Nutrient analysis is based on three full tree samples (aboveground compartments) for *Quercus* and three foliage and branch samples from different crown layers per plot and species. Foliage was collected in August 2011 to ensure sampling fully developed leaves. $HNO_3/HClO_4$ extraction according to ÖN L 1085 [48] followed by ICP-OES analysis was used to determine nutrient contents.

4.2. Aboveground biomass

Aboveground biomass pools increased with stand age in both forest management systems as a consequence of steady accumulation (Figure 4). HF follows a typical pattern with high increments in the aggregation phase and marginal accumulation rates after 50 years.

Thinning concentrates additional growth on selected individuals with the highest possible quality, while low quality stems are harvested and utilized as fuelwood or further chipped. Hence the silvicultural activities aim at refining the product instead of maximizing biomass production. Approximately 2/3 of the aboveground biomass corresponds to understorey in the youngest HF site (11 years), while its share decreases steadily with increasing stand age (Table 1). The slightly rising share in the oldest stand (from ~3 to ~5 %) could be explained by initiation of generative regeneration as the canopy opens after thinning operations, allowing seeds to germinate and initiate regeneration.

Site	Age [years]	Biomass stocks [t/ha^{-1} dry mass]			Biomass stocks [%]	
		Overstorey	Understorey	Sum	Overstorey	Understorey
HF1	11	9.8	4.9	14.7	33.3	66.7
HF2	32	81	18.1	99.1	81.7	18.3
HF3	50	111.9	13.9	125.8	88.9	11.1
HF4	74	137.4	4.1	141.5	97.1	2.9
HF5	91	130.3	7.1	137.4	94.8	5.2
CS1	1	133.2	0	133.2	100	0
CS2	15	73.8	31	104.8	70.4	29.6
CS3	26	147.7	36.7	184.4	80.1	19.9
CS4	31	138.7	65	203.7	68.1	31.9
CS5	50	167.5	87.5	255	65.7	34.3

Table 2. Aboveground biomass stocks in tons per ha^{-1} dry mass for HF and CS, separated into overstorey and understorey compartments. In HF, overstorey represents individuals with DBH > 8 cm, while understorey represents individuals with DBH< 8 cm respectively. In CS, overstorey represents standards and understorey the vegetative coppice regeneration with some individuals being the result of generative regeneration.

In CS, we found a steady increase until the end of the rotation as the stand is still in the aggradation phase. The 15 year old stand is an exception because standards were previously harvested (irregular cut) resulting in lower biomass stocks as compared to the one year old stand where the total biomass equals that of standards. The relationship between overstorey and understorey biomass stocks is typical for coppice with standards forest management. While the overstorey stocks remain relatively constant (between 133 and 168 t.ha^{-1}), except in the 11 year old CS site where standards were recently harvested, the understorey coppice biomass pool constantly increases to 88 t.ha^{-1} at an age of 50 years (see Table 1). The relative share of coppice biomass increases from 20% in a 26-year-old stand to 34% in the oldest stand. This is an example for adaptive forest management since the demand for fuelwood has been low for decades and we were consequently able to find outgrown CS plots (50 years) and the share of coppice biomass is still relatively low. It could be increased under a different demand structure, where biomass for energetic utilization is in demand and commercialization becomes an interesting option for the forest owner.

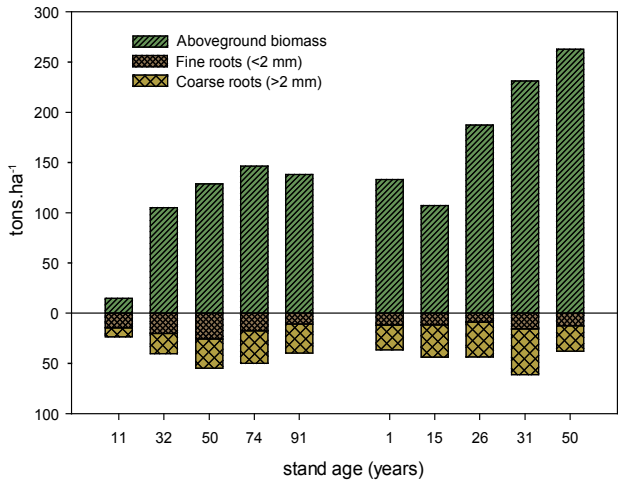

Figure 2. Aboveground (shoot) and belowground (root) biomass (dry mass) in two different forest management systems (high forest and coppice with standards). Data from Bruckman et al. [13].

On average, approximately 40% less biomass is stored in the HF system aboveground, and 7% less belowground (roots). Net primary production (NPP) is higher in CS which compensates lower basal area of the overstorey (DBH > 8 cm) with higher stand density [13]. The main reason of elevated NPP in CS is the higher fertility of chernozems as compared to cambisols in combination with a more effective water holding capacity as compared to the Eutric Cambisol in HF as a consequence of the coarse material content. The underlying silvicultural practices contribute to this biomass pool structure as thinning is performed at regular intervals in HF while typically only one intervention takes place in CS when harvesting coppice and selected standards at the end of the rotational cycle. As a consequence, additional C sequestration may only be achieved in CS when extending the rotation period. The second argument leads to higher productivity of the CS system, which allows higher C sequestration rates.

4.3. Belowground biomass

As previously mentioned, fine root turnover might be the most important pathway of C sequestration in forest ecosystems. Therefore, it is crucial to study root dynamics and turnover in the context of forest management. Our case study confirms that on average, fine root biomass (FRB) decreased with increasing stand age in HF ($R = -0.28$; $p<0.01$) but remained constant in CS. This basically reflects aboveground biomass dynamics where the CS system has a relatively balanced stand structure throughout rotational cycles. It is partly a consequence of shorter rotation cycles and therefore retains the aggradation phase [49]. Another reason lays in the continuous growing stock, since standards are kept on site during and after understorey harvest. Considering a finer resolution one may observe dynamic changes in FRB corresponding to stand development stages. FRB increased after stand reorganization, culminated at an age of 31 (CS) and 50 years (HF) and subsequently decreased as stands aged. In accordance with increasing aboveground biomass stores,

coarse root C pools increased with age in HF (R= 0.87; p= 0.53), accounting for 8.0 (0.9) % of total C pool and no trend was observed in CS, where coarse root C pools accounted for 7.8 (1.0) % respectively [13]. Although on average HF has lower total belowground biomass stores (7 % less), the FRB is 32% higher as compared to CS. The root-to-shoot ratio indicates higher belowground relative to aboveground biomass accumulation rates in early successional phases. A direct comparison between HF and CS reveals two major differences:

1. In comparison with HF, there was no initial major decrease of the ratio observed in CS
2. The ratio is always lower in CS than in HF

These differences may be due to significant aboveground biomass stocks represented by standards in CS and therefore comparatively low ratios, even in the stand reorganization phase. Consequently, root/shoot ratios are in equilibrium throughout the rotation period. More favourable soil conditions in CS may lead to lower ratios throughout stand development. It was shown that drought and limited soil resources (nutrient) availability promote FRB production [50, 51]. The effect of standards harvesting was observed in our case study as a slightly higher ratio in the 15-year old stand compared with the one-year old stand in CS. On average, the root C pool represented 28.0(3.0)% of total phytomass C stores when excluding the youngest HF stand where the root C pool was 1.6 times as high as aboveground phytomass stores [13].

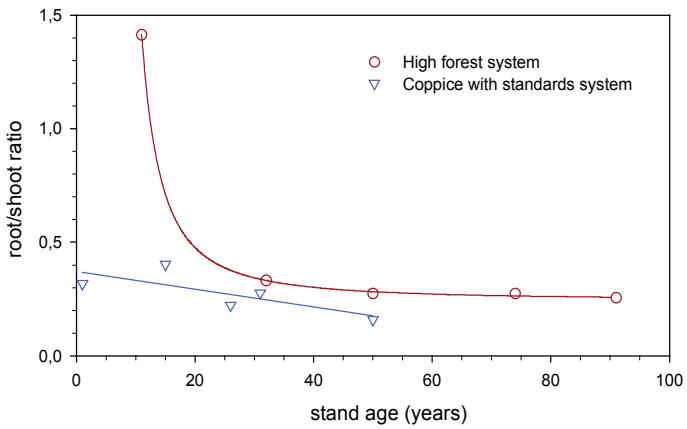

Figure 3. Root/shoot ratios of High forest system (HF) and Coppice with standards system (CS). The solid lines represent a hypothetic pattern. Source: Bruckman et al. [13].

4.4. Nutrient analysis

A comprehensive elemental analysis was performed for the HF system in both soil horizons and biomass compartments in the context of the framework for biomass investigations in northeastern Austria. HF was chosen because of lower soil fertility in comparison to the CS sites, which implies a higher sensitivity with regard to nutrient extraction.

The nutrient balance at a given site is an essential factor for stand productivity, species composition and biodiversity. Gains of nutrients that are in plant available forms and

therefore could be incorporated in new plant tissue are limited to originate from weathering of bedrock material, atmospheric deposition as well as fertilization. The major processes of decreasing nutrient availability to plants are removal (biomass extraction and leaching) or chemical transformation processes, resulting in recalcitrant and thus plant unavailable fractions. Soil microbes play an important role in these processes and there is even a competition for some elements, e.g. N between microbes and plant roots [52]. Interfering nutrient cycles, e.g. by changing forest management practices therefore influences a complex system. The consequences are not usually recognized immediately, depending on the nutritional status of the soil. If nutrient pools and cation exchange capacity (CEC) are low, the consequences are seen within just a few years, as is the case in tropical soils, for instance.

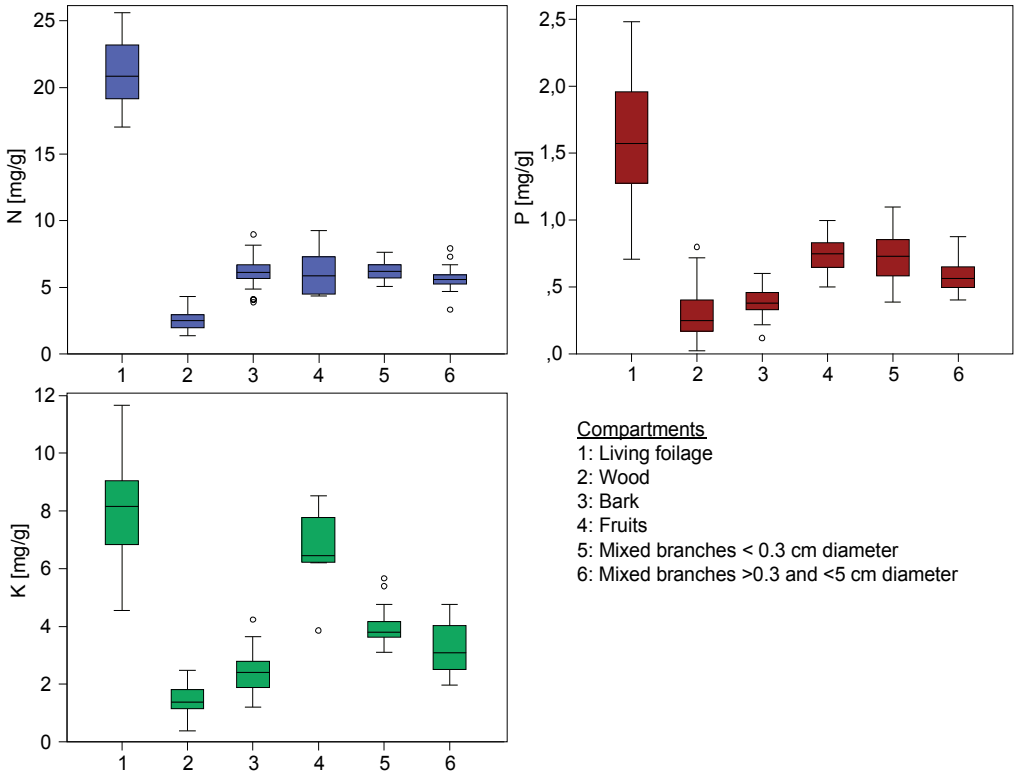

Figure 4. Nitrogen (N), Phosphorous (P) and Potassium (K) content in mg/g dry mass of different compartments for the dominating species, *Quercus petraea* in the HF system. Note that foliage is the compartment with the highest content of all macronutrients. Boxplots show the median (solid horizontal line), the bottom and the top of the box represent the 25th and the 75th percentile (interquartile range) and the whiskers show the highest and lowest values that are not outliers (< 1.5 times the interquartile range).

Based on an analysis of nutrient contents in various compartments (Figure 5), the aim was to compare plant available (exchangeable) pools of nutrient elements in soil with aboveground pools. This was done in order to determine potential nutritional bottlenecks when the management goal shifts towards biomass production as a source for energy, which implies

higher nutrient extraction rates as compared to conventional forestry or intermediate types (see Table 1). We focussed on the dominant tree species (*Quercus petraea*, *Carpinus betulus* and *Corylus avellana*) where we sampled whole trees on each plot to account for local differences. Only foliage and branches were sampled from less abundant species (e.g. *Fagus sylvatica*, *Betula pendula* and *Prunus avium*) where they occurred.

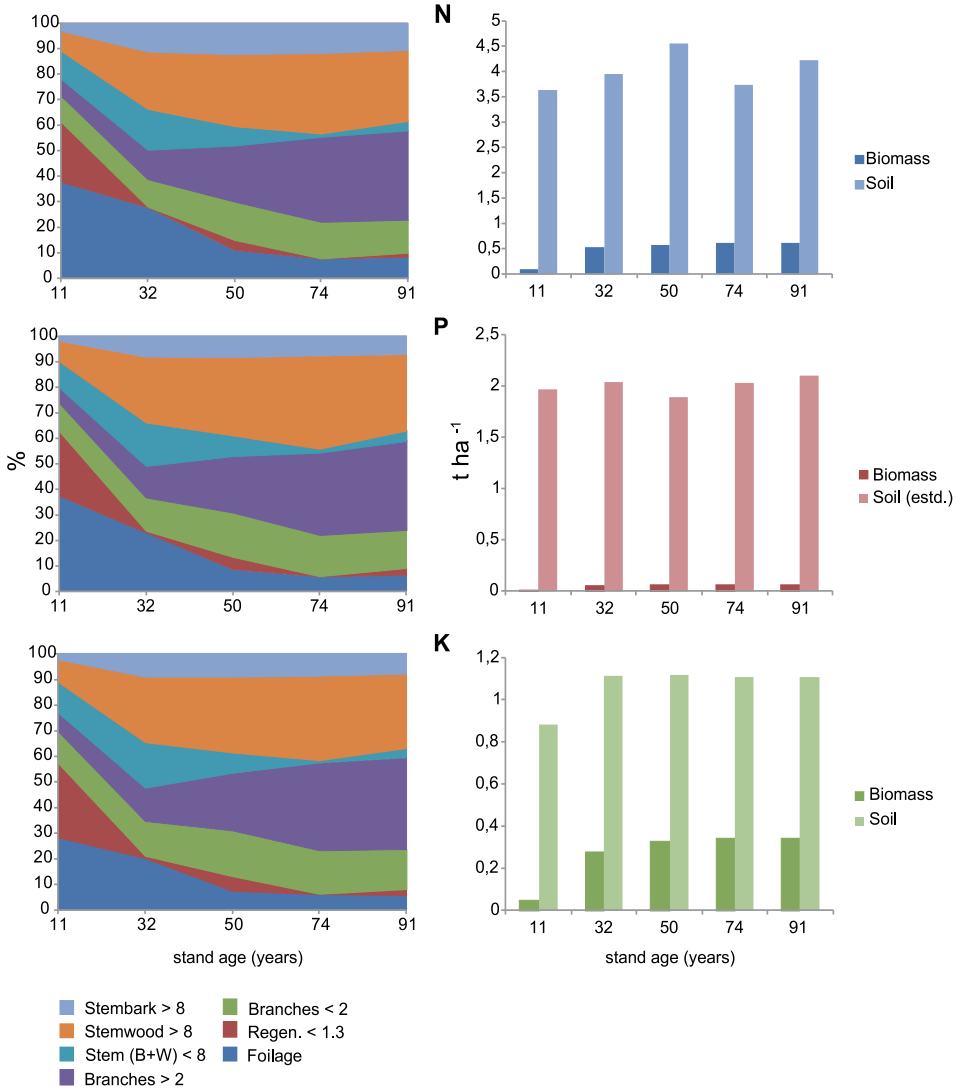

Figure 5. Relative amount of macronutrients in different compartments of *Quercus petraea* along the HF chronosequence (left) and a comparison of macronutrient contents in aboveground biomass and exchangeable soil pool (right). Category explanation (same order as legend): Bark of stems > 8 cm DBH, stemwood excluding bark of stems > 8 cm DBH, stems < 8 cm DBH (wood and bark), branches > 2 cm diameter (wood and bark), branches < 2 cm diameter (wood and bark), regeneration < 1.3 m height (total value), foliage (living). Soil exchangeable P pools were not actually measured, but estimated from data of the Austrian forest soil inventory [47].

Figure 5 illustrates the macronutrients nitrogen, phosphorous and potassium (NPK) contents of different compartments. Foliage clearly has the highest contents of macronutrients, followed by bark and thin branches. In the 91-year-old stand, foliage only accounts for 1.7% of the aboveground biomass, but represents 8.2% of the N pool, while 23.3% represent 37.5% in the youngest stand respectively. Wood (sapwood and heartwood) had the lowest contents. Similar patterns of nutrient distribution were previously reported for the same species [53]. The nutrient content of composite samples (wood and bark) depends on the respective proportions of wood and bark. However, it seems different for the case of P where higher contents in the composite as compared to separate wood and bark samples indicate higher contents of P in bark of thin branches (Figure 5). The bark sample consists of bark from branches and stem where the latter is therefore expected to have lower P contents. Approximately 40% of the macronutrients are stored in stems > 8 cm in diameter from an age of 50 years onwards (Figure 6) while representing approximately 60% of the stand aboveground biomass. Bark accounts for another 10% of the 40% stem pools. Consequently it was suggested to consider oak stem debarking to limit nutrient exports (especially Ca in the case of *Quercus* bark) from the stand [53]. A comparison with exchangeable soil pools revealed sufficient potential supplies from the soil matrix as the soil pools of macronutrients are well above the stand biomass pools. However, a simple comparison of pools does not necessarily represent the nutritional status of the vegetation since plant availability, stress and soil biogeochemical processes may cause uptake limitations of certain nutrients. For instance, the N:P ratio is well acknowledged as an indicator for either N or P limitation and values of < 14 indicate N deficiency where values of > 16 designate P limitation [54]. Obviously pools of soil exchangeable P are very high (Figure 6) which is also represented in our foliar N:P ratios. They are very stable at 14.2 for the 50, 74 and 91 year old stands and close to the threshold value (13.9) in the 32 year old stand. Interestingly the youngest stand (11 years) shows signs of N limitation with a ratio of 10.3. We suggest a combination of reasons for this observation. High rates of increment have a great N demand in the stand organization and subsequent aggradation phase. Herbaceous vegetation on this specific site might compete with woody species for topsoil N and the relatively high coarse material content contributes to low water holding capacities. In combination with low rates of precipitation, water availability might inhibit mobilisation and uptake of N at this site as suggested in a comprehensive review [34]. It is also shown that although the forest is surrounded by intensively managed agricultural land with associated atmospheric deposition of aerosols (dust), it has not led to P limitation as recently suggested [54]. In summary, despite evidence for N limits in the youngest stand, the macronutrient supply meets the demand under current forest management. However, it could be problematic if lower diameter compartments are extracted as biomass for energetic utilization. For instance, if the crown biomass is utilized at the final harvest and branches with a diameter > 2 cm are being extracted, it will account for a twofold extraction of N as compared to stem-only harvests. In a scenario of whole tree harvesting, close to 90% of N in biomass will be extracted, compared to approximately 40% in the stem-only scenario. Stem debarking could further reduce N extraction to below 30% of the total aboveground biomass pool. This example demonstrates the importance of assessing the dynamic nutrient pools in

order to provide profound recommendations for sustainable forest management, as was concluded in a previous study [53]. Forest nutrition not only has implications on species diversity, sequestration of carbon, and provision for a magnitude of environmental services, but it has distinct implications on site productivity and thus earning capacity of a given management unit. Sustainable nutrient management is therefore an essential component of successful forest management, especially if management aims at harvests that are more intensive for bioenergy production.

5. Sustainable coppice biomass production: the Japanese example of Satoyama

Satoyama forests have a long tradition in Japan. Directly translated "sato" means "village" and "yama" "mountain" [55]. The translation points at the conceptual meaning of Satoyama, which describes the typical landscape between villages and mountains (Okuyama). Although there are many definitions, one probably finds a suitable one in the Daijirin dictionary: "the woods close to the village which was a source of such resources as fuelwood and edible wild plants, and with which people traditionally had a high level of interaction" [56]. Satoyama could be understood as an integrative approach of landscape management, including the provision of raw materials such as wood, natural fertilizer (see the transfer from nutrients from forests to agricultural systems as previously mentioned), drinking water and recreational opportunities. Besides its inherent economic and ecological values, it provides a sphere for human-nature interactions and as such, it opens a window to see how Japanese perceive and value their natural environment over time [56]. As a consequence of small-scale structures and specific management, Satoyama woodlands represent hotspots of biodiversity [55]. They are pegged into a landscape of paddy fields, streams and villages. From a silvicultural point of view, Satoyama woodlands consist of mainly deciduous species, such as *Quercus acutissima* and *Quercus serrata* (however, Japanese cedar is sometimes used to delimit parcels of different ownership), which are coppiced on rotational cycles of 15-20 years, creating a mosaic of age classes [33]. Along with their scenic beauty and recreational capabilities which is doubtlessly a strong asset close to urban megacities, Satoyama woodlands are capable of providing goods and services for the society; even under conditions of changing demands and specific needs of generations [57]. In contrast to Satoyama, coppice forests in Austria were traditionally managed to obtain fuelwood and because of suitable environmental conditions for coppicing. Low levels of precipitation (~500 mm yr^{-1}) promote coppicing, since a fully established root system is present at any time, stimulating re-sprouting even under periods of drought. However, the holistic approach of landscape management is by far not considered to the same extent as it is for Satoyama. The importance of coppice forests for biomass provision ceased with the utilization of fossil fuels. Likewise, Satoyama was devaluated as a result of the "fuel revolution" in Japan [57], during which large areas were abandoned, leading to dismissed or poorly managed Satoyama woodlands. This is comparable with the previously mentioned divergent silvicultural structures with diffuse standards in Austria [39]. Satoyama woodlands are now back in the public interest, starting in the 1980s when volunteer groups

formed, being part of the Satoyama movement which urged for development of adequate environmental policies [33]. Although most groups are focussed on recreational activities in urban and peri-urban areas, the potential of Satoyama to provide biomass for energetic utilization has recently gained attention. Terada et al. [33] calculated a C reduction potential of 1.77 t ha^{-1} yr^{-1} of C for the Satoyama woodlands if coppiced and the biomass is utilized in CHP power plants. Considering the total study area (including settlements, agricultural areas and infrastructure, the C reduction potential is still 0.24 t ha^{-1} yr^{-1}. In the context of nutrient budgets, practices such as cyclic litter removal have to be evaluated with regard to the base cation removal rates and subsequent acidification, which ultimately leads to lower ecosystem productivity as was previously shown. Litter removal may cause substantial nutrient extractions of the compartment with the highest contents (Figure 5). The Fukushima Daiichi nuclear incident clearly showed the threat of nuclear fission energy sources. In combination with efforts to cut carbon emissions, paired with rising demands for energy, the situation caused a shift towards sustainable, clean and safe forms of energy provision. If a sustainable management is applied, especially in the context of nutrient budgets, Satoyama perfectly fulfils these requirements and might be a good choice to include in the future energy system while being neutral in terms of GHG release. Satoyama could represent a good model for sustainable resource management that the rest of the world can learn from [55, 56]. A study to evaluate Satoyama landscapes on a global basis was started under the "Satoyama initiative", launched by the Japanese Ministry of the Environment[*].

6. Conclusion

Specific types of biomass, i.e. wood and wood-derived fuels, have a long history of being the major source of thermal energy since humanity learned to control fire, which was a turning point in human development. These sources have not lost their significance in many developing countries, especially in domestic settings. However, over-population, climatic conditions and low efficiency cause shortages of fuelwood in many regions, e.g. Ethiopia or Northern India. This would not be the major problem in developed nations, where biomass as a source for thermal energy and raw materials for industrial processes has recently gained increased attention as a renewable and greenhouse-friendly commodity. Hence, sustainable management is required to prevent adverse consequences for society and the environment. Paradoxically biomass is tagged to be sustainable per se, although this is by no means substantiated since it depends on the local conditions and management used. Compared to conventional fossil sources of energy where "sustainability" is only directed at a wise and efficient use of a finite resource, sustainability of biomass from forests has to be considered in a much wider context. Biomass represents just one of a multitude of other ecosystem services and the potential for its provision depends on the conditions of any given ecosystem. As a matter of fact, ecosystems are extremely heterogeneous from global to forest stand scale, mainly controlled by environmental conditions, such as climate, soils and resulting species composition and anthropogenic impacts. Likewise, society's demands for specific ecosystem services are highly diverse. While recreation and the provision of a clean

[*] See http://satoyama-initiative.org/en/ for more details.

environment (water, air) are likely the most important service close to urban areas and settlements, the provision of wood and by-products as economic commodities might be important in more remote, but accessible regions. This also implies one of the major differences to the current energy system. Besides the fact that bio-energy is not capable of providing the same amount of sustainable energy we are currently receiving from fossil fuels, the infrastructure has to be decentralized, directed at local demands and supplies. Both, the economic stability as well as the benefits for environment and society are at risk in the case of large-scale bioenergy power plants. Based on a global review, Lattimore et al. [58] identified six main areas of environmental constraints with regard to wood fuel production:

1. Soils
2. Hydrology and water quality
3. Site productivity
4. Forest biodiversity
5. Greenhouse gas balances
6. Global and supply-chain impacts of bioenergy production

This listing elucidates the challenge of applying sustainability criteria to biomass production, since a large set of criteria and indicators for bioenergy production systems has to be implied. Consequently, sustainability comes at the cost of a high complexity of ensuring mechanisms. Nonetheless, a set of regionally adaptable principles, criteria, indicators and verifiers of sustainable forest management, as suggested by Lattimore et al. [58] might be inevitable to ensure best practices according to the current status of scientific knowledge. They propose an adaptive forest management framework with continuous monitoring in certification systems to ensure efficacy and continual improvement. Policy has to ensure that such regulations are implemented in binding regulations. Corruption and greed for short-term profit maximization are still major problems, providing a base for unsustainable use of resources, especially in countries with unstable political situations. Apart from the general challenges of biomass production, we showed a number of examples of different *Quercus* dominated forest management systems and their considerations for sustainable biomass production.

Short rotation woody crops (SRWC) have the potential to maximize biomass production, which comes at certain environmental costs. Utilizing the maximum possible increment, by using fast growing species including *Quercus* and by reducing rotation periods, soils have to provide substantial amounts of nutrients, which must be returned by fertilization. Economic considerations (economies of scale) and high levels of mechanization lead to uniform structures and monocultures. Hence, biodiversity and a number of other ecosystem services are threatened. Fertilization in agriculture is always associated with considerations of groundwater pollution. Likewise, fertilization can have the same effects in SRWC if soil properties are ignored and improper fertilization strategies are applied. However, there are indications that SRWC may better utilize the nutrients from fertilization and leaching could be minimized [36]. This might be a consequence of perennial crops in comparison to conventional crops in agriculture and deeper rooting of the biomass crops. However,

another potential problem linked to agriculture is the fact that SRWC are often established on former agricultural land. Together with the trend towards other kinds of agrarian non-woody biomass crops for energy production (biofuels, biogas), it should be noted that agricultural land should be given priority for food and feed production under current predictions for population growth. This conversion of use is especially problematic in some developing countries and was recently discussed extensively [59].

Quercus dominated high forest (HF) represents a system of conventional forestry and it was expedited as a consequence of reduced demands for fuelwood and charcoal as they were substituted by fossil sources of energy. Another reason was increasing demands for higher timber qualities for construction and trade. This reversal in forest management towards longer rotation periods of > 100 years in combination with limited utilization of small diameter compartments such as branches and twigs resulted in improving soil conditions in many cases in Europe, especially close to urban areas. However, the potential for biomass production for energetic utilization is limited as this system aims at biomass refinement rather than maximizing outputs in terms of quantities. Potential sources for bioenergy production are harvests of silvicultural interventions (thinning) as well as crown and stump biomass at the end of the rotation period. We showed that crown biomass removal in addition to stem utilization (full tree harvest) will extract 50% more N as compared to stem-only harvesting. On the contrary, stem debarking could retain 70% of the total aboveground N pool in the system under a scenario of stem-only extraction. In accordance with aboveground stand development, the root-to-shoot ratio follows a distinct pattern. While the woody vegetation invests more in root growth in the youngest stand to explore soil nutrient and water reserves, the ratio reaches a value below 1 (i.e. aboveground biomass prevails) in the 32 year old HF stand and subsequently levels off. This observation was supported by the N:P ratio in the youngest HF indicating N limitations, potentially priming root growth. In general, the biomass potential for energetic utilization is lower as compared to the CS system, which is mainly expressed by higher NPP as a consequence of more fertile soils and different management aims. If more intensive biomass extractions are considered to be sustainable or not therefore depends on soil nutrient and water budgets and has to be assessed at a local scale. Our HF sites showed sufficient nutrient pools despite some indication for N deficiency in the youngest plot.

Coppice with standards (CS) holds an intermediate position between SRWC and HF. It is capable of providing both higher quality timber logs and biomass for energetic utilization. The system has a long tradition in our study region and it is very flexible concerning different demands of the respective qualities and quantities. It was demonstrated that CS has the advantage of a fully functional root system at all times during the rotational cycles which is represented in the relatively stable root-to-shoot ratio and this is an advantage in relatively dry climates. Re-sprouting occurs relatively fast and is likely to be more successful as compared to planting or natural generative regeneration, especially under conditions of seasonal droughts. However, standards also act as seed trees where genetic selection is possible (promotion of individuals with high stem quality). They act as a backup if vegetative regeneration is unsuccessful and provide shade during summer. Nutritional bottlenecks are not expected

under current management practices as the soils in our CS plots are relatively fertile and the forestland is surrounded by extensively managed agricultural land. However, if the management strategy is changed towards schemes of increased biomass extraction, the effects on soils have to be studied in order to ensure sustainability.

The Satoyama woodlands in Japan are an example of traditional sustainable woodland management, carefully balancing ecosystem services in regions with relatively high population density, thus implying a high level of social-nature interaction. Since this form of landscape management proved to be successful over centuries with a high degree of flexibility with regard to changing demands over time, it might be a model of sustainable land use for other regions in the world. However, the effects of cyclic litter removal from soil nutrient pools should be investigated since we demonstrated that foliage is the compartment holding the highest contents of macronutrients.

A sustainable future, entirely based on renewable sources of energy without harming our environment is possible. It will certainly base on decentralized structures with a large pool of different sources of renewable energy, which has another great advantage of a high level of resilience in comparison to our current system. Biomass can play a significant role in areas with sufficient supplies, as long as the production follows sustainability criteria and does not interfere with other environmental services essential for that region. The optimal future energy system consists of a range of different sources, in which biomass is eligible along with other renewable sources as long as it is produced in a sustainable manner. Certainly, changing lifestyles (reduced energy consumption, less meat in diets, higher efficiency) especially in the developed regions of the world may be a very important and effective step to reducing resource consumption, which should be taken immediately.

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Chapter 4

Biomass Production in Northern Great Plains of USA – Agronomic Perspective

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Additional information is available at the end of the chapter

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1. Introduction

The development of biofuel is an important measure to meet America's energy challenges in the future. In the 2007 Energy Independence and Security Act, the U.S. government mandates that 136 billion liters of biofuel will be produced by 2022, of which 60 billion liters will be cellulosic ethanol derived from biomass [1-3]. Currently, ethanol is one of the biofuels that has been developed extensively. In the U.S., initial efforts for ethanol production were focused on fermentation of sugars from grains (especially maize). However, there have been criticisms for ethanol production from maize because of low energy efficiency, high input cost and adverse environmental impacts [4-5]. Biofuels from biomass feedstocks are more attractive because biomass is a domestic, secure and abundant feedstock. There are at least three major benefits for using biofuels. The very first benefit is national energy security. To reduce the reliance of imported oil for transportation, alternative energy options must be developed. Economically, a biofuel industry would create jobs and ensure growing energy supplies to support national and global prosperity. Environmentally, producing and using more biofules will reduce CO_2 emission and slow down the pace of global warming and climate change.

There are several sources of biomass feedstocks in forest and agricultural lands. The agricultural resources for biomass include annual crop residues, perennial crops, and miscellaneous process residues and manure [2, 3, 6]. Among the agricultural sources, the dedicated biofuel crops based on perennial species have been considered to the future of the biofuel industry and are the focus of intense research [2, 3, 6-8]. In addition, perennial biofuel crops also can provide other environmental and ecological benefits such as improving soil health, providing wild life habitat, increasing carbon sequestration, reducing soil erosion and enhancing water conservation [2, 9]. A key factor for meeting the government's goal is the development of biomass feedstocks with high yield as well as ideal quality for conversion to liquid fuels and valuable chemicals [2-3, 6-8,10].

The Northern Great Plains (NGP) of USA has been identified as an important area for biomass production. In particular, North Dakota is ranked first in potential for producing perennial grasses and other dedicated biofuel crops among the 50 states [10]. With about 1.2 million ha of CRP (Conservation Reserve Program) land and over 2.8 million ha of marginal land that are not suitable for cropping, the state has great potential for liquid biofuel production from biomass crops such as perennial grasses [11]. Before the great potential for biofuel production can be realized, questions still remain for developing management practices and their economic and environmental benefits for biofuel crops, such as appropriate species in certain areas, biomass yield potential and quality, harvesting scheduling (e.g., annual vs. biennial harvest), and effects on soil health and carbon sequestration.

In this paper, we review the current research progress for developing perennial biofuel crops in the NGP, primarily based on long-term field studies. We start to briefly discuss the species selections for biofuel crops in the USA and Europe. Then, we focus on development of crop management strategies for high yield as well as ideal quality. Finally, some possible environmental and ecological benefits from perennial biofuel crops are briefly discussed.

2. Appropriate species for biofuel crops

2.1. Ideal biomass crop for biofuels

There are mainly three goals to develop biomass crop for biofuels: (1) maximizing total biomass yield per year; (2) maintaining sustainability while minimizing inputs; (3) maximizing the fuel production per unit of biomass. To achieve the above goals, an ideal biomass crops should have some attributes as followings: high photosynthesis efficiency (e.g., C4 plants), long canopy (green leaf) duration, low inputs, high water-use efficiency, winter hardiness, no known pests and disease, noninvasive, and uses of existing farm equipment [2]. Based on above criteria, perennial forage crops would be ideal candidates for biofuel crops. The primary purpose for growing perennial crops for biomass production is reducing input and maintenance costs. Economically, using perennial species is more cost effective than annual ones, given the current high costs of fertilizers, pesticides (mainly herbicides) and operation fuels, and low values of lands for growing biomass crops.

2.2. Species for potential biofuel crops

Over the years, many species have been or being evaluated for potential of biofuel crops in the USA and Europe, in which the perennial grasses are dominant (Tables 1 and 2) [12]. In the USA, switchgrass was determined as a model species. In Europe, miscanthus, reed canarygrass, giant reed and switchgrass were chosen for more extensive research programs [12]. In addition, legume species and mixture of multi-species also been evaluated as bioenergy crops [5,13].

2.2.1. Switchgrass and miscanthus

Switchgrass and miscanthus are two dominant species reported in literatures for potential biofuel crops. Switchgrass, a C4 perennial grass, has been designated by the U.S. DOE as

primary bioenergy crop and has been extensively studied for over two decades. Several reviews have addressed current research and development issues in switchgrass, from biology and agronomy to economics, and from production to policies [6, 14-18]. The attributes of switchgrass for biofuel production included high productivity under a wide range of environments, suitability for marginal and erosive land, relatively low water and nutrient requirements, and positive environmental benefits [17]. For biofuel purpose, switchgrass can be used to produce ethanol [2, 7, 18]. It also can be used as combustion to co-fire with coal in power plant for electricity. Currently, switchgrass production in southern Iowa is mainly used for combustion [19].

Miscanthus is another C4 tall perennial grass originated in East Asia and has been studied extensively throughout the Europe from the Mediterranean to southern Scandinavia [20]. Comparing with other C4 species (such as maize), miscanthus is more cold tolerance and winter hardy in temperate regions of Europe. It also has a low requirement of nitrogen fertilizer and pesticides. In general, miscanthus has a very high biomass yield potential when it is well established. Lewandowski et al. (2000) [20] reported that the irrigated miscanthus yield can be as high as 30 Mg/ha, and yield under rainfed conditions ranged from 10 to 12 Mg/ha. When compared biomass production in US for switchgrass and Europe for miscanthus, the average yield of miscanthus (22 Mg/ha) was twice as much as the average yield of switchgrass (10 Mg/ha), given the similar temperature, nitrogen and water regimes [21]. A side-by-side study in Illinois showed that average biomass yield in miscanthus (30 Mg/ha) can be 3 times as much as switchgrass (10 Mg/ha) [22]. Compared to switchgrass, miscanthus may require higher input costs because it must be established using rhizome cuttings, which delays full production until the second or third year [20, 21]. In Europe, the primary use of miscanthus biomass is for combustion because of the ideal chemical composition [20]. However, little information is known for the conversion of ethanol from miscanthus.

2.2.2. Reed canarygrass and alfalfa

In addition to switchgrass and miscanthus, two other species, reed canarygrass and alfalfa, have also been studied considerably for biofuel crops. Reed canarygrass is a C3 grass commonly used for hay and grazing in temperate agricultural ecosystems, and can yield 8-10 Mg/ha in the Midwest of USA and northern Europe [6, 12]. Similar to switchgrass, reed canarygrass is difficult to establish and normally has a low yield in the seeding year [6].

Alfalfa, one of the oldest forage crops in the world, has traditionally been used as high quality forage. However, alfalfa may also have some values for biofuel feedstock [13]. In an alfalfa biomass energy production system, the forage could be fractionated into stems and leaves. The stems could be processed to generate electricity or biofuel (ethanol), and the leaves could be sold as a supplemental protein feed for livestock. Currently, researchers in Minnesota are conducting experiments to select dual-use alfalfa varieties and developing management systems [13

English name	Scientific name	Photosynthetic pathway	Yields reported Mg DM/ha/year
Crested wheatgrass	*Agropyron desertorum* (Fisch ex Link) Schult.	C3	16.3
Redtop	*Agrostis gigantea* Roth	C3	Not available
Big bluestem	*Andropogon gerardii* Vitman.	C4	6.8-11.9
Smooth bromegrass	*Bromus inermis* Leyss	C3	3.3-6.7
Bermudagrass	*Cynodon dactylon* L.	C4	1.0-1.9
Intermediate wheatgrass	*Elytrigia intermedia* [Host] Nevski.	C3	Not available
Tall wheatgrass	*Elytrigia pontica* [Podp.] Holub.	C3	Not available
Weeping lovegrass	*Eragrostis curvula* (Schrad.) Nees .	C4	6.8-13.7
Tall Fescue	*Festuca arundinacea* Schreb.	C3	3.6-11.0
Switchgrass	*Panicum virgatum* L.	C4	0.9-34.6
Western wheatgrass	*Pascopyrum smithii* (Rydb.) A. Love	C3	Not available
Bahiagrass	*Paspalum notatum* Flugge.	C4	Not available
Napiergrass (elephant grass)	*Pennisetum purpureum* Schum.	C4	22.0-31.0
Reed canary grass	*Phalaris arundinacea* L.	C3	1.6-12.2
Timothy	*Phleum pratense* L.	C3	1.6-6.0
Energy cane	S*accharum* spp	C4	32.5
Johnsongrass	*Sorghum halepense* (L.) Pers.	C4	14.0-17.0
Eastern gammagrass	*Tripsacum dactyloides* (L.) L.	C4	3.1-8.0

Table 1. The 18 perennial grass species that were screened by the US herbaceous energy crop research program [12].

2.2.3. Others

Compared to the above four widely studied species, many other species for potential biofuel crops are more regional specific and related to local climatic conditions. In the southern region of the U.S., subtropical and tropical grasses such as bermudagrass and napiergrass have been evaluated as biomass crops [6]. In southwestern Quebec, Canada, a short growing season environment, Madakadze et al. (1998) [23] evaluated 22 warm-season grasses in 5 species (sandreed, switchgrass, big bluestem, Indian grass and cordgrass). They found that the most productive entries were cordgrass and several entries of switchgrass. Switchgrass from high latitude tended to produce less biomass. The sandreed showed little potential for forage or biomass production. This study was conducted using space-planted nursery conditions and these data represent individual plant potential. Thereafter, their studies were only focused on switchgrass under solid sward conditions [23-25].

English name	Scientific name	Photosynthetic pathway	Yields reported Mg DM/ha/year
Meadow Foxtail	*Alopecurus pratensis* L.	C3	6-13
Big Bluestem	*Andropogon gerardii* Vitman	C4	8-15
Giant Reed	*Arundo donax* L.	C3	3-37
Cypergras, Galingale	*Cyperus longus* L.	C4	4-19
Cocksfoot grass	*Dactylis glomerata* L.	C3	8-10
Tall Fescue	*Festuca arundinacea* Schreb.	C3	8-14
Raygras	*Lolium* ssp.	C3	9-12
Miscanthus	*Miscanthus* spp.	C4	5-44
Switchgrass	*Panicum virgatum* L.	C4	5-23
Napier Grass	*Pennisetum purpureum* Schum	C4	27
Reed canary grass	*Phalaris arundinacea* L.	C3	7-13
Timothy	*Phleum pratense* L.	C3	9-18
Common Reed	*Phragmites communis* Trin.	C3	9-13
Energy cane	*Saccharum officinarum* L.	C4	27
Giant Cordgrass/	*Spartina cynosuroides* L.	C4	9
Salt Reedgrass			5-20
Prairie Cordgrass	*Spartina pectinata* Bosc.	C4	4-18

Table 2. Perennial grasses grown or tested as energy crops in Europe [12].

3. Biofuel crops in Northern Great Plains (NGP)

3.1. Species and biomass yields

In NGP, species evaluated for biofuel crops include switchgrass, big bluestem, Indian grass, tall wheatgrass, intermediate wheatgrass, wild rye, alfalfa and sweet clover [11, 26-33]. Switchgrass still remains in most of the studies in NGP. In South Dakota, switchgrass has been evaluated under both conventional farmland and CRP land, and the biomass yield ranged from 2 to 11 Mg/ha [28-30]. In North Dakota, cultivars of switchgrass have been tested in western and central areas in small research plots (Dickinson and Mandan) and biomass yield ranged between 2 to 13 Mg/ha, depending on cultivar [26-27]. In another site (Upham), biomass yield of switchgrass ranged from 2.4 to 10.8 Mg/ha [32]. In an on-farm scale trial, switchgrass yield ranged from 4.6 to 9.9 Mg/ha in Streeter and Munich [8, 34].

For selecting species for biofuel crops, switchgrass still has more advantages than any other species. This is because: (1) the species has been studied extensively in the US in last two decades and the germplasm pool is larger than other species; (2) it is a warm season species and has greater water use efficiency and drought resistance; (3) it is native to North America

and there are no concerns about the invasiveness; (4) there are many environmental benefits for growing switchgrass.

Switchgrass plot following the 2011 harvest at Central Grasslands Research Extension Center, Streeter, ND. Photography by Rick Bohn.

In addition to species, environmental factors (e.g., precipitation, temperature, soil type etc.) have large effects on yield and quality in biofuel crops. To address the interactions of species and environment, a ten-year long-term study was initiated and established in 2006 to evaluate ten cool and warm season grasses and mixtures across North Dakota [11]. The 10 entries of species and mixtures were shown in Tables 3. These grasses/mixtures were grown in six environments in five locations across North Dakota. Among the five locations, long term growing season precipitation varies from 318 mm at Williston in the west to 431 mm at Carrington in the east. In general, western ND has a semi-arid environment but eastern ND is more humid [11, 35].

Initial biomass yield data indicated Basin and Altai wildrye showed lower biomass yields than either switchgrass or wheatgrass species (Table 4). Tall wheatgrass and intermediate wheatgrass performed well across environments in North Dakota. In contrast, performance of switchgrass was largely related to environment, particularly the seasonal precipitation. For dryland conditions, studies are still needed to address both establishment and persistence of switchgrass in the future.

Harvesting perennial grasses plots in fall 2007, Streeter, ND.

Entry	Species/mixtures
1	Switchgrass (Sunburst)
2	Switchgrass (Trailblazer or Dakota)
3	Tall wheatgrass (Alkar)
4	Intermediate wheatgrass (Haymaker)
5	CRP Mix [Intermediate wheatgrass (Haymaker) + Tall wheatgrass (Alkar)]
6	CRP Mix [Intermediate wheatgrass (Haymaker) + Tall wheatgrass (Alkar) + alfalfa + Yellow sweetclover]
7	Switchgrass (Sunburst) + Tall wheatgrass (Alkar)
8	Switchgrass (Sunburst) + Big Bluestem (Sunnyview)
9	Switchgrass (Sunburst) + Altai Wildrye (Mustang)
10	Basin Wildrye (Magnar) + Altai Wildrye (Mustang)

Table 3. Species/mixtures of perennial grasses in ten entries used for biomass study across five locations in North Dakota (names in parenthesis are cultivars) [11].

3.2. Chemical composition

Chemical composition of biomass feedstock affects the efficiency of biofuel production and energy output. The major parts of the chemical composition in the perennial biomass feedstocks are lignocellulose including cellulose, hemicellulose, and lignin; and mineral elements such as ash [3, 36-38]. Biomass may be converted into energy by direct combustion or by producing liquid fuels (mainly ethanol) using different technologies. For converting

cellulosic biomass into ethanol, the conversion technologies generally fall into two major categories: biochemical and thermochemical [3, 37, 38]. Biochemical conversion refers to the fermentation of carbohydrates by breakdown of feedstocks. Thermochemical conversion includes the gasification and pyrolysis of biomass into synthetic gas or liquid oil for further fermentation or catalysis. Currently, the U.S. Environmental Protection Agency (USEPA) listed six conversion categories from different companies for ethanol from biomass [3]. Different conversion technologies may require different biomass quality attributes. For ethanol production from biochemical process (fermentation), ideal biomass composition would contain high concentrations of cellulose and hemicellulose but low concentration of lignin [37-38]. While for gasification-fermentation conversion technology, low lignin may not be necessary. For direct combustion and some thermochemical conversion processes, high ash content can reduce the effectiveness and chemical output [3, 37-38].

Entry	Hettinger	Williston-dryland	Williston-irrigated	Minot	Streeter	Carrington
	----------Mg/ha ----------					
1	0.0 c†	0.2 c	13.0 ab	5.2 cde	4.0 c	12.1 ab
2	0.0 c	0.7 bc	9.6 cd	2.9 e	4.3 c	13.7 a
3	3.4 a	2.2 a	11.2 bc	10.1 a	7.4 a	10.5 bcd
4	1.8 abc	2.7 a	9.2 cd	7.4 bc	6.0 b	10.1 cd
5	3.4 a	2.5 a	10.1 cd	9.4 ab	7.6 a	9.6 d
6	4.0 a	1.8 ab	8.7 d	8.5 ab	5.8 b	10.3 bcd
7	2.0 abc	2.2 a	12.8 ab	9.4 ab	8.3 a	11.4 bc
8	0.0 c	0.7 bc	11.2 bc	4.7 de	3.6 c	12.1 ab
9	0.0 c	0.7 bc	14.3 a	5.8 cd	3.6 c	11.4 bc
10	0.9 bc	0.7 bc	9.0 d	5.8 cd	3.4 c	9.0 d
Mean	**1.5**	**1.4**	**10.9**	**6.9**	**5.4**	**11.0**
LSD (0.05)	**2.5**	**1.3**	**2.0**	**2.2**	**1.8**	**1.1**

†In each column, values followed by the same letter were not significantly different based on LSD test at P=0.05.

Table 4. Biomass yields in ten entries with different species/mixtures of perennial grasses harvested in 2007 at five locations in North Dakota (the species/mixture for each entry is shown in Table 3) [11].

Among the perennial grasses for biofuel production, chemical composition of switchgrass has been investigated in many studies [19, 29-31, 35, 39]. There is little information in the lignocellulose contents in other species such as tall and intermediate wheatgrass when they are harvested at fall as biomass feedstocks because these species have been mainly used as forage. As with yield, biomass composition is affected by genetic and environmental factors as well as by management practices such as nitrogen (N) fertilization and harvest timing. In a study in the southern Iowa, both yield and quality traits were different among 20 switchgrass cultivars. The high yielding cultivars generally had low ash content [19]. In NGP, we reported the chemical composition of the above 10 perennial grasses and mixtures shown in Table 3 in 2007 harvest. The contents of neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL), hemicellulose (HCE), cellulose (CE) and

ash were determined. Biomass chemical composition was affected by environment and species/mixtures, and their interaction. Biomass under drier conditions had higher NDF, ADL and HCE contents but lower CE contents. Tall and intermediate wheatgrass had higher NDF, ADF and CE but lower ash contents than the other species and mixtures. Switchgrass and mixtures had higher HCE. Tall wheatgrass and Sunburst switchgrass had the lowest ADL content. Biomass with higher yield had higher cellulose content but lower ash content. Combining with higher yields, tall and intermediate wheatgrass and switchgrass had optimal chemical compositions for biomass feedstocks production (Table 5) [35]. In another study in NGP, Karki et al. (2011) showed that tall wheatgrass had similar composition to switchgrass and has potential for ethanol production [39].

Entry	NDF	ADF	ADL	HCE	CE	Ash
	---------- g/kg ----------					
1	733.4 bcd†	475.1 c	116.0 e	258.4 bcd	359.1 cd	79.2 ab
2	736.8 bcd	468.5 cd	139.1 bc	268.3 ab	329.4 f	81.2 a
3	792.6 a	535.2 a	116.3 e	257.4 bcd	418.9 a	68.8 de
4	753.5 b	507.1 b	154.5 a	246.4 d	352.6 de	71.3 cde
5	753.6 b	503.8 b	145.9 ab	249.8 cd	358.1 d	70.7 cde
6	745.5 bc	518.0 ab	140.4 bc	227.5 e	377.6 bc	69.5 cde
7	781.9 a	515.9 b	121.3 de	266.1 abc	394.6 b	64.3 e
8	736.8 bcd	459.9 cd	132.5 cd	276.9 a	327.4 f	73.9 bcd
9	723.7 cd	456.2 d	124.7 de	267.1 ab	331.5 f	74.8 abcd
10	715.4 d	461.9 cd	124.2 de	253.5 bcd	337.7 ef	76.3 abc
Mean	**747.3**	**490.2**	**131.5**	**257.1**	**358.7**	**73.0**
LSD (0.05)	**23.6**	**18.6**	**12.5**	**16.9**	**18.9**	**7.1**

†In each column, values followed by the same letter were not significantly different based on LSD test at P=0.05.

NDF: Neutral detergent fiber; ADF: Acid detergent fiber; ADL: Acid detergent lignin;

HCE: Hemicellulose (NDF-ADF); CE: Cellulose (ADF-ADL).

Table 5. Biomass compositional parameters in different species/mixtures averaged across six environments (the species/mixture for each entry is shown in Table 3) [35].

3.3. Mixture of multiple species

From a long-term sustainability perspective, the reliance on a single species of perennial crops (monoculture) for biomass production may be risky because of less diversity and more chance to prone to certain pests and diseases. Mixture of multiple species may overcome some problems encountered in monoculture crops. In terms of dedicated biofuel crops such as switchgrass and miscanthus, most previous and current studies are focused on

monoculture. Little information is known about the mixture of multiple species and their productivity as compared to monoculture.

In ecology studies, the benefits of mixtures of species over monocultures in terms of sustainability and biodiversity have been recognized in both annual and perennial species [6, 40, 41]. For biofuel purpose, specifically, Tilman et al. (2006) argued that the mixtures of different perennial grasses are more stable, more reliable and more productive than monoculture. Also, the mixtures are more environmentally friendly in terms of energy inputs and greenhouse gas emission. From agronomic standpoint, growing mixtures of multiple species in a large farm scale will face challenges such as selecting species, seeding methods, seeds costs, harvesting and so on. In addition, biomass feedstock quality will be an important factor when considering harvesting mixtures.

4. Management strategies for perennial biofuel crops

4.1. Establishment

Many perennial warm season grasses such as switchgrass are difficult to establish [17]. In an on-farm scale study, net energy value of switchgrass is largely determined by the biomass yield in established year [8]. Therefore, improving crop establishment is a very important step to successfully manage biofuel crops. There are many factors affecting establishment of perennial grasses; however, soil moisture and temperature are the most important ones, and many management practices are related to maintenance of adequate moisture and optimum temperature for seedling development and growth.

Seeding rate (pure live seeds): Typically recommended seeding rate in the US is 4-10 kg/ha for switchgrass based on the review of Parrish and Fike (2005) [17]. Sedivec et al. (2001) provided a detail recommendation for grass varieties for ND, ranging from 2 to 24 lb/ac, depending on species or varieties [42].

Seeding depth: The seeding depth may vary with soil types. However, seeding depth of grasses is generally shallower than cereal crops. For switchgrass in NE, seeding depth ranged from 1.5 cm to 3.0 cm in silt loam soil [43]. In SD, Nyoka et al. (2007) recommended not seeding deeper than 2.5 cm regardless of soil type [44].

Seeding date: Seeding date is largely determined by soil temperature and moisture. For warm season grasses, the ideal temperature for seed germination is between 20-30 oC if no dormancy [44, 46]. In SD, the recommended seeding date is early May to mid-June [44]. In VA, the planting date for switchgrass is much later than for corn but similar to that for millet or sorghum-sudangrass. In conventionally prepared seedbeds, June 1-15 was recommended [47]. In NE, study showed that planting switchgrass in mid-March can significantly increase seedling size as compared to late April and May [48]. Under NGP conditions, early seeding may provide benefit in terms of adequate soil moisture [48]. However, low soil temperature may be a factor for limiting germination and emergence of warm season grasses.

Timing an appropriate seeding date is also important for weed control. In a study conducted in Mississippi, Holmberg and Baldwin (2006) seeded switchgrass monthly from April to October and found that the months with minimum weed biomass were April and June. In addition, rainfall is also a very important factor for determining weed suppression for seeding switchgrass [49].

Seeding methods: switchgrass and other warm season grasses can be seeded under both conventional and no-till conditions. The ideal condition for conventional seeding should be a smooth, firm, clod-free soil for optimum seed placement with drills or culti-packer seeders [44]. The seedbed should be firm enough for good seed-soil contact and a consistent seeding depth [44, 47]. Since switchgrass requires warm weather for seeding, water loss during tillage could be a problem under dry and warm days. As a result, conventional seeding may not be ideal [47].

No-till helps to conserve soil moisture, requires less time and fuel, and eliminates the soil crusting frequently encountered in conventional seedbed [47]. In the literature, the results of comparison of conventional and no-till planting for warm season grass establishment are controversial. However, no-till planting frequently showed advantages over conventional tillage, in terms of soil and water conservation [17].

The warm season grasses can be seeded by drilling as well as broadcasting. For broadcasting method, cultipacking or rolling the seedbed after broadcasting is required to ensure that seeds are sufficiently covered by soil and to improve seed-to-soil contact [44].

Seed size (seed mass): Seed size varies considerably within cultivars as well as seedlots of a single cultivar [50]. In general, seed size is linearly related to seed mass or weight in many grasses and cereal crops. Large seeds normally have advantage over small seeds for germination and emergence [51], and seedling development [52]. Switchgrass seedlings grown from larger seeds developed adventitious roots more quickly than those from small seeds [52]. Even the seedling size associated to seed size was only evident at early stage [53], Vogel (2000) still suggested that selection of populations with larger seeds may improve seedling establishment in switchgrass [18].

Seedling vigor: Seedling establishment can be quantified by a more general term, seedling vigor. Greater seedling vigor refers to larger seedling size, greater ground cover and higher biomass at early stage. In addition to environmental factors, seedling vigor is believed to the single most important trait controlled by genetic variability in establishment capacity of perennial forage crops. Many researchers have used some measure of seedling vigor as a selection criterion to improve establishment capacity, while others have used more indirect measures, such as seed mass or germination rate [54]. As mentioned in the above, seed size is positively related to seeding vigor. However, other factors are also related to seedling vigor. For example, studies in cereal crops in Australia showed that embryo size significantly contributed to seedling vigor in barley [55]. In spring wheat, high protein content also contributed to seeding vigor.

Others: Application of arbuscular mycorrhizal fungi (AMF) has been shown to be effective for enhancing seedling yield and nutrient uptake in switchgrass [56-58]. Hanson and

Johnson (2005) showed that soil PH affected switchgrass germination and the optimum PH is 6.0 [46].

4.2. Weed control during establishment

Weed competition is often a major cause of establishment failure in grasses [16, 17, 44]. Although the weed species varies from region to region and even between nearby locations, perennial forbs and warm-season grass species provide the most severe competition for warm season crops like switchgrass [17].

Application of herbicides generally provides very effective weed control. In switchgrass and other warm season grasses, atrazine has been used almost universally as both pre- and post-emergence herbicides for improving establishment [17]. However, atrazine is only labeled for roadside and CRP lands in some states, not for large area of switchgrass except for a special use in Iowa [17]. Alternatively, switchgrass was companion-planted with corn or sorghum-sudangrass using atrazine [59-60].

There are several other chemicals showing to be effective for controlling weed during switchgrass establishment. For pre-emergence application, Mitchell and Britton (2000) [61] used metolachlor for control of several warm season annual grasses. Chlorsulfuron and metsulfuron showed some efficacy in switchgrass [62]. For post-emergence application, imazapyr, sulfometuron, quincloric, 2, 4-D and dicamba have been reported or recommend for weeds control in switchgrass and other warm season grasses [17, 44]. Non-selective herbicides (e.g, glyphosate and paraquat) have been used to prepare seedbeds for no-till plantings for establishing grasses. In addition, Buhler et al. (1998) listed a few more herbicides that showed potential to provide selective weed control to improve establishment of perennial warm season grasses [63].

Herbicides are generally effective and largely available in the market. However, many herbicides are not currently registered for perennial crops for biomass production [16, 63, 71]. As a result, weed control during the establishment year can not be solely relied on chemical applications. Other control methods must be adopted to achieve the best weed control. Buhler et al. (1998) reviewed weed management in biofuel crops and provided several non-chemical control options. These options include timing seeding date, tillage and cropping practices, using companion crops and clipping. Ultimately, the best weed management strategy will be the integration of various options [63].

The overall goal of non-chemical options for weed control is to create an environment that favors to crop growth and development but disfavors weeds. A typical example is manipulation of seeding date to minimize the weed competition, by changing the relative emergence of crop and weed. In general, if crops emerged earlier than weeds, they would have advantage to acquire resources. Therefore, seeding crops before the weeds emergence is an effective way to avoid weeds pressure.

Several other management practices have been successfully used to increase crop competitive ability. In western US, Canada and Australia, increasing seeding rate has been

an effective measure to suppress wild oat in barley and spring wheat [64-66]. Using large seeds also provided competitive benefit in sparing wheat against wild oat [67. 68]. Choosing cultivars with more competitive ability also provided benefit to weed control during establishment stage.

4.3. Nitrogen (N) management and N use efficiency

Like any other crops, optimizing biomass yield and maintaining quality stands require fertilizer inputs for biofuel crops. Currently, nitrogen (N) remains the primary fertilizer used in biofuel crops; therefore, most studies just consider the N application. Although some perennial species such as swithgrass and miscanthus are tolerant to low soil fertility conditions, studies showed that biomass yield responded to N application [16, 69]. Lemus et al. (2008) used 4 N rates (0, 56, 112 and 224 kg/ha) in switchgrass southern Iowa. They found that N application generally improved the biomass yield but the yield response declined as N level increased [19].

The amount of N fertilizer required for any biofuel crop is a function of several factors including yield potential of the site, cultivar, management practices, soil types, and so on. Therefore, the optimum N rate can vary from place to place. For example, a study in Texas using lowland switchgrass cultivar 'Alamo' determined that the optimum N rate was 168 kg/ha [70]. In another study in CRP land of NGP, however, the N rate of 56 kg/ha was optimum for upland switchgrass cultivars [30]. Gunderson et al. (2008) [71] summarized the response of biomass yield of upland switchgrass cultivars to N fertilizer rate. They showed that switchgrass yield even decreased as N rate was over 100 kg/ha (Figure 1). Among the management practices, perennial grass rotating with legume crops or mixture of grass and legumes may reduce N fertilizer inputs and improve their energy balance [71].

Some perennial grasses (e.g., switchgrass and miscanthus) can recycle N from the aboveground shoots to the crown, rhizome, and root in the fall for use in over-wintering and regrowth in the following spring [72]. This mechanism makes an efficient use and reuse of N by plant. However, there is still little information on when and how much of N recycles among plant organs, and how much the N cycling can contribute to over N balance in biofuel crops [7].

Another factor affecting crop N balance is fertilizer use efficiency and N use efficiency (NUE). Take switchgrass as an example, biomass yield varied considerably (up to 5 fold) at the same N application level (Figure 1). Certainly, N was not used efficiently at low yield level. Therefore, improving both fertilization use efficiency and NUE is very important for increasing biomass yield in biofuel crops. In addition, increased efficiency will ultimately reduce the N inputs.

4.4. Water management and Water Use Efficiency (WUE)

In NGP, soil water deficit occurs very frequently during crop growing season because of the highly variable and uneven distribution of seasonal precipitation. In general, biomass yield

of switchgrass increased as the amount of seasonal precipitation increased. However, at a given seasonal precipitation level (e.g., 500-600 mm), switchgrass yield ranged from 2 to 25 Mg/ha (Figure 2) [71], indicating the importance of crop WUE and precipitation use efficiency. Ideally, the figure 2 should be converted to the biomass yield as a function of seasonal evapotranspiration (ET) or transpiration (T), not precipitation because crop yield is more closely related to ET or T. Although most field studies have included precipitation information in NGP, there is no detailed information of crop ET, transpiration and water-use efficiency (WUE). The quantification of ET and WUE in biofuel crops under various environmental conditions and management practices will lead to identify the best management strategies. Because both water and N are critical for crop growth, the interaction of water and N becomes important, particularly under dryland conditions. However, there are very few studies on the interactive effects of N and soil moisture on biomass yield and quality in biofuel crops.

4.5. Harvest management

Proper harvest management is important for biofuel crops for high yields and ideal qualities. The harvest management practices include harvest frequency, timing and stubble height. Currently, most studies for harvest management are focused on switchgrass [29, 69, 73]. Although switchgrass can be harvested in 2 times a year in south part of USA [73, 74], swithcgrass in NGP can only be harvested once a year either after anthesis (summer) or killing frost (fall). For maximizing the biomass yields and chemical compositional attributes for biofuels, harvesting in killing frost is an ideal harvest management [29]. Another harvest practice in the NGP is harvesting every other year (biennial harvest). Comparing annual harvest and biennial harvest, average annual biomass yield is generally lower for biennial harvest. The only benefit for biennial harvest is reducing machine operation cost. However, biennial harvest improved the switchgrass stand health if harvested in summer [29]. The reduction of annual biomass yield in biennial harvest was related to species and mixtures in our long-term field study. The reduction in annual biomass yield due to biennial harvesting ranged between 20 to 50 percent. In general, biomass yield of intermediate wheatgrass reduced the most in biennial harvest. However, there was one dryland site that Sunburst switchgrass + Altai wildrye had higher yield on the biennial harvest [11, 75]. Cutting height during harvest also affect biomass yield in perennial grasses. In general, lower cutting stubble resulted in higher biomass yield than higher cutting [75].

4.6. The role of biofuel crops in cropping systems

Given emerging markets for biofuels and increasing production of biofuel crops, new and improved cropping systems are needed to maintain overall productivity as well as sustainability. Introducing perennial crops to the existing cropping systems will face challenges. Boehmel et al. (2008) [76] studied annual and perennial biofuel cropping systems in Germany. They compared 6 systems: short rotation willow coppice, miscanthus, switchgrass, energy corn and 2 annual crop rotation systems (oilseed rape, winter wheat and triticale). The results showed that perennial biomass systems based on *Miscanthus*,

switchgrass, or willows could be as productive as energy corn with lower energy inputs. Energy corn had the best energy yield performance but a relatively high energy input.

Anex et al. (2007) [77] proposed that the development of new biofuel crops and cropping systems, in conjunction with nutrient recycling between field and biorefinery, comprise a key strategy for the sustainable production of biofuels and other commodity chemicals derived from plant biomass. Such systems will allow N nutrient to be recovered and reduce fertilizer inputs.

Currently, little information is known how perennial crops interact with annual crops and their benefit in NGP. Perennials, however, are rarely permanent and some annual cropping or innovative combinations of annual and perennial biofuel crops strategically deployed across the farm landscape and combined into synergistic rotations may be necessary in the future. Combining annual biofuel crops such as corn and sorghum into rotations with perennial biofuel crops may benefit biofuel cropping systems [77].

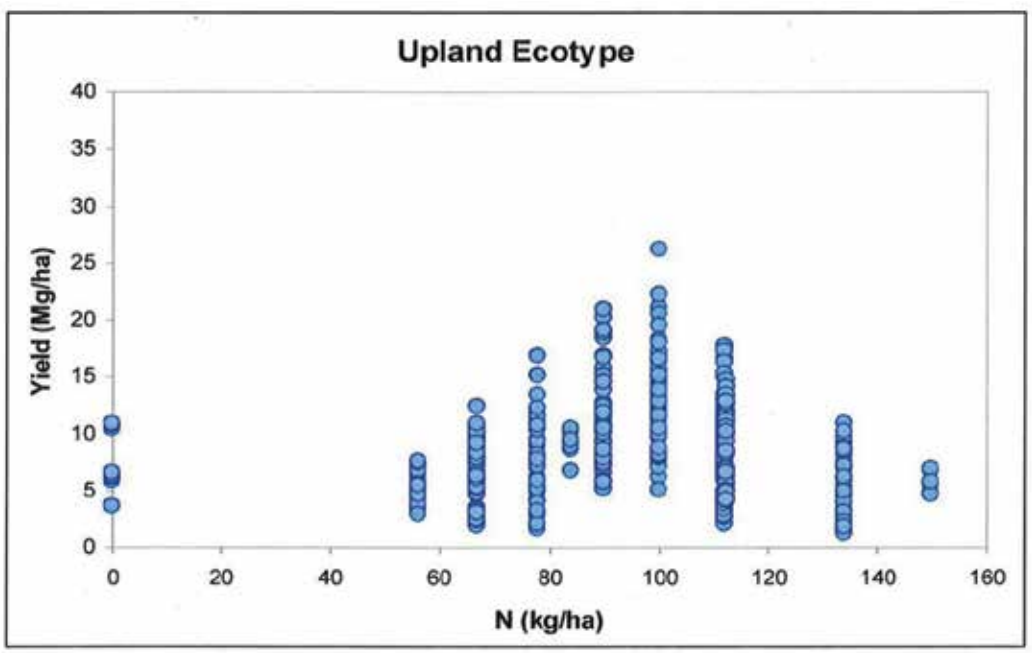

Figure 1. Biomass yield in upland switchgrass as a function of total nitrogen application during the growing season [71].

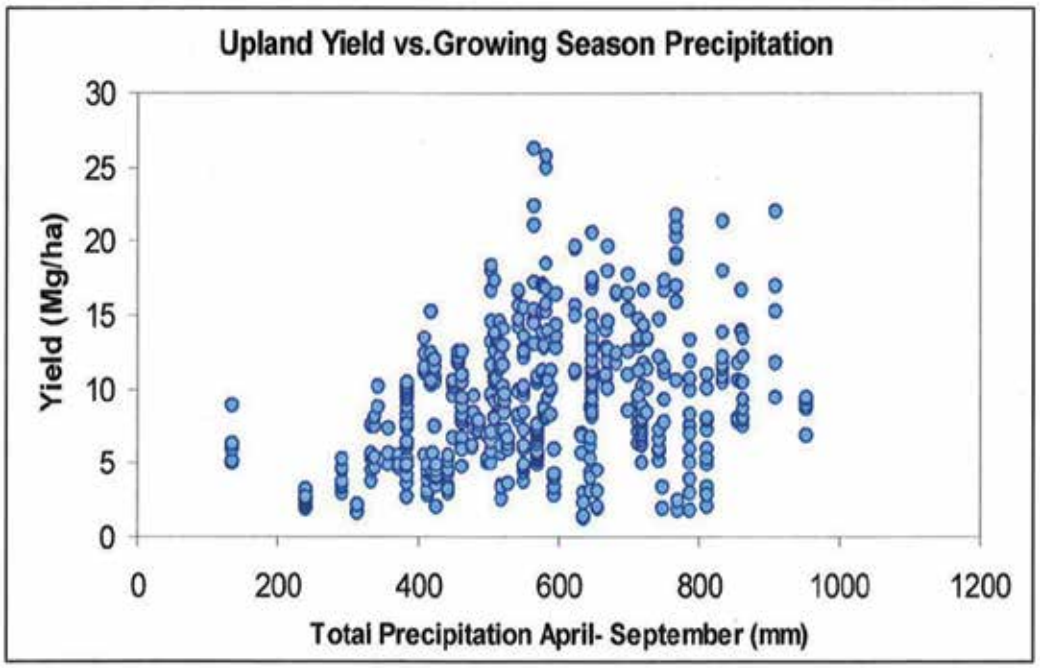

Figure 2. Biomass yield in upland switchgrass as a function of precipitation from April to September [71].

5. Ecological and environmental benefits of biofuel crops

Development of perennial biofuel crops may provide long-term sustainability on these lands by reducing soil erosion, increasing soil organic matter, reducing greenhouse gases and enhancing carbon sequestration [35]. Studies have shown that perennial crops provided many ecological and environmental benefits. Switchgrass and other warm season grasses can be used to control soil erosion, reduce runoff losses of soil nutrients, improve water quality (facilitate the breakdown or removal of soil contaminants), diversify wild life habitats and so on [17, 44]. Roth et al. (2005) [78] showed that proper managing switchgrass harvest can significantly increase grassland birds diversity. More importantly, perennial crops such as switchgrass have been shown to increase carbon sequestration and improve soil quality [9].

The environmental benefits for producing biofuel crops include high energy efficiency and reducing greenhouse gas (GHG) emission. Schmer et al. (2008) [8] evaluated the net energy efficiency and economic feasibility of switchgrass and similar crops in North and Central Great Plains. Switchgrass produced 540% more renewable than nonrenewable energy consumed. Switchgrass monocultures managed for high yield produced 93% more biomass yield and an equivalent estimated NEY than previous estimates from human-made prairies that received low agricultural inputs. Estimated average GHG emissions from cellulosic ethanol derived from switchgrass were 94% lower than estimated GHG from gasoline.

6. Future perspectives for biomass production in the northern great plains

The Northern Great Plains has over 4 million hectares of highly erodible and saline crop land. Development of perennial biofuel crops may provide long-term sustainability on these lands by reducing soil erosion, increasing soil organic matter, reducing greenhouse gases and enhancing carbon sequestration. Although studies are on-going in long-term field experiments, the best management practices are still needed to be developed for producers. The long-term ecological and environmental benefits are also needed to be quantified in the area.

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Chapter 5

Design of a Cascade Observer for a Model of Bacterial Batch Culture with Nutrient Recycling

Miled El Hajji and Alain Rapaport

Additional information is available at the end of the chapter

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1. Introduction

Microbial growths and their use for environmental purposes, such as bio-degradations, are widely studied in the industry and research centres. Several models of microbial growth and bio-degradation kinetic have been proposed and analysed in the literature. The Monod's model is one of the most popular ones that describes the dynamics of the growth of a biomass of concentration X on a single substrate of concentration S in batch culture [15, 18]:

$$\dot{S} = -\frac{\mu(S)}{Y}\, X, \quad \dot{X} = \mu(S)\, X\,. \tag{1}$$

where the specific growth rate $\mu(\cdot)$ is:

$$\mu(S) = \mu_{max}\, \frac{S}{K_s + S}, \tag{2}$$

with μ_{max}, K_s and $Y$are repsectively the maximum specific growth rate, the affinity constant and the yield coefficient. Other models take explicitly into account a lag-phase before the growth, such as the Baranyi's [1–3] or the Buchanam's [6] ones. These models are well suited for the growth phase (i.e. as long as a substantial amount of substrate remains to be converted) but not after [18], because the accumulation of dead or non-viable cells is not taken into account. Part of the non-viable cells release substrate molecules, in quantities that are no longer negligible when most of the initial supply has been consumed. The on-line observation of the optical density of the biomass provides measurements of the total biomass, but not of the proportion among dead and viable cells. Some tools allow the distinction between viable and dead cells but do not detect non-viable non-dead ones [22].

In this work, we consider an extension of the model (1) considering both the accumulation of dead cells and the recycling of part of it into substrate, and tackle the question of parameters

and state reconstruction. To our knowledge, this kind of question has not been thoroughly studied in the literature. Models of continuous culture with nutrient recycling have already been studied [4, 5, 9, 12–14, 16, 20, 21, 24, 25] but surprisingly few works considers batch cultures. A possible explanation comes from the fact that only the first stage of the growth, for which cell mortality and nutrient recycling can be neglected, is interested for industrial applications. Nevertheless, in natural environment such as in soils, modelling the growth end is also important, especially for biological decontamination and soil bioremediation.

Moreover, we face a model for which the parameters are not identifiable at steady state. Then, one cannot apply straightforwardly the classical estimation techniques, that usually requires the global observability of the system. Estimation of parameters in growth models, such as the Baranyi's one, are already known to be difficult to tackle in their differential form [11]. In addition, we aim here at reconstructing on-line unmeasured state variables (amounts of viable and non-viable cells), as well as parameters. For this purpose, we propose the coupling of two non-linear observers in cascade with different time scales, providing a practical convergence of the estimation error. Design of cascade observers in biotechnology can be found for instance in [17, 23], but with the same time scale.

2. Derivation of the model

We first consider a mortality rate in the model (1):

$$\dot{X} = \mu(S)X - mX$$

where parameter $m > 0$ becomes not negligible when $\mu(S)$ takes small values. In addition, we consider an additional compartment X_d that represents the accumulation of dead cells:

$$\dot{X}_d = \delta m X,$$

where the parameter $\delta \in (0, 1)$ describes the part of non-viable cells that are not burst. We assume that the burst cells recycle part of the substrate that has been assimilated but not yet transformed. Then, the dynamics of the substrate concentration can be modified as follows:

$$\dot{S} = -\frac{\mu(S)}{Y}X + \lambda(1-\delta)mX,$$

where $\lambda > 0$ is recycling conversion factor. It appears reasonable to assume that the factor λ is smaller that the growth one:

Assumption A1. $\dfrac{1}{Y} > \lambda$.

In the following we assume that the growth function $\mu(\cdot)$ and the yield coefficient Y of the classical Monod's model are already known. Typically, they can be identified by measuring the initial growth slope on a series of experiments with viable biomass and different initial concentrations, mortality being considered to be negligible during the exponential growth. We aim at identifying the three parameters m, δ and λ, and on-line reconstructing the variables

X and X_d, based on on-line observations of the substrate concentration S and the total biomass $B = X + X_d$.

Without any loss of generality, we shall assume that the growth function $\mu(\cdot)$ can be any function satisfying the following hypotheses.

Assumption A2. The function $\mu(\cdot)$ is a smooth increasing function with $\mu(0) = 0$.

For sake of simplicity, we normalise several quantities, defining

$$s = S,\ x = X/Y,\ x_d = X_d/Y,\ a = (1-\delta)m \text{ and } k = \lambda Y\,.$$

Then, our model can be simply written as

$$\begin{cases} \dot{s} &= -\mu(s)x + kax, \\ \dot{x} &= \mu(s)x - mx, \\ \dot{x}_d &= mx - ax, \end{cases} \tag{3}$$

along with the observation vector $y = \begin{pmatrix} s \\ x + x_d \end{pmatrix}$. Typically, we consider known initial conditions such that

$$s(0) = s_0 > 0, \quad x_d(0) = 0 \quad \text{and} \quad x(0) = x_0 > 0\,.$$

Our purpose is to reconstruct parameters m, a and k and state variable $x(\cdot)$ or $x_d(\cdot)$, under the constraints $m > a$ and $k < 1$, that are direct consequences of the definition of a and Assumption A1. Moreover, we shall assume that a priori bounds on the parameters are known i.e.

$$(m, a, k) \in [m^-, m^+] \times [a^-, a^+] \times [k^-, k^+]\,. \tag{4}$$

3. Properties of the model

Proposition 1. *The dynamics (3) leaves invariant the 3D-space $\mathcal{D} = \mathbb{R}^3_+$ and the set*

$$\Omega = \left\{ (s, x, x_d) \in \mathcal{D} \mid s + x + \frac{(m - k\,a)}{(m-a)}\, x_d = s_0 + x_0 \right\}\,.$$

Proof. The invariance of $\mathbb{R}^3_+$ is guaranteed by the following properties:

$$\begin{aligned} s = 0 \quad &\Rightarrow \quad \dot{s} = k\,a\,x \geq 0, \\ x = 0 \quad &\Rightarrow \quad \dot{x} = 0, \\ x_d = 0 \quad &\Rightarrow \quad \dot{x}_d = (m-a)\,x \geq 0. \end{aligned}$$

Consider the quantity $M = s + x + \dfrac{(m - k\,a)}{(m-a)} x_d$. One can easily check from equations (3) that one has $\dot{M} = 0$, leading to the invariance of the set Ω. ∎

Let $\bar{s}$ be the number $\bar{s} = \mu^{-1}(m)$ or $+\infty$.

Proposition 2. *The trajectories of dynamics (3) converge asymptotically toward an equilibrium point*

$$E^\star = \left(s^\star, 0, \frac{m-a}{m-ka}(s_0 + x_0 - s^\star) \right)$$

with $s^\star \leq \min(s_0 + x_0, \bar{s})$.

Proof. The invariance of the set Ω given in Proposition 1 shows that all the state variables remain bounded. From equation $\dot{x}_d = (m-a)x$ with $m > a$, and the fact that x_d is bounded, one deduces that $x(\cdot)$ has to converge toward 0, and $x_d(\cdot)$ is non increasing and converges toward $x_d^\star$ such that $x_d^\star \in [0, (s_0 + x_0)(m-a)/(m-ka)]$. Then, from the invariant defined by the set Ω, $s(\cdot)$ has also to converges to some $s^\star \leq s_0 + x_0$. If $s^\star$ is such that $s^\star > \bar{s}$, then from equation $\dot{x} = (\mu(s) - m)x$, one immediately see that $x(\cdot)$ cannot converge toward 0. ∎

4. Observability of the model

We recall that our aim is to estimate on-line both parameters and unmeasured variables x, x_d, based on the measurements. One can immediately see from equations (3) that parameters (m, a, k) cannot be reconstructed observing the system at steady state. Nevertheless, considering the derivative μ' of μ with respect to s and deriving the outputs:

$$\begin{cases} \dot{y}_1 = (-\mu(y_1) + k\,a)\,x, \\ \dot{y}_2 = (\mu(y_1) - a)\,x, \\ \ddot{y}_1 = (\mu(y_1) - m)\,\dot{y}_1 - \mu'(y_1)\,x\,\dot{y}_1, \\ \ddot{y}_2 = (\mu(y_1) - m)\,\dot{y}_2 + \mu'(y_1)\,x\,\dot{y}_1., \end{cases}$$

one obtains explicit expression of the parameters and unmeasured state variable as functions of the outputs and its derivatives, away from steady state:

$$\begin{cases} m &= \mu(y_1) - \dfrac{\ddot{y}_1 + \ddot{y}_2}{\dot{y}_1 + \dot{y}_2}, \\ x &= \dfrac{\ddot{y}_2 - (\mu(y_1) - m)\dot{y}_2}{\mu'(y_1)\dot{y}_1}, \\ x_d &= y_2 - x, \\ a &= \mu(y_1) - \dfrac{\dot{y}_2}{x}, \\ k &= \dfrac{\mu(y_1)}{a} + \dfrac{\dot{y}_1}{a\,x}, \end{cases} \tag{5}$$

from which one deduces the observability of the system.

5. Design of a practical observer

Playing with the structure of the dynamics, we are able to write the model as a particular cascade of two sub-models. We first present a practical observer for the reconstruction of the parameters a and k using the observation y_1 only, but with a change of time that depends on y_1 and y_2. We then present a second observer for the reconstruction of the parameter m

and the state variables x and x_d, using both observations y_1 and y_2 and the knowledge of the parameters a and k. Finally, we consider the coupling of the two observers, the second one using the estimations of a and k provided by the first one. More precisely, our model is of the form

$$\dot{Z} = F(Z,P) \quad , \quad y = H(Z)$$

where F is our vector field with the state, parameters and observation vectors Z, P and y of dimension respectively 3, 3 and 2. We found a partition

$$Z = \begin{pmatrix} Z_1 \\ Z_2 \end{pmatrix}, P = \begin{pmatrix} P_1 \\ P_2 \end{pmatrix} \text{ s.t. } \begin{cases} \dim Z_1 = 1, \dim P_1 = 2 \\ \dim Z_2 = 2, \dim P_2 = 1 \end{cases}$$

$$y = \begin{pmatrix} y_1 \\ y_2 \end{pmatrix} = \begin{pmatrix} H_1(Z_1) \\ H_2(Z_2) \end{pmatrix}$$

and the dynamics is decoupled as follows

$$\dot{Z}_1 = \frac{1}{\frac{d\phi(y)}{dt}} F_1(Z_1, P_1)$$
$$\dot{Z}_2 = F_2(Z_2, y_1, P_1, P_2)$$

with $d\phi(y)/dt > 0$. Moreover, the following characteristics are fulfilled:

i. (Z_1, P_1) is observable for the dynamics (F_1, H_1) i.e. without the term $d\phi(y)/dt$,

ii. (Z_2, P_2) is observable for the dynamics (F_2, H_2) when P_1 is known. Then, the consideration of two observers $\hat{F}_1(\cdot)$ and $\hat{F}_2(P_1, \cdot)$ for the pairs (Z_1, P_1) and (Z_2, P_2) respectively, leads to the construction of a cascade observer

$$\frac{d}{d\tau} \begin{pmatrix} \hat{Z}_1 \\ \hat{P}_1 \end{pmatrix} = \hat{F}_1(\hat{Z}_1, \hat{P}_1, y_1),$$
$$\frac{d}{dt} \begin{pmatrix} \hat{Z}_2 \\ \hat{P}_2 \end{pmatrix} = \hat{F}_2(\hat{P}_1, \hat{Z}_2, \hat{P}_2, y_2)$$

with $\tau(t) = \phi(y(t)) - \phi(y(0))$, that we make explicit below. Notice that the coupling of two observers is made by $\hat{P}_1$, and that the term $d\phi(y)/dt$ prevents to have an asymptotic convergence when $\lim_{t \to +\infty} \tau(t) < +\infty$.

Definition 1. *An estimator $\hat{Z}_\gamma(\cdot)$ of a vector $Z(\cdot)$, where $\gamma \in \Gamma$ is a parameter, is said to have a* practical exponential convergence *if there exists positive constants K_1, K_2 such that for any $\epsilon > 0$ and $\theta > 0$, the inequality*

$$||\hat{Z}_\gamma(t) - Z(t)|| \leq \epsilon + K_1 e^{-K_2 \theta t}, \quad \forall t \geq 0$$

is fulfilled for some $\gamma \in \Gamma$.

In the following we shall denote by $\text{sat}(l, u, \iota)$ the saturation operator $\max(l, \min(u, \iota))$.

5.1. A first practical observer for k and a

Let us consider the new variable

$$\tau(t) = y_1(0) - y_1(t) + y_2(0) - y_2(t)$$

that is measured on-line. From Proposition 1, one deduces that $\tau(\cdot)$ is bounded. One can also easily check the property

$$\frac{d\tau}{dt} = (1-k)\, a\, x(t) > 0\,, \quad \forall t \geq 0\,.$$

Consequently, $\tau(\cdot)$ is an increasing function up to

$$\bar{\tau} = \lim_{t \to +\infty} \tau(t) < +\infty \tag{6}$$

and $\tau(\cdot)$ defines a diffeomorphism from $[0, +\infty)$ to $[0, \bar{\tau})$. Then, one can check that the dynamics of the variable s in time τ is decoupled from the dynamics of the other state variables:

$$\frac{ds}{d\tau} = \alpha - \beta\mu(s)$$

where α and β are parameters defined as combinations of the unknown parameters a and k:

$$\alpha = \frac{k}{1-k}\,,$$
$$\beta = \frac{1}{a(1-k)}$$

and from (4) one has $(\alpha, \beta) \in [\alpha^-, \alpha^+] \times [\beta^-, \beta^+]$. For the identification of the parameters α, β, we propose below to build an observer. Other techniques, such as least squares methods, could have been chosen. An observer presents the advantage of exhibiting a innovation vector that gives a real-time information on the convergence of the estimation.

Considering the state vector $\xi = \begin{bmatrix} s & \dfrac{ds}{d\tau} & \dfrac{d^2s}{d\tau^2} \end{bmatrix}^T$, one obtains the dynamics

$$\frac{d\xi}{d\tau} = A\xi + \begin{pmatrix} 0 \\ 0 \\ \varphi(y_1, \xi) \end{pmatrix} \text{ with } y_1 = C\xi\,,$$

$$\varphi(y_1, \xi) = \frac{\xi_3^2}{\xi_2} + \xi_2\xi_3 \frac{\mu''(y_1)}{\mu'(y_1)}\,,$$

and the pair (A, C) in the Brunovsky's canonical form:

$$A = \begin{pmatrix} 0 & 1 & 0 \\ 0 & 0 & 1 \\ 0 & 0 & 0 \end{pmatrix} \text{ and } C = \begin{pmatrix} 1 & 0 & 0 \end{pmatrix}\,. \tag{7}$$

The unknown parameters α and β can then be made explicit as functions of the observation y_1 and the state vector ξ:

$$\alpha = l_\alpha(y_1, \xi) = \xi_2 - \frac{\xi_3 \mu(y_1)}{\xi_2 \mu'(y_1)},$$
$$\beta = l_\beta(y_1, \xi) = -\frac{\xi_3}{\xi_2 \mu'(y_1)}\,.$$

One can notice that functions $\varphi(y_1, \cdot)$, $l_\alpha(y_1, \cdot)$ and $l_\beta(y_1, \cdot)$ are not well defined on $\mathbb{R}^3$, but along the trajectories of (3) one has $\xi_3/\xi_2 = -\beta\mu'(y_1)$ and $\xi_2 = \alpha - \beta\mu(y_1)$, that are bounded. Moreover Assumption A2 guarantees that $\mu'(y_1)$ is always strictly positive . We can consider (globally) Lipschitz extensions of these functions away from the trajectories of the system, as follows:

$$\begin{aligned}
\tilde{\varphi}(y_1, \xi) &= \xi_3 \left(h_1(y_1, \xi) + \frac{\mu''(y_1)}{\mu'(y_1)} h_2(y_1, \xi) \right), \\
\tilde{l}_\alpha(y_1, \xi) &= \xi_2 - h_1(y_1, \xi) \frac{\mu(y_1)}{\mu'(y_1)}, \\
\tilde{l}_\beta(y_1, \xi) &= -\frac{h_1(y_1, \xi)}{\mu'(y_1)}
\end{aligned}$$

with

$$\begin{aligned}
h_1(y_1, \xi) &= \text{sat}\left(-\beta^+ \mu'(y_1), -\beta^- \mu'(y_1), \frac{\xi_3}{\xi_2} \right), \\
h_2(y_1, \xi) &= \text{sat}\left(\alpha^- - \beta^+ \mu(y_1), \alpha^+ - \beta^- \mu(y_1), \xi_2 \right).
\end{aligned}$$

Then one obtains a construction of a practical observer.

Proposition 3. *There exist numbers $b_1 > 0$ and $c_1 > 0$ such that the observer*

$$\begin{aligned}
\frac{d\hat{\xi}}{d\tau} &= A\hat{\xi} + \begin{pmatrix} 0 \\ 0 \\ \tilde{\varphi}(y_1, \hat{\xi}) \end{pmatrix} - \begin{pmatrix} 3\theta_1 \\ 3\theta_1^2 \\ \theta_1^3 \end{pmatrix} (\hat{\xi}_1 - y_1) \\
(\hat{\alpha}, \hat{\beta}) &= \left(\tilde{l}_\alpha(y_1, \hat{\xi}), \tilde{l}_\beta(y_1, \hat{\xi}) \right)
\end{aligned} \tag{8}$$

guarantees the convergence

$$\max\left(|\hat{\alpha}(\tau) - \alpha|, |\hat{\beta}(\tau) - \beta| \right) \leq b_1 e^{-c_1 \theta_1 \tau} ||\hat{\xi}(0) - \xi(0)|| \tag{9}$$

for any θ_1 large enough and $\tau \in [0, \bar{\tau})$.

Proof. Consider a trajectory of dynamics (3) and let $O_1 = \{y_1(t)\}_{t \geq 0}$. From Proposition 1, one knows that the set O_1 is bounded.

Define $K_{\theta_1} = -\left(3\theta_1 \;\; 3\theta_1^2 \;\; \theta_1^3 \right)^T$. One can check that $K_{\theta_1} = -P_{\theta_1}^{-1} C^T$, where P_{θ_1} is solution of the algebraic equation

$$\theta_1 P_{\theta_1} + A^T P_{\theta_1} + P_{\theta_1} A = C^T C.$$

Consider then the error vector $e = \hat{\xi} - \xi$. One has

$$\frac{de}{d\tau} = (A + K_{\theta_1} C)e + \begin{pmatrix} 0 \\ 0 \\ \tilde{\varphi}(y_1, \hat{\xi}) - \tilde{\varphi}(y_1, \xi) \end{pmatrix}$$

where $\tilde{\varphi}(y_1, \cdot)$ is (globally) Lipschitz on $\mathbb{R}^3$ uniformly in $y_1 \in O_1$. We then use the result in [10] that provides the existence of numbers $c_1 > 0$ and $q_1 > 0$ such that $||e(\tau)|| \leq q_1 e^{-c_1 \theta_1 \tau} ||e(0)||$ for θ_1 large enough. Finally, functions $\tilde{l}_\alpha(y_1, \cdot)$, $\tilde{l}_\beta(y_1, \cdot)$ being also (globally) Lipschitz on $\mathbb{R}^3$ uniformly in $y_1 \in O_1$, one obtains the inequality (9). ∎

Corollary 1. *Estimation of a and k with the same convergence properties than (9) are given by*

$$\hat{k}(\tau), \hat{a}(\tau) = sat\left(k^-, k^+, \frac{\hat{\alpha}(\tau)}{1 + \hat{\alpha}(\tau)}\right), sat\left(a^-, a^+, \frac{1 + \hat{\alpha}(\tau)}{\hat{\beta}(\tau)}\right)$$

Remark. The observer (8) provides only a practical convergence since $\tau(t)$ does not tend toward $+\infty$ when the time t get arbitrary large. For large values of initial x, it may happens that $\mu(t) > t$ for some times $t > 0$. Because the present observer requires the observation y_1 until time τ, it has to be integrated up to time $\min(\tau(t), t)$ when the current time is t.

5.2. A second observer for m and x

We come back in time t and consider the measured variable $z = y_1 + y_2$. When the parameters α and β are known, the dynamics of the vector $\zeta = \begin{bmatrix} z & \dot{z} & \ddot{z} \end{bmatrix}^T$ can be written as follows:

$$\dot{\zeta} = A\zeta + \begin{pmatrix} 0 \\ 0 \\ \psi(y_1, \zeta, \alpha, \beta) \end{pmatrix} \text{ with } z = C\zeta$$

$$\text{and } \psi(y_1, \zeta, \alpha, \beta) = \frac{\zeta_3^2}{\zeta_2} + \zeta_2^2 \mu'(y_1)(\beta\mu(y_1) - \alpha)$$

Parameter m and variable $x(\cdot)$ can then be made explicit as functions of y_1 and ζ:

$$m = l_m(y_1, \zeta) = \mu(y_1) - \frac{\zeta_3}{\zeta_2}, \; x = -\beta\zeta_2$$

Functions $\psi(y_1, \cdot, \alpha, \beta)$ and $l_m(y_1, \cdot)$ are not well defined in $\mathbb{R}^3$ but along the trajectories of the dynamics (3), one has $\zeta_3/\zeta_2 = \mu(y_1) - m$ and $\zeta_2 = -x/\beta$ that are bounded. These functions can be extended as (globally) Lipschitz functions w.r.t. ζ:

$$\begin{aligned} \tilde{\psi}(y_1, \zeta, \alpha, \beta) &= h_3(y_1, \zeta)\zeta_3 + \min(\zeta_2^2, z(0)^2/\beta^2)\mu'(y_1)(\beta\mu(y_1) - \alpha) \\ \tilde{l}_m(y_1, \zeta) &= \mu(y_1) - h_3(y_1, \zeta) \end{aligned} \tag{10}$$

with

$$h_3(y_1,\zeta) = \text{sat}\left(\mu(y_1) - m^+, \mu(y_1) - m^-, \frac{\zeta_3}{\zeta_2}\right) .$$

Proposition 4. *When α and β are known, there exists numbers $b_2 > 0$ and $c_2 > 0$ such that the observer*

$$\frac{d}{dt}\hat{\zeta} = A\hat{\zeta} + \begin{pmatrix} 0 \\ 0 \\ \tilde{\psi}(y_1,\hat{\zeta},\alpha,\beta) \end{pmatrix} - \begin{pmatrix} 3\theta_2 \\ 3\theta_2^2 \\ \theta_2^3 \end{pmatrix} (\hat{\zeta}_1 - y_1 - y_2) \tag{11}$$

$$(\hat{m},\hat{x}) = \left(\tilde{I}_m(y_1,\hat{\zeta}), -\beta\hat{\zeta}_2\right)$$

guarantees the exponential convergence

$$\max\left(|\hat{m}(t) - m|, |\hat{x}(t) - x(t)|\right) \leq b_2 e^{-c_2\theta_2 t}||\hat{\zeta}_2(0) - \zeta_2(0)||$$

for any θ_2 large enough and $t \geq 0$.

Proof. As for the proof of Proposition 3, it is a straightforward application of the result given in [10]. ∎

5.3. Coupling the two observers

We consider now the coupling of observer (11) with the estimation $(\hat{\alpha},\hat{\beta})$ provided by observer (8). This amounts to study the robustness of the second observer with respect to uncertainties of parameters α and β.

Proposition 5. *Consider the observer (11) with (α,β) replaced by $(\tilde{\alpha}(\cdot),\tilde{\beta}(\cdot))$ such that*

$$(\tilde{\alpha}(t),\tilde{\beta}(t)) \in [\alpha^-,\alpha^+] \times [\beta^-,\beta^+], \quad \forall t \geq 0 ,$$

then there exists positive numbers $\bar{b}_2$, $\bar{c}_2$, $\bar{d}_2$ such that for any $\epsilon > 0$ there exists θ_2 large enough to guarantee the inequalities

$$|\hat{m}(t) - m| \leq \epsilon + \bar{b}_2 e^{-\bar{c}_2 t}||\hat{\zeta}(0) - \zeta(0)|| \tag{12}$$

$$|\hat{x}(t) - x(t)| \leq \epsilon + \bar{d}_2|\tilde{\beta}(t) - \beta| + \bar{b}_2 e^{-\bar{c}_2 t}||\hat{\zeta}(0) - \zeta(0)|| \tag{13}$$

for any $t \geq 0$.

Proof. As for the proof of Proposition 3, we fix an initial condition of system (3) and consider the bounded set $O_1 = \{y_1(t)\}_{t\geq 0}$. The dynamics of $e = \hat{\zeta} - \zeta$ is

$$\dot{e} = (A + K_{\theta_2}C)e + (\tilde{\psi}(y_1,\hat{\zeta},\tilde{\alpha},\tilde{\beta}) - \tilde{\psi}(y_1,\zeta,\alpha,\beta))v$$

where (A, C) in the Brunovsky's form (7), $v = \begin{pmatrix} 0 & 0 & 1 \end{pmatrix}^T$ and $K_{\theta_2} = -P_{\theta_2}^{-1} C^T$ with

$$P_{\theta_2} = \begin{pmatrix} \theta_2^{-1} & -\theta_2^{-2} & \theta_2^{-3} \\ -\theta_2^{-2} & 2\theta_2^{-3} & -3\theta_2^{-4} \\ \theta_2^{-3} & -3\theta_2^{-4} & 6\theta_2^{-5} \end{pmatrix} \tag{14}$$

solution of the algebraic equation

$$\theta_2 P_{\theta_2} + A^T P_{\theta_2} + P_{\theta_2} A = C^T C. \tag{15}$$

Consider then $V(t) = ||e(t)||^2_{P_{\theta_2}} = e^T(t) P_{\theta_2} e(t)$. Using (15), one has

$$\begin{aligned} \dot{V} &= -\theta_2 e^T P_{\theta_2} e - e^T C^T C e + 2\delta e^T P_{\theta_2} v \\ &\leq -\theta_2 ||e||^2_{P_{\theta_2}} + 2\delta ||e||_{P_{\theta_2}} ||v||_{P_{\theta_2}} \end{aligned} \tag{16}$$

where $\delta = |\tilde{\psi}(y_1, \hat{\zeta}, \tilde{\alpha}, \tilde{\beta}) - \tilde{\psi}(y_1, \zeta, \alpha, \beta)|$.

One can easily compute from (14) $||v||_{P_{\theta_2}} = \sqrt{6}\theta^{-5/2}$.

From the expression (10) and the (globally) Lipschitz property of the map $\zeta \mapsto \tilde{\psi}(y_1, \zeta, \alpha, \beta)$ uniformly in $y_1 \in O_1$, we deduce the existence of two positive numbers c and L such that

$$\begin{aligned} \delta &\leq |\tilde{\psi}(y_1, \hat{\zeta}, \tilde{\alpha}, \tilde{\beta}) - \tilde{\psi}(y_1, \hat{\zeta}, \alpha, \beta)| + |\tilde{\psi}(y_1, \hat{\zeta}, \alpha, \beta) - \tilde{\psi}(y_1, \zeta, \alpha, \beta)| \\ &\leq |\tilde{\psi}(y_1, \hat{\zeta}, \alpha^-, \beta^+) - \tilde{\psi}(y_1, \hat{\zeta}, \alpha^+, \beta^-)| + L||e|| \\ &\leq c + L||e|| \end{aligned} \tag{17}$$

Notice that one has $||e||_{P_{\theta_2}} = \theta_2 ||\tilde{e}||_{P_1}$ with $\tilde{e}_i = \theta_2^{-i} e_i$ and $||\tilde{e}||^2 \geq \theta_2^{-6} ||e||^2$ for any $\theta_2 \geq 1$. The norms $||\cdot||_{P_1}$ and $||\cdot||$ being equivalent, there exists a numbers $\eta > 0$ such that $||\tilde{e}||_{P_1}|| \geq \eta ||\tilde{e}||$, and we deduce the inequality

$$||e||_{P_{\theta_2}} \geq \eta \theta_2^{-5/2} ||e|| \,. \tag{18}$$

Finally, gathering (16), (17) and (18), one can write

$$\frac{d}{dt} ||e||_{P_{\theta_2}} \leq \left(-\frac{\theta_2}{2} + \frac{\sqrt{6}L}{\eta} \right) ||e||_{P_{\theta_2}} + \sqrt{6}\theta_2^{-5/2} c$$

For θ_2 large enough, one has $-\theta_2/2 + \sqrt{6}L/\eta < 0$ and then, using again (18), obtains

$$\frac{d}{dt} ||e|| \leq \left(-\frac{\theta_2}{2} + \frac{\sqrt{6}L}{\eta} \right) ||e|| + \frac{\sqrt{6}}{\eta} c$$

from which we deduce the exponential convergence of the error vector e toward any arbitrary small neighbourhood of 0 provided that θ_2 is large enough.

The Lipschitz continuity of the map $l_m(\cdot)$ w.r.t. ζ uniformly in $y_1 \in O_1$ provides the inequality (12).

For the estimation of $x(\cdot)$, one has the inequality

$$|\hat{x} - x| = |\hat{\beta}\hat{\zeta}_2 - \beta\zeta_2| \leq |\hat{\beta} - \beta||\zeta_2| + \beta^+|\hat{\zeta}_2 - \zeta_2|$$

provided the estimation (13), the variable ζ_2 being bounded. ∎

Corollary 2. *At any time $t > 0$, the coupled observer*

$$\frac{d\hat{\xi}}{ds_1} = A\hat{\xi} + \begin{pmatrix} 0 \\ 0 \\ \tilde{\varphi}(y_1, \hat{\xi}) \end{pmatrix} - \begin{pmatrix} 3\theta_1 \\ 3\theta_1^2 \\ \theta_1^3 \end{pmatrix} (\hat{\xi}_1 - y_1)$$

$$\frac{d\hat{\zeta}}{ds_2} = A\hat{\zeta} + \begin{pmatrix} 0 \\ 0 \\ \tilde{\psi}(y_1, \hat{\zeta}, \hat{\alpha}(s_2), \hat{\beta}(s_2)) \end{pmatrix} - \begin{pmatrix} 3\theta_2 \\ 3\theta_2^2 \\ \theta_2^3 \end{pmatrix} (\hat{\zeta}_1 - y_1 - y_2)$$

integrated for $s_1 \in [0, \min(t, \tau(t))]$ and $s_2 \in [0, t]$, with

$$\begin{aligned} &\tau(t) = y_1(0) - y_1(t) + y_2(0) - y_2(t), \\ &\hat{\alpha}(s_2) = sat(\alpha^-, \alpha^+, \tilde{I}_\alpha(y_1(\min(s_2, \tau(t)))), \hat{\xi}(\min(s_2, \tau(t)))), \\ &\hat{\beta}(s_2) = sat(\beta^-, \beta^+, \tilde{I}_\beta(y_1(\min(s_2, \tau(t)))), \hat{\xi}(\min(s_2, \tau(t)))), \end{aligned}$$

provides the estimations

$$\begin{aligned} &\hat{m}(t) = \tilde{I}_m(y_1(t), \hat{\zeta}(t)) \,, \\ &(\hat{x}(t), \hat{x}_d(t)) = \left(-\hat{\beta}(t)\hat{\zeta}_2(t), y_2(t) + \hat{\beta}(t)\hat{\zeta}_2(t)\right) \,. \end{aligned}$$

The convergence of the estimator is exponentially practical, provided θ_1 and θ_2 to be sufficiently large.

6. Numerical simulations

We have considered a Monod's growth function (2) with the parameters $\mu_{max} = 1$ and $K_s = 100$ and the initial conditions $s(0) = 50$, $x(0) = 1$, $x_d(0) = 0$. The parameters to be reconstructed have been chosen, along with a priory bounds, as follows:

parameter	δ	k	m
value	0.2	0.2	0.1
bounds	[0.1, 0.3]	[0.1, 0.3]	[0.05, 0.2]

Those values provide an effective growth that is reasonably fast ($s(0)$ is about $K_s/2$), and a value $\bar{\tau}$ (see (6)) we find by numerical simulations is not too small. For the time interval $0 \leq t \leq t_{max} = 80$, we found numerically the interval $0 \leq \tau \leq \tau_{max} = \tau(tmax) \simeq 37.22$ (see Figure 1). For the first observer, we have chosen a gain parameter $\theta_1 = 3$ that provides

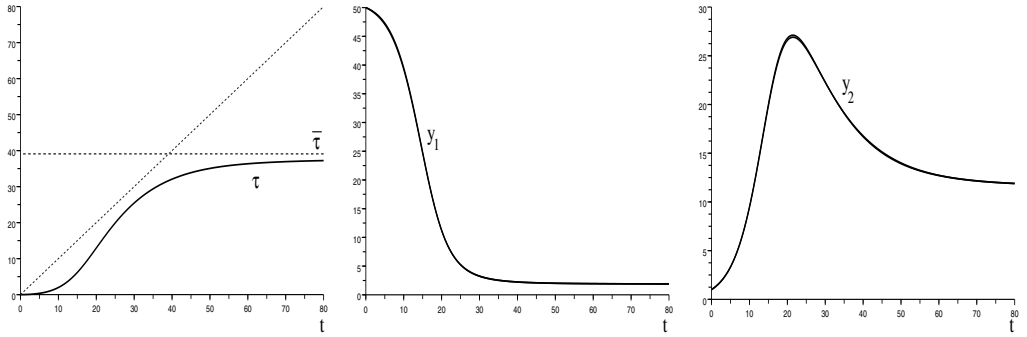

Figure 1. Graphs of function τ and observations y_1, y_2.

a small error on the estimation of the parameters α and β at time τ_{max} (see Figures 2 and 3). These estimations have been used on-line by the second observer, with $\theta_2 = 2$ as a choice

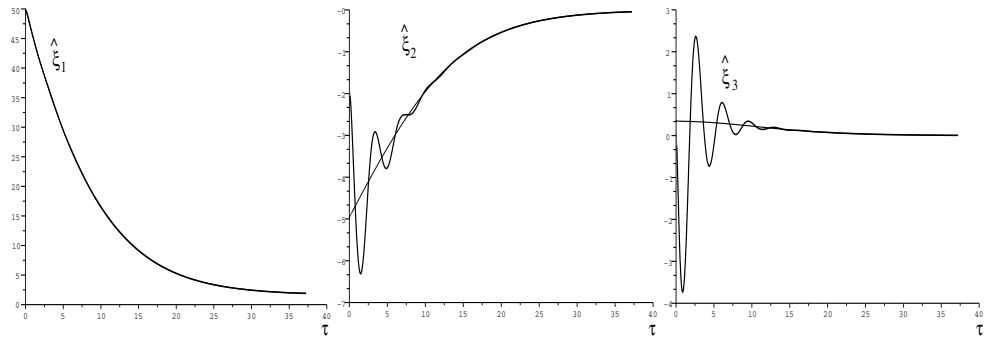

Figure 2. Internal variables $\hat{\xi}$ of the first observer in time τ (variables ξ of the true system in thin lines).

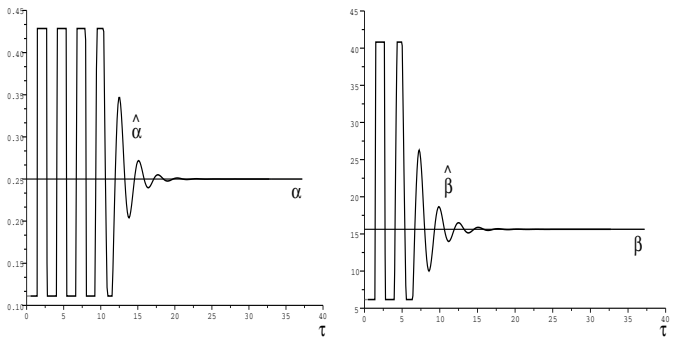

Figure 3. On-line estimation of parameters α and β.

for the gain parameter. On Figures 4 and 5, one can see that the estimation error get small when the estimations provided by the first observer are already small. Simulations have been also conducted with additive noise on measurements y_1 and y_2 with a signal-to-noise

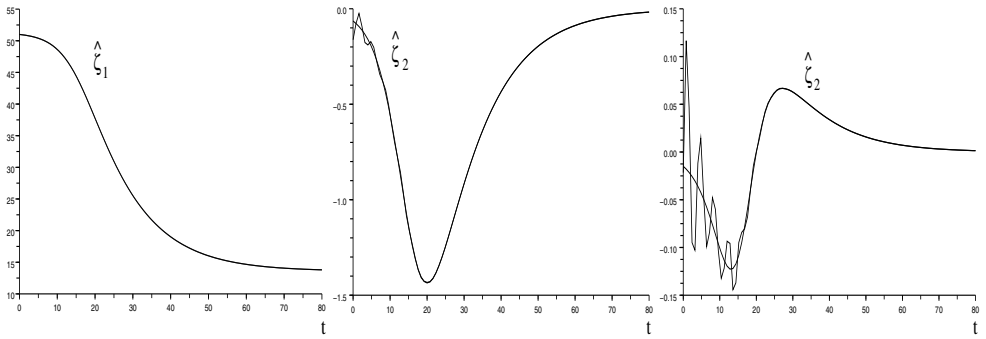

Figure 4. Internal variables $\hat{\zeta}$ of the second observer in time t (variables ζ of the true system in thin lines).

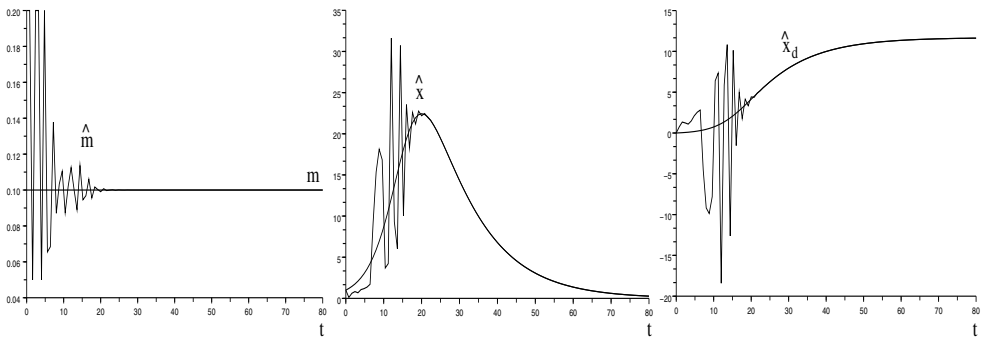

Figure 5. On-line estimation of parameter m and state variables x and x_d.

ratio of 10 and a frequency of $0.1Hz$ (see Figures 6 and 7). In presence of a low frequency

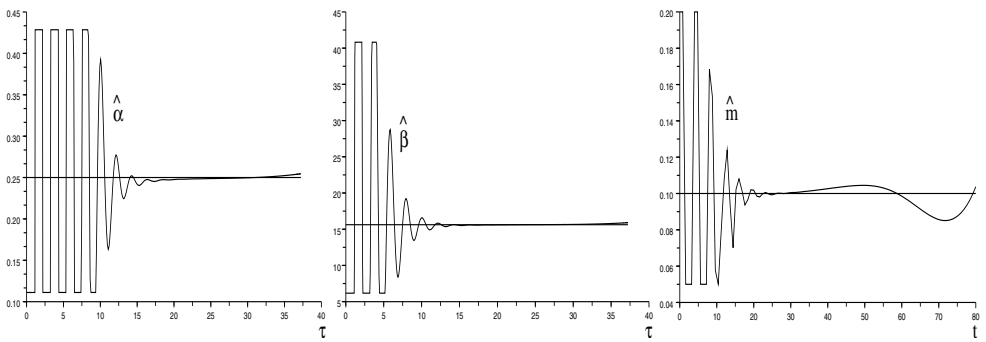

Figure 6. Estimation of the parameters α, β and m in presence of measurement noise.

noise (as it can be usually assumed in biological applications), one finds a good robustness of the estimations of parameters α, β and variables x and x_d. Estimation of parameter m is more affected by noise. This can be explained by the structure of the equations (5): the estimation of m is related to the second derivative of both observations y_1 and y_2, and consequently is more sensitive to noise on the observations.

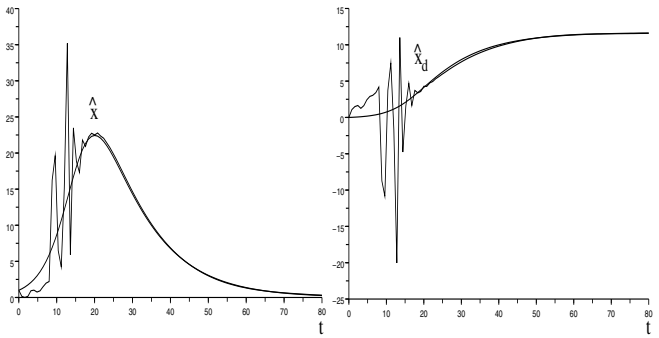

Figure 7. Estimation of the state variables x and x_d in presence of measurement noise.

7. Conclusion

The extension of the Monod's model with an additional compartment of dead cells and substrate recycling terms is no longer identifiable, considering the observations of the substrate concentration and the total biomass. Nevertheless, we have shown that the model can be written in a particular cascade form, considering two time scales. This decomposition allows to design separately two observers, and then to interconnect them in cascade. The first one works on a bounded time scale, explaining why the system is not identifiable at steady state, while the second one works on unbounded time scale. Finally, this construction provides a practical convergence of the coupled observers. Each observer has been built considering the variable high-gain technique proposed in [10] with an explicit construction of Lipschitz extensions of the dynamics, similarly to the work presented in [19]. Other choices of observers techniques could have been made and applied to this particular structure. We believe that such a decomposition might be applied to other systems of interest, that are not identifiable or observable at steady state.

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Chapter 6

Harvest Systems and Analysis for Herbaceous Biomass

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Additional information is available at the end of the chapter

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1. Introduction

Biomass is a distributed energy resource. It must be collected from production fields and accumulated at storage locations. Previous studies of herbaceous biomass as a feedstock for a bioenergy industry have found that the costs of harvesting feedstocks are a key cost component in the total logistics chain beginning with a crop on the field and ending with a stream of size-reduced material entering the biorefinery. Harvest of herbaceous biomass is seasonal and the window of harvest is limited. Biomass needs to be stored at a central location. Normally, several or many of these central storage locations in a certain range of a biorefinery are needed to ensure 24 hours a day and seven days a week supply. These centralized storage locations are commonly called satellite storage locations (SSL). The size and number of SSLs depend on the size of the biorefinery plant, availability of biomass within a given radius, window of harvest, and costs.

It is convenient to envision the entire biomass logistics chain from fields to biorefineries with three sections. The first section is identified as the "farmgate operations", which include crop production, harvest, delivery to a storage location, and possible preprocessing at the storage location. This section will be administrated by farm clientele with the potential for custom harvest contracts. The second section is the "highway hauling operations," and it envisions commercial hauling to transport the biomass from the SSL to the biorefinery in a cost effective manner. The third section is the "receiving facility operations," and it includes management of the feedstock at the biorefinery, control of inventory, and control of the commercial hauler contract holders to insure a uniform delivery of biomass for year-round operation.

Agricultural biomass has low bulk density, and it is normally densified in-field with balers, or chopped with a self-propelled forage harvester. Currently, there are four prominent harvesting technologies available for biomass harvesting [1]. They are: (1) round baling, (2)

rectangular baling, (3) chopping with a forage harvester, and separate in-field hauling, and (4) a machine that chops and loads itself for in-field hauling (combined operation). Large round and large rectangular balers are two well-known and widely accessible harvesting technologies [2], which offer a range of advantages and disadvantages to farmgate operations. Round bales have the ability to shed water. When these bales are stored in ambient storage, they will store satisfactorily without covering and storage cost is significantly reduced. The round baler, because it is a smaller machine with fewer trafficability issues, can be used for more productive workdays during an extended harvest season over the winter months [3]. Large rectangular bales have greater bulk density, ease of transport, and increased baler productivity (Mg/h). However, the increased capital cost for the large rectangular baler and the bales' inability to shed water limit its use on farms in Southeastern United States. Bale compression machines are available to compress a large rectangular bale and produce high-density packages [4]. The densified package has two or three times higher density than the field density of large rectangular bales [5].

The goal of an effective logistics system is to streamline storage, handling, and preserve the quality of the biomass through the entire logistics chain. This goal will minimize average feedstock cost across year-round operation. The farmer shares the goal to preserve the quality of the biomass, and also desires to produce the biomass at minimum cost. To assist in the accomplishment of the mutual objectives of both parties, this chapter will discuss major logistics and machine systems issues starting from the farmgate to the receiving facilities at a biorefinery. Constraints in this biomass supply chain will also be discussed. The impact of different harvest scenarios for herbaceous biomass harvest will be shown. Logistics systems have been designed for many agricultural and forest products industries. Thus, it is wise to use the lessons learned in these commercial examples. Each of these industries faces a given set of constraints (length of harvest season, density of feedstock production within a given radius, bulk density of raw material, various storage options, quality changes during storage, etc.), and the logistics system was designed accordingly. Typically, none of these systems can be adopted in its entirety for a bioenergy plant at a specific location, but the key principles in their design are directly applicable. Commercial examples will be used in this chapter to interpret these principles.

2. Biomass harvesting and the field performance of harvest machine systems

2.1. Introduction to herbaceous biomass harvesting

Harvesting of cellulosic biomass, specifically herbaceous biomass, is done with a machine, or more typically a set of machines, that travel over the field and collect the biomass. These machines are designed with the traction required for off-road operation, thus they typically are not well suited for highway operation. Therefore, the transition point between "in-field hauling" and "highway hauling" is critical in the logistics system. In-field hauling is defined as the operations required to haul biomass from the point a load is created in-field to a

storage location chosen to provide needed access for highway trucks. This hauling includes hauling in-field plus some limited travel over a public road to the storage location.

Harvesting systems can be categorized as coupled systems and uncoupled systems. Ideal coupled systems have a continuous flow of material from the field to the plant. An example is the wood harvest in the Southeast of the United States. Wood is harvested year-round and delivered directly to the processing plant. Uncoupled systems have various storage features in the logistics system.

Sugarcane harvesting is an example of a coupled system for herbaceous crops. The sugar cane harvester cuts the cane into billets about 38-cm long and conveys this material into a trailer traveling beside the harvester (Figure 1). The harvester has no on-board storage. Thus, a trailer has to be in place for it to continue to harvest. The trailer, when full, travels to a transfer point where it empties into a truck for highway hauling (Figure 2). Each operation is coupled to the operation upstream and downstream. It requires four tractors, trailers, and operators to keep one harvester operating. The trucks have to cycle on a tight schedule to keep the trailers moving. One breakdown delays the whole operation.

A "silage system" can be used to harvest high moisture herbaceous crops for bioenergy. With this system, a forage harvester chops the biomass into pieces about one inch (25.4 mm) in length and blows it into a wagon beside the harvester. This wagon delivers directly to a silo (storage location), if the field is close to the silo, or it dumps into a truck for a longer haul to the silo. All operations are coupled. That is, a wagon must be in place to keep the harvester moving, and a truck must be in place at the edge of the field to keep the wagons cycling back to the chopper. It is a challenge to keep all these operations coordinated.

A coupled system can work very efficiently when an industry is integrated like the sugarcane industry in South Florida, USA. Because the sugar mill owns the production fields surrounding the mill and the roads through these fields, the mill controls all operations (harvesting, hauling, and processing). Sugarcane has to be processed within 24 hours after harvest so the need for a tightly-controlled process is obvious.

Figure 1. Sugar cane harvester delivering material into a dump trailer for delivery to edge of field (Photo by Sam Cooper, courtesy of Sugar Journal, P.O. Box 19084, New Orleans, LA 70179).

Figure 2. Transfer of sugar cane from in-field hauling trailers to highway-hauling trucks.

An example of an uncoupled system is cotton production using the cotton harvester that bales cotton into 7.5-ft diameter by 8-ft long round bales of seed cotton. This system was developed to solve a limitation of the module system. With the module system, in-field hauling trailers (boll buggies), have to cycle continuously between the harvester and the module builder at the edge of the field. The best organized system can typically keep the harvester processing cotton only about 70% of the total field time. Harvesting time is lost when the harvester waits for a trailer to be positioned beside the harvester so the bin on the harvester can be dumped.

Baling is an uncoupled harvest system and this offers a significant advantage. Harvesting does not have to wait for in-field hauling. Round bales, which protect themselves from rain penetration, can be hauled the next day or the next week. Rectangular bales have to be hauled before they are rained on.

2.2. Field capacity and efficiency of biomass harvest machines

The equipment used for baling and in-field hauling is a critical issue to the farm owners. More efficient harvest systems coupled with well-matched harvesting technologies specific to farm size and crop yield can minimize costs. The importance of understanding the linkage between various unit operations in the logistics chain was illustrated [6,7]. Similarly, researchers have quantified the handling and storage costs for large square bales at a bioenergy plant [8]. However, in both of these evaluations the details of field operations and field capacities of machines involved in the field harvesting and handling were not available [9]. Instead, costs of bales at the farm gate were used to analyze bioenergy production costs. To maximize the field efficiency of field machine systems, it is essential for farm managers to know the field capacity of each machine involved in harvesting. In addition, quantitatively understanding the capacity of biomass harvest machines is essential to assess daily production and supply rate for a biorefinery or a storage facility.

The field efficiency of rectangular balers can be determined by calculating the theoretical material capacity of the baler and actual field capacity [10]. Calculation of material capacity can be demonstrated using a large rectangular baler as an example. The end dimensions of the large bales were 1.20 m × 0.90 m; bale length was 2.44 m. The depth and width of the chamber were 0.9 m and 1.2 m, respectively. Plunger speed was 42 strokes per minute. Measured bale density was 146 kg/m^3, and the thickness of each compressed slice in bale

was 0.07 m. Thus, calculated theoretical bale capacity using equation 11.60 in [10] is 27.83 Mg/h. The plunger load could be set higher and produce higher density bales.

The theoretical capacity was obtained from a baler manufacturer under ideal conditions. Ideal conditions exist when a baling operation has [11]:

1. Long straight windrows
2. Windrows prepared with consistent and recommended density (mass/length)
3. Properly adjusted and functioning baler
4. Experienced operator

Actual field capacity of a baler will be impacted by the size and shape of the field, crop type, yield and moisture content of the crop at harvest, and windrow preparation. Typical field efficiencies and travel speeds can be found from ASAE Standards D497 [12]. Cundiff et al. [11] analyzed the field baler capacity and considered the effect of field size on baler field capacity. They found that the field capacities of round and large rectangular balers were 8.5 Mg/h and 14.4 Mg/h, respectively.

Another example of testing the baling capacity of a large rectangular would be the field tests conducted on wheat straw and switchgrass fields [13,14]. Results showed that actual field capacity of a large rectangular baler was between 11 and 13 Mg/h. This indicates that the field capacity of a large rectangular baler could be 50% or less compared to its theoretical capacity.

2.3. Power performance of harvest machine systems

Machine system field efficiency is limited by tractor power performance, machine field capacity, and field conditions. Field conditions limit operational parameters and the percentage of the maximum available power. Since the high cost of harvest and in-field handling is still one of the main roadblocks of utilizing biomass feedstocks to produce biofuels, increasing machine system field efficiency through designing or selecting suitable machine systems is the challenge to machinery design and management professionals.

Power performance of a 2WD rear drive tractor was presented in a format of flow chart in ASAE standards [12,15]. Total power required for a tractor is the sum of PTO power (P_{pto}), drawbar power (P_{db}), hydraulic power (P_{hyd}), and electric power (P_{el}) as expressed by equation (1). Depending on the type of implement, components in equation (1) may vary. Total power calculated with equation (2) is defined as equivalent PTO power, which can be used to estimate the tractor fuel consumption under specific field operations.

$$P_T = \frac{P_{db}}{E_m E_t} + P_{pto} + P_{hyd} + P_{el} \quad (1)$$

Where E_m and E_t are mechanical efficiency of the transmission and tractive efficiency, respectively. Each of the power requirements in Equation (1) can be estimated using recommended equations in [12]. Example of using this standard to estimate power requirements for a large rectangular baler system is available [13].

2.4. System analysis case study - Large rectangular bale handling systems

2.4.1. Baling and bale collection

Baling, bale collecting, and bale storing of 650-ha wheat straw field at a commercial farm was studied as a typical large rectangular bale harvesting system. Factors that affect large rectangular bale production and handling logistics were quantified through observing complete field operations and then the system performance was analyzed and field capacities of all machines in this system was determined. System limitations were quantified, and means to reduce production costs were discussed [14].

Bales were stored in covered storage facilities. The equipment used included one large rectangular baler, two bale handlers, and three flatbed bale trucks (Figure 3). The straw was raked with a twin rotary rake. The rake was used to form uniform and evenly-spaced windrows from the straw that had been expelled by the combines. If the straw was rained on before baling, the rake fluffed the straw to enhance drying. The operator adjusted the swath of the rake in order to form windrows of proper size.

Figure 3. The large square baler, bale handler, and bale truck used in straw harvesting.

The field crew included a person operating the baler, two persons operating wheel loaders, and three truck drivers. The baler ran continuously throughout the day with the exception of operator breaks. Since no bales were left in the field overnight, the bales were collected at about the same pace as produced. A truck driver would bring the truck into the field and locate two bales. The wheel loader operator followed the truck through the field and loaded two bales at a time. When the truck was full, the driver would exit the field and transport the load to a storage facility. Bales were loaded in an interlocking manner, and the bales were not strapped down, a procedure that saved a lot of time.

Because there were three truck drivers and two trucks, a driver was not present while the truck was unloaded. The driver of the fully loaded truck would drive into the storage facility and position the truck to be unloaded. By the time this truck arrived with a full load of straw, the previous truck at the site would be empty. This process was repeated, with minor delays when a driver waited for a truck to be unloaded. Capacity of each operation was measured in terms of number of large rectangular bales (Table 1). Baling rate was the limiting factor in the system.

2.4.2. Bale compression

To reduce long distance hauling cost of forage/hay bales, compression of these bales into ultra-dense bales was adopted by some producers to prepare the hay for overseas transport.

Commercial bale compression equipment has potential to be used for the densification of baled biomass.

Field operation	Number of bales per hour (St. dev.)
Raking	85 (equivalent)
Baling	43 (9)
Accumulating into stacks of bales in field	93 (28)
Loading truck in field from accumulated bales	204 (29)
Loading truck in field from not first accumulated bales	143 (46)
Stacking bales in storage	93 (12)

Table 1. Field capacities of large rectangular baler and bale handlers (Straw M.C., 14% w.b.) [14].

Ten large rectangular switchgrass bales were compressed using the compressor (Figure 4) [9]. The input bale had a dimension of 0.86×1.22×2.21m, and the resulting compressed bales were 0.53×0.46×0.38m. When stacked in groups of 20 per pallet, the package had a dimension of 1.02×1.17×1.70m. The initial bales had a volume of 23.27m^3. When accounting for a 7.0% loss of material and water, this volume was 21.67m^3. The bales had average moisture of 14% (w.b.) at the time of compression. The compressed package had a volume of 13.13m^3, a reduction of 60.6% in volume.

Figure 4. Commercial large rectangular bale slicer/compressor.

2.5. System analysis case study – Round bale handling system

The advantage of round bales is that the rounded top sheds water and bales can be stored in ambient storage without the expense of covered storage. A second important advantage of round bales is that round balers are conventional technology throughout the United States. They are widely used to harvest forage. Compared to a large rectangular baler, a round baler has lower capital cost.

A significant advantage is realized by the farmers if their round balers can be used for both their existing livestock enterprise and a bioenergy enterprise. Warm-season grasses are harvested during the winter for the bioenergy market. Thus, the biomass harvest does not conflict with the hay harvest. The advantage gained by the biorefinery is that no need for their feedstock producers to invest in new equipment. Requiring capitalization of new equipment will make the contacting with feedstock producers more difficult.

The disadvantage of round bales is that they are hard to stack and thus the handling and transport costs are higher. System performance was analyzed and field capacities were determined based on field measurements [16]. Individual handling of bales (either round or rectangular) is not cost effective. The high cost is caused by long loading and unloading times. Multi-bale handling units have been designed, and these units are discussed in Section 9 of this chapter.

3. Field working days – Harvesting limitations

Determining the schedule time required for harvest operations is an essential prerequisite to determine herbaceous biomass priority for securing inventory and effectively matching the transport of biomass from SSLs (satellite storage locations) to a biorefinery. Estimating the number of days expected to be available for baling is difficult because agricultural field work is heavily weather dependent and information concerning winter field operations is not well established. Harvest costs depend in part on the investment required in harvest machines (number of machines purchased), and this investment depends on the field capacity of the machines and the number of field workdays available during the harvest window. Therefore, an estimate of the number of harvest days is necessary to determine the total investment in harvest machines required to support a biorefinery.

The optimal perennial grass harvest strategy for a biorefinery remains to be determined. One potential strategy is to delay harvest until the plants standing in the field have transitioned from a non-dormant to a dormant state. Transitional events within the plant include translocation of nutrients from the above-ground plant parts to below-ground plant parts as initiation of degeneration (senescence) of above-ground tissues occur. Delaying harvest until the end of this transitional period, and harvesting over a short time period, can result in near maximum biomass yield, reduced moisture content, and reduced amount of nutrients removed with the biomass. This strategy would suggest an optimum harvest window for switchgrass from October to December. However, a harvest season this short would require substantial investment in harvest machines to complete harvest within a 3-month period. It also requires a large storage for inventory to supply the plant for year-around operation. By delaying harvest until nutrients have translocated, biomass tonnage will be decreased by this loss of mineral content, however since the relative amount of carbon in the material is increased, conversion efficiency and combustion quality may be improved [17]. Moreover, the translocated nutrients stored in the roots can be used for growth and development by the plant year after year, thus, reducing the need for and cost of supplementing the soil with nutrients through fertilization.

A second strategy is to extend harvest over as many months as possible. This would enable more economical use of harvest machines, reduce the quantity required in SSL storage, and potentially better match truck transport with SSL operations. With an extended harvest strategy, harvest would begin in July and extend through the following March. This extended harvest season would allow for spreading the fixed costs of the harvest machines over more Mg harvested per year thus reducing the $/Mg harvest cost. However, the earlier

harvests before senescence (July through September) will remove more nutrients, and they will potentially conflict with other farm operations (i.e. grain/hay harvest, small grain seeding). To maintain productivity, additional fertilizer will be required, and this represents an additional cost to the farmer whose fields are harvested prior to October. The farmer whose fields are harvested January through March will have a lower yield because of leaf loss from the standing biomass exposed to winter conditions.

A delayed harvest raises an important issue relative to the payment to a farmgate contract holder. As previously explained, a certain loss occurs when the crop is left standing in the field for delayed harvest (December through March). Also, the delayed harvest increases the risk that a weather event will cause the crop to lodge so the harvest machine leaves material, and the "yield" is reduced. If these same fields were harvested during the optimum window (October through December) and stored longer in an SSL, there is a storage loss that increases with time in storage [18]. Considering the total reliance on stored biomass during the non-harvest months, which strategy is the better choice for the farmgate contract holder (i.e., generates the maximum quantity of biomass to sell to the biorefinery)? Is it better to delay harvest or build a larger SSL and invest in more harvesting machine capacity to complete the entire harvest within the 3-month optimum window? Epplin et al. [19] estimated differences in switchgrass harvest costs for both a 4-month and a 8-month harvest window. They accounted for differences in biomass yield across months, but they did not adjust for fertilizer requirements. They found that the estimated harvest costs varied from $25 per Mg for a 4-month harvest season to $11 per Mg for a 8-month harvest season. These results show that the length of the harvest season is a significant factor in determining the costs of harvesting biomass.

Epplin et al. [19] did not have refined estimates of the number of days that biomass could be harvested. They based their estimates of available harvest days on a study designed to determine the number of days that farmers in Southwestern Oklahoma of United States could conduct tillage operations. To determine more precise estimates of the number of harvest machines required to harvest, and thus obtain a more precise estimate of harvest costs, a more precise estimate of the number of harvest days is required.

Hwang et al. [20] determined the number of suitable field workdays in which switchgrass could be mowed and the number of days that mowed material can be baled. Empirical distributions of the days available for mowing and for baling switchgrass were determined for nine counties in Oklahoma. Distributions were determined for each month and for two potential harvest seasons (short, October–December and extended, July - February). Several conditions are necessary for safe baling of switchgrass. First, the soil must be sufficiently dry to support the weight of harvest machines. Second, prior to baling, the moisture content of the cut switchgrass must be at a level for safe storage. They provided evidence of the difference in harvest days across strategies.

Determining the time available for required harvest operations is a necessary, but not sufficient, prerequisite for determining the optimal harvest strategy. Additional information will also be required. As previously explained, if harvest begins in July prior to maturity

and subsequent senescence in October, additional fertilizer will be required (the amounts are undefined) to offset the nutrients removed from the pre-senescence harvest. If harvest is delayed into December or later, the harvestable yield will be less (the amounts are undefined) than if it is harvested in October and November. Thus, the information regarding differences in harvest days across months must be combined with information regarding differences in fertilizer requirements, and biomass yields across harvest months, to determine an optimal harvest strategy.

4. Constraints for a typical biomass logistics system

4.1. Constraints set by resource

Any biomass crop that provides an extended harvest season has an advantage because most biorefineries want to operate as many hours per year as possible. Continuous operation yields the maximum product per unit of capital investment. Herbaceous crops cannot be harvested during the growing season. Thus, storage is always a component in a single-feedstock logistics system design.

Two examples are discussed from opposite ends of the length-of-harvest-season spectrum. In the Southeastern United States, wood is harvested year-round. This is referred to as "stored on the stump." Weather conditions in the Southeast are such that few harvest days are lost in the winter due to ice and snow. On the other end of the spectrum, consider the harvest of corn stover in the Upper Midwest of the United States. The grain harvest is completed in the fall, and then the stover is collected. In some years, there are less than 15 days between the completion of the grain harvest and the time when the fields are covered with snow, and no stover can be collected. All feedstock required for year-round operation of a bioenergy plant, if it uses only corn stover, must be harvested in a three- to five-week period. This is a significant challenge for a logistics system.

4.2. Constraints set by purchaser of raw biomass

Biorefinery operators are interested in the cost of the feedstock as it enters their plant. The plant can burn the biomass directly to produce heat and power, or it can use the biomass as a feedstock for some more complex conversion processes to produce a high-value product. The term "feedstock" is used to refer to any raw biomass before its chemical structure is modified by a conversion process, which can be direct combustion, thermo-chemical, or biological.

Feedstock cost ($/dry ton) is defined as the cost of the stream of size-reduced material entering the reactor at the Bioenergy plant for 24/7 operation. The reactor is defined as the unit operation where an initial chemical change in the feedstock occurs. The reason for choosing this reference point for the biomass logistics system is two-fold.

1. The plants that can operate continuously, 24/7, have an advantage. Maximum production (tons/y) per unit of capital investment gives a competitive advantage in the market place – cost to produce the product is lower.

2. Some logistics systems do size reduction with the harvesting machine, while some size reduce at a transfer point between in-field hauling machines and highway hauling machines (perhaps as a pre-requisite to a densification step), and some size reduce at the entrance to the processing plant. In order to compare the several systems, it is necessary to have a consistent end point for the system analysis.

The readers should be aware that many studies in the literature select a different analysis endpoint than used here. A typical endpoint is the cost of feedstock when a truckload of raw biomass enters the plant gate. This reference point is favorable as many agricultural and forest industries pay the producer when the raw material is delivered to the plant.

5. Field loss

The yield losses of different herbaceous biomass feedstocks while standing in the field will impact the harvest systems and the windows of opportunities to gather the feedstocks into storage. The inventory losses will be impacted by herbaceous biomass feedstocks, package configurations and integrity, storage facilities and time in storage. Loss of dry matter is also an important parameter in biomass collection and transportation.

Biomass loses dry matter due to its high moisture or dryness. Leaves and other fragile parts of the plant are broken and lost in the wind or mixed with soil. Some of the losses occur during storage due to fermentation and breakdown of carbohydrates to carbon dioxide and other volatiles. Unfortunately the exact account of switchgrass losses in the field or during storage is not available. Sanderson et al. [21] reported dry matter loss in baling and storage of switchgrass and stated that the overall losses were less than for legume hay. They estimated that switchgrass bales stored outside without protection resulted in a dry matter loss of 13% of the original bale dry weight. They also estimated that dry matter loss of 1-5% during baling depending on the moisture content. Kumar and Sokhansanj estimated field and storage losses for straw and stover [22]. Other studies have also estimated the dry matter loss of biomass during storage, collection and transport [23-26]. Turhollow [27] estimated the losses from switchgrass to be similar to losses in alfalfa. The study estimated 8% losses for a mower-conditioner, 3% for a rake, 10% for a round baler, and 0.1% for a round bale wagon. He estimated average loss of 15% over 6 months of storage. A recent study showed that the dry matter loss in switchgrass collection (including storage) is less than 2% for different collection methods.

Moisture causes damage (microorganism growth) and subsequent dry matter losses in stored switchgrass bales. Several studies have shown that dry matter losses in switchgrass bales are greater for bales stored outside as compared to bales stored inside [18,22,28]. Moreover, dry matter losses are far greater for covered rectangular bales than uncovered round bales [18]. Large uncovered round bales had a better economic return than covered rectangular bales, when considering the cost due to mass loss during storage [18]. However, another study highlighted their successful use of rectangular bales [29]; the cost of covered storage was more than offset by the reduction in hauling cost for the square bales.

6. Computer model of biomass logistics systems

When a series of operations are linked into a long chain of events, it is often difficult to see which operation is having the most impact on cost-effectiveness. Computer-assisted decision making tools are useful to select the most cost-effective logistics system. Recent studies [30-33] compared a number of different scenarios to determine the best unit operation options and identify bottlenecks. Mathematical programming approaches have been used to develop optimization models that are applicable to a variety of cases studies [34-37]. More recently, focus has shifted to simulation models using object-oriented programming [38] or discrete event simulation [39, 40]. The object-oriented approach [22, 41-43] simplifies scenario building, particularly related to various equipment options available for the same operation, since data pertaining to different options are stored in a standard object-oriented format.

The biomass logistics subject area can be divided into two types of modeling approaches depending on the environment encountered: stochastic or deterministic, and integer or continuous. In the areas of stochastic and deterministic environment, ISBAL and BioFeed capture the stochastic biomass logistics issues such as variability in processing as a result of weather, time, equipment breakdowns, etc. Additional work in this area can be found in [34; 40; 44-45]. In the deterministic environment, most work utilizes a geographic information system (GIS) interactively with optimization. This allows the GIS framework to work as a data management tool, which may call the optimization software directly. Most of the deterministic models are mixed-integer programs [37, 46-49]. One of the principles of designing an effective logistics system is starting with the feedstock resources distribution, and the simplest method is to utilize a GIS framework for data management.

Continuous models assume that all the land within a region is utilized for biomass production [50-52]. This approach uses average haul distances and cost. Hence, the solution obtained is difficult to implement due to lack of detail, as opposed to the solution obtained using an integer model formulation, where each production field is considered as a specific entity with specific costs, thereby resulting in precise decisions for implementation.

6.1. Modeling of biomass harvesting and handling systems for field operations

There are numerous studies developing for the optimum set of field equipment where the unit operations affect the performance of units upstream or downstream. Review of this literature is beyond the scope of this chapter. However, several simulation programs are highlighted that specifically addresses a biomass harvest and delivery system.

The Integrated Biomass Supply Analysis and Logistics Model (IBSAL) is a simulation model which simulates operations in the field [22,38,41]. ISBAL is very useful for the simulation of operations that collects feedstock from the field and examines the flow of biomass into storage. IBSAL is best used for drawing conclusions about a sequential set of events. For example, if the user has a defined number of fields, a defined set of equipment, and a target number of tons to be harvested each month (each week) then IBSAL can provide valuable

guidance for optimum biomass collection. The influence of weather, equipment breakdowns, and other disturbances to the biomass system can be "played" with a series of simulations.

The BioFeed model [43] determines the overall system optimum, and integrates the important operations in the biomass chain into a single framework. It is possible that the optimal solution recommended by BioFeed may not be implemented in a real system, either due to unforeseen disturbances such as weather or due to the actions of independent stakeholders such as farmers. However, an integrated model such as Bio-Feed determines the optimal configuration, system bottlenecks, and potential improvements. Such a model can be useful in quantifying the systemic impacts of technology improvement [42].

The BioFeed model results were compared to recent studies in literature [22,31,33]. The scope of the BioFeed model was similar to the scenarios developed in [22], where the delivered cost was estimated to be about $35/Mg (d.b.) excluding biomass size reduction. The major differences between ISBAL and BioFeed were harvesting and storage costs. While Kumar and Sokhansanj [22] ignored the storage costs, they also considered a self-propelled forage harvester which had a higher throughput capacity than the mower-conditioner considered in BioFeed, thereby reducing the cost. Khanna et al. [31] reported the switchgrass delivered cost of $64.84/Mg, which was similar to the BioFeed cost estimate. The study by Duffy [33] estimated the delivered cost to be $124.30/Mg. However, the major difference in the estimates was due to a much higher establishment cost.

Since the scope of the analyses and the assumptions differed for these studies, it is impossible to make specific comparisons. However, these comparisons illustrate that the overall model predictions agree reasonably well.

6.2. Modeling of biomass delivery systems

Linear programming models have been used to analyze system interactions in biomass delivery systems. Dunnett et al. [53] proposed a program to optimize scheduling of a biomass supply system for direct combustion. This model simulated storage on farms and delivery to one location with a variable demand for heat. They suggest that costs of biomass handling can be improved 5 to 25% with the model recommendations. Bruglieri and Liberti proposed a "branch and bound" nonlinear model to determine biorefinery locations as well as the optimum transport method [54]. Their model focused on multiple feedstocks but did not use actual equipment performance data.

A model comprised for multiple purposes can bring attributes of benchmarking, simulation, and linear programming together to solve for the best solution. Leduc et al. a system of wood gasification plants optimized [55]. Their model focused on establishing a biorefinery plant in a location that is suitable for distribution of the product being manufactured (in this case, methanol). Other similar models have focused on silage handling operations [56].

A number of models have proven that a single chain of handling procedures can be optimized, but fail to adequately address the "disconnect" caused by storage, specifically

satellite storage. Unfortunately, few models consider different harvest systems (or feedstock) supplying a single biorefinery. For example, one equipment system delivers chopped material directly from the field to the plant (probably from fields close to the plant), and a second set of equipment bales material and places it in storage which will be delivered during months when direct delivery of field chopped material is not possible.

As described earlier, a satellite storage location (SSL) is a pre-designated location that is used as a storage location for the biomass collected by the producers within a defined geographic region. SSLs are a logical transition point between "agricultural" and "industrial" operations and thus are critical elements in a logistics system design.

6.3. Study of Satellite Storage Locations (SSLs)

An SSL should be established where sufficient feedstock density will ensure the investment is justified. Location affects the costs associated with transporting the biomass from the production field to the SSLs and from the SSLs to the biorefinery. An additional factor that needs to be considered is how often a SSL is filled and unloaded. If a SSL is emptied only once a year, then the total SSL storage area would be doubled as compared to a twice-per-year unload schedule. Note, multiple fillings of a SSL is possible if harvest can be extended over several months and is properly matched with the SSL unload sequence.

Specialized equipment, with high productivity (ton/h) will be utilized to empty each SSL. This equipment can be permanent equipment for each SSL, or the equipment may be mobile and move from one SSL to the next. Questions that need to be answered to implement the mobile option are: 1) how many sets of equipment should be used to service the entire area? 2) What is the sequence of SSLs that each equipment set should unload? 3) Is an SSL ready to be unloaded? This last question directly affects the hauling company's contract. These questions are best considered in a virtual environment where scenarios can be compared and contrasted.

A case study to characterize feedstock resources and establish the SSL's was analyzed by Resop et al. [57] for a 48-km radius around Gretna, VA, USA. The GIS analysis identified potential production fields based on current land utilization determined using aerial photography and landuse classification (Figure 5). The analysis selected fields such that the production area was 6% of the total land area within the radius. The biomass produced is sufficient to supply the demand for a 1,944 Mg/d biorefinery, assuming 47 operating weeks per year. SSLs were established at 199 locations, and the existing road network (GIS database) was used to determine the travel distance from each SSL to the proposed plant location in Gretna. A weighted Mg-km parameter for transportation from the SSLs to the plant was computed to be 44.8 km, which implies that, averaged across all 199 SSLs, each Mg traveled an average of 44.8 km to the biorefinery.

Judd et al. studied three equipment options for the operations performed at a SSL [58]. Two options utilize the rack concept [59]. Ravula et al. analyzed a round bale logistics system utilizing this rack system; the rack size emulate a 20-ft (6.1-m) ISO container providing two

levels of 8 bales, total of 16 bales, to be handled as a single unit [40]. A tractor-trailer truck can haul two racks (32 bales, 14.4 Mg). The results showed the rack hauling had higher transportation costs due to not loading the truck to maximum allowable weight.

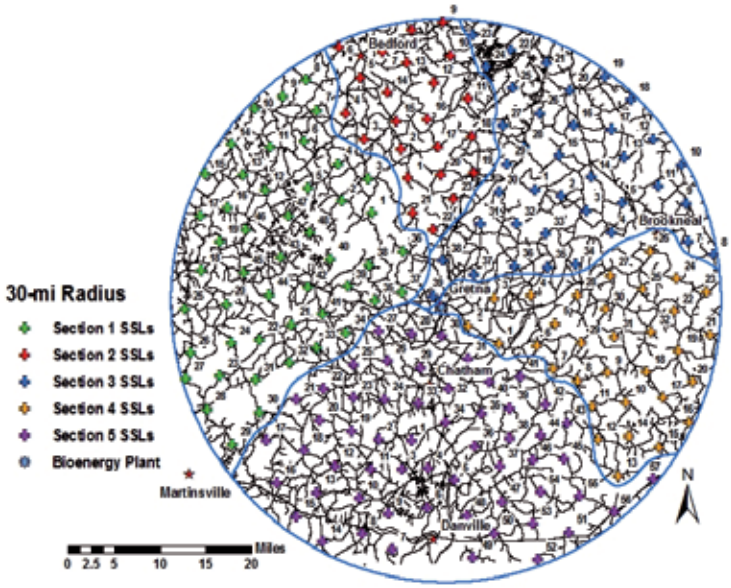

Figure 5. Example of Satellite Storage Locations (SSLs) located over a 30-mi (48-km) radius around a chosen bioenergy location. The refinery is located in the center of the circle. Each cross represents an SSL location with access to the public road system. The smallest SSL was a storage that can store biomass from 60 ac (24 ha) of production fields and the largest stores biomass from 1200 ac (486 ha) of production fields.

At a SSL, the round bales could be handled in one of two ways. The first option loads bales into the rack from the rear and is referred to as the "rear-loading." The second option, referred to as the "side-loading," loads the bales into a rack from the side. These two options were compared to grinding the bales at the SSL and compacting the ground material into a briquette (maximizes over-the-road load) for delivery [50]. Judd et al. [58] found that it was more cost effective to use the "side-load" option and haul the round bales than to form briquettes at the SSL, if the haul distance was less than 50 miles (81 km).

The large biorefinery concept calls for delivery of feedstock from a large geographic area. The results [58] suggest that some type of intermediate processing step, referred to as an Advanced Regional Biomass Processing Depot (ARBPD) by Eranki et al. [60], may be needed. These depots will convert the raw biomass to a more energy dense (higher value) product, and this product will then be delivered to a large biorefinery for final processing.

Why was the expense of size reduction and densification into briquettes at the SSL considered? Independent studies [61-63] reported that the tradeoff between the additional cost for densification and the reduction in transportation cost is significant for the hauling of logging residues. For additional work in the area of densification, see [64-65].

6.4. Application of information technologies

The inclusion of a hauling contractor in the business plan provides the best opportunity for all of the technology developed for other logistics systems to be applied to feedstock logistics. The information technologies applied include a GPS receiver in every truck and a bar code on every load as well as geographic mapping and routing management tools. Thus, time and location information can be observed at every SSL and the weigh-in scale at the receiving facility. The data collected can be used to optimize asset utilization in real time, and it will also feed needed data into the accounting software to compensate the farmgate and haul contracts.

It is expected that the collected data will be presented in real time. The information will provide a "Feedstock Manager," maps showing the location of all assets with updates at suitable time intervals. The goal is to provide an opportunity for the Feedstock Manager to make optimization decisions in real time. Examples are: trucks rerouted to avoid traffic delays, assets redeployed during breakdowns, increase at-plant inventory when inclement weather is forecasted, and a turn-down of plant consumption when a delay in feedstock deliveries cannot be avoided.

Some perspective of the logistics complexity, as presented to the Feedstock Manager, can be gained from the following sample parameters. This example presumes that operations will be in the Upper Southeast of the United States where switchgrass is harvested over an 8-month harvest season. Suppose the supply area has 199 SSLs within a 48-km radius of the plant, and each SSL has a different amount of material stored as shown in Figure 6. The producer desires to fill their SSLs at least twice during the harvest season to minimize per-Mg SSL investment. Suppose there are five SSL crews under contract and each of them wants the same opportunity to earn income (total Mg mass hauled per year). The Feedstock Manager's job is to treat all farmgate and haul contractors fairly.

6.5. Benchmarking models for biomass logistics

It is appropriate to benchmark a proposed feedstock delivery system against existing commercial systems; for example, woodchips, grain, hay, sugarcane, and cotton. Ravula et al. [37] studied delivery to a cotton gin from over 2,100 field locations to better understand logistics system design for a biorefinery.

Switchgrass, corn stover, and other energy crops present two major challenges when benchmarked against other commercial examples. First, energy crops are spread over a greater number of sites when compared with woodchips harvested as byproducts from logging. Second, energy crops have low value when compared with hay, grain, sugarcane, or cotton. Applying benchmarked models to energy crop systems provides a starting point, but optimization techniques must be applied to "fine tune" the logistics system design.

6.6. Simulation of mixed harvesting options for supply of single plant

Brownell and Liu [66] developed a computer model to select the best option, or the best combination of options, for supply of a given bioenergy plant. The options include a direct

chop and delivery option, two bale options (round and large rectangular), and a variation of the large rectangular bale option where these bales are compressed at a stationary location before shipment. Specifically, the options are: (1) loose biomass harvest using a self-loading forage wagon; (2) round bale harvest; (3) large rectangular bale harvest; and (4) large rectangular bale with compression before hauling.

The results of the model provide users a decision matrix which shows the optimized handling scenario for all four handling options analyzed by this program. Cost calculation was based on three plant demands (2,000, 5,000, and 10,000 Mg DM/d) to compare costs as the required production area expanded. Satellite storage locations were established based on the distribution of production fields and the existing road network.

The key constraints used for the simulation are:

1. Only loose biomass can be directly hauled from the field to the biorefinery.
2. Biomass cannot be stored at the plant, 20,000 tons (18,144 Mg) will "stand in field" in surrounding area of the plant and collected on demand as loose material.
3. Baled biomass will be transported to and stored at satellite storage locations.
4. Large rectangular bales may be compressed before transportation.
5. Biomass will be grown on one third of the available acreage, with an average yield of 3 Mg/ha (15% average moisture content).

The land close to the plant is more valuable, from the biorefinery viewpoint, for the production of feedstock. The study found that a plant can afford to pay more for feedstock (landowner gets a higher price) if a farm happens to be closer to the chosen plant location.

The results showed that the size of SSLs is sensitive to the amount of material demanded by the plant. The program tests various sizes, distances between SSLs, and possible overlaps of production fields "served" by each SSL. The programming optimizes the simulation by slowly changing the size and location of SSLs. The program solves for the maximum amount of acreage available in the simulation model. The output is a distance material in each SSL can be efficiently hauled based on if it is harvested and hauled directly from the field or if it is baled and stored before hauling. The program also solves for the lowest cost for a set acreage when the closest fields are field chopped and remaining fields are baled.

7. Commercial examples of logistics systems

7.1. Typical linkage in logistics chain

As previously stated, biomass logistics is "a series of unit operations that begin with biomass standing in the field and ends with a stream of size-reduced material entering a bioenergy plant for 24/7 operation." This concept can be visualized as a chain (Figure 6), where the links are unit operations. The example in Figure 6 shows a logistics system moving bales from the field to SSLs, and then from these SSLs to a bioenergy plant. The dotted lines show various segments of the chain that are assigned to the several entities in the business plan. In this example, the segment identified "farmgate" is performed by the

feedstock producer, the segment labeled "SSL" is performed by a load-haul contractor; the segment identified as "Receiving Facility" is performed by the bioenergy plant. Figure 6 is one example; several other options are used in commercial practice. In next section, three commercial examples are given to show readers the range of options in a logistics system.

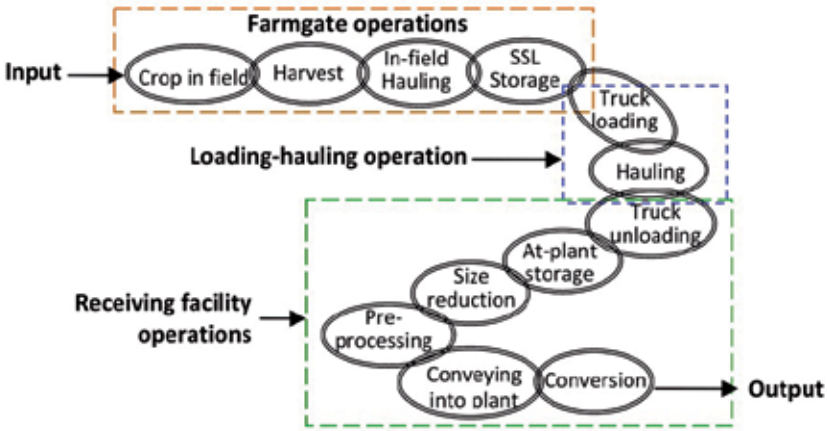

Figure 6. Logistics chain for delivery of round bales to a bioenergy plant (The dotted lines identify segments of the chain which are performed by different business entities).

7.2. Examples of commercial logistics models

7.2.1. Traditional model

The producer grows, harvests, stores, and delivers raw material (biomass) in accordance with a contract with the processing plant. Deliveries are made to insure the plant has a supply for continuous operation during the processing season. For many agricultural industries (i.e. cotton, grain, sugar cane, fruits and vegetables), the processing season approximately coincides with harvest season, and it is only part of the year.

The advantage of the traditional model, from a processor's point of view, is that all quality issues reside with the producer. There is no question who is responsible if a quality standards are not met. Business people planning the operation of a bioenergy plant tend to prefer this model, though they typically balk at paying a feedstock price that adequately compensates the producer for their additional risk.

7.2.2. Cotton model

A cotton producer grows, harvests, and stores the raw material (seed cotton) in modules at an edge-of-field location with suitable access for highway hauling trucks (Figure 7). The gin (processing plant) operates a fleet of trucks to transport the modules as required for operations during the ginning season. Farmers are paid for the seed cotton that crosses the scale at the gin. The gin operates a warehouse and stores bales of ginned cotton for periodic delivery to provide a year-round supply for their textile mill customers.

Figure 7. Module hauler picking up module stored at edge of field.

The advantage of the cotton model is that the specialized equipment used for hauling the modules is owned, scheduled, and managed by the gin. Thus, the producer does not own a piece of equipment they will use only a few times a year. The gin deploys the module haulers to their customers, thus the haulers accumulate more hours per years over what a producer would use, and the hauling cost ($/Mg) is minimized [40]. Typically, gins haul modules in the order they are "called in" by the producers. Module storage time in the field, and any subsequent losses (at the gin), are not dealt with in the producer-gin contract. The producer is paid the contract price for the mass of cotton fiber (and co-products) that the gin produces from a particular module.

7.2.3. Sugarcane (Texas model)

The producer grows the sugarcane. The sugar mill takes ownership of the crop in the field and harvests and transports it for continuous operation during the processing season. Sugarcane must be processed within 24 hours of harvest so storage is not an option within the sugarcane system.

The advantage of the Texas model is that the producer does not own any harvesting or hauling equipment. The disadvantage of the system is the producer does not have control over the time of harvest. Sugar content peaks (point of maximum compensation) in the middle of the season and a producer who has their cane harvested early, or late, sells less sugar. Fortunately, the issue is addressed in their contract.

8. Development of concept for multi-bale handling unit

8.1. Modulization of bales

Individual handling of bales (round or square) is not cost effective because of long loading and unloading times. Several concepts for a multi-bale handling unit are being developed. A verified multi-bale system is not available at this time to use as an example.

Permission was received [67] to present a concept that is far enough along in development that some field tests have been done. The concept was developed by a consortium led by FDC Enterprises and is shown in Figure 8. The self-loading trailer loads six stacks of six large rectangular bales, referred to "6-packs," for a total load of 36 bales. The bale size is 0.91

m × 1.22 m × 2.44 m. The length of the load is 6 × 2.44 = 14.6 m (48 ft), the height is 3 × 0.91 = 2.74 m (9 ft), and the width is 2 × 1.22 m = 2.44 m (8 ft).

Figure 8. Multi-bale handling unit concept developed for 3x4x8 large rectangular bales by FDC Enterprises-led Consortium (Reprinted with permission [67]).

The trailer built to implement the concept [68] is shown in Figure 9. Estimated load time is 5 min, which is about the same load time for the cotton module (Figure 7). The 36-bale unit can be off-loaded by the truck directly onto the conveyor into a bioenergy plant, or it can be off-loaded into at-plant storage to be used later, just as is done with cotton modules at a cotton gin.

A similar concept known as the "Rack System" envisions that round bales will be loaded into a rack in-field or at an SSL. This rack is lifted off a trailer at the plant, emptied as the bales are needed, and then returned to be refilled. The racks are cycled multiple times each week within the closed logistics system. Cundiff et al. [59] developed the rack system concept, and it will now be used as an example to illustrate how the principles required for a logistics system are implemented.

All the various concepts and options cannot be discussed. However, we will apply a specific example to help the reader think through a process of developing a logistics system. The selection of the "Rack System" for this example implies no criticism of other ways of implementing the multi-bale handling unit concept currently being developed.

Figure 9. Self-loading trailer built by Kelderman Mfg. to implement multi-bale handling unit concept for 36-bale stack of large rectangular bales.

8.1.1. Receiving facility

When a truckload of feedstock arrives at the bioenergy plant, what happens? Does this material go directly into the plant as in a just-in-time processing model? Or, does it go into some type of at-plant storage? If it goes into at-plant storage, is there an efficient procedure for placing it into storage and retrieving it? All these questions relate directly to the cost of Receiving Facility operations, thus, when a bioenergy plant commissions a logistics system, it first specifies how it wants to receive material. Key questions for a receiving facility are:

1. Do I want each load to be the same size with the truck loaded the same way (i.e. are bales need to be in the same configuration)?
2. What are the hours of operation, and can I schedule approximately the same number of deliveries each hour of the workday?
3. What size at-plant storage must be maintained?

Design of a receiving facility is beyond the scope of this chapter; but, it is hoped that by posing these questions, the reader will begin to think about the constraints that a bioenergy may have when specifying a logistic system. Two criteria used in this example are:

1. Weigh and unload a truck in 10 min, and
2. Cost effective flow of material in-and-out of at-plant storage to support 24/7 operation.

Design of a logistics system is directly linked to the design of the receiving facility – neither is designed independent of the other.

8.1.2. Farmgate contract

Creation of a multi-bale handling unit will require specialized equipment. It is not a cost-effective to require each farmgate contract holder to own this equipment The rack system, for example, envisions a farmgate contract whereby the contract holder grows the crop, harvests in round bales, and places these bales in storage at a specified SSL. The contract holder owns and maintains the SSL and is paid a storage fee for each unit of feedstock that is stored. The biomass is purchased by the bioenergy plant in the SSL. All agricultural operations are now "sequestered" in the farmgate contract which gives those seeking a farmgate contract a well-defined process to prepare their bid.

A SSL must be graded to a minimum slope specification, must be near a main road, and must have a compacted gravel base. The gravel reduces bale degradation from water damage on the bottom of the bale and provides a suitable surface for equipment operations. Each SSL will receive material from either a single large production field or multiple, smaller fields as proposed by Cundiff et al. [59].

8.1.3. Hauling contract

The rack system envisions that the hauling contractor will invest in industrial equipment needed for year-round operation. Because the hauling contractor is hauling year-round, they

can a) afford to invest in higher capacity industrial-grade equipment designed for up to 5,000 hour/year (or more) operation, and b) their labor force will develop expertise at the operations, and the Mg handled per unit of equipment investment will be a maximum. These two factors together create the potential to minimize hauling cost ($/Mg).

8.1.4. Storage

The logistics system has three storage features. Round bale packages act as self-storage and protect the biomass. Rounded top sheds water, so the round bale can be left in the field for a short time before in-field hauling. This provides the advantage of uncoupling the harvest and in-field hauling operations, and thus provides an opportunity for improving the cost efficiency of both operations. The farmgate contract holder has the opportunity to bale when the weather is right and haul later.

The second storage features are the SSLs. These locations provide the needed transition between in-field hauling and highway hauling. The SSLs will be located so that the Mg-km parameter for each SSL will be not more than 4 km. This means that, averaged across all Mgs stored at that SSL, each Mg will be hauled less than 4 km from the production field to the SSL. This constraint gives the producer an upper bound for calculating in-field hauling cost and all farmgate contractors are treated the same relative to in-field hauling cost.

The third storage feature is the inventory in at-plant storage, which provides the needed feedstock buffer at the plant. Those building a bioenergy plant would like to operate with just-in-time (JIT) delivery of feedstock as this gives them the lowest cost for receiving facility operation. If JIT is not possible, they want the smallest at-plant inventory for cost effective operation. There is obviously a trade-off in the logistics system design between the higher cost to purchase JIT delivery, and the cost of at-plant storage operations.

8.1.5. At-plant storage

In day-to-day management, it will be very difficult to achieve JIT delivery of any feedstock for 24/7 operation. All multi-bale handling unit concepts must include some at-plant storage. Even when known quantities of feedstock are stored in a network of SSLs, and a Feedstock Manager is controlling the deliveries, there will be delays.

To give a frame of reference, suppose 3 days of at-plant storage is the design goal. This inventory amount may suffice in the Southeast where ice and snow on the roads is not typically a significant problem for winter operations. But in the Midwest, additional days of at-plant storage will be required. A visualization of 3-day at-plant storage is shown in Figure 10. The number of racks shown is not part of the "cost analysis" example given later.

Bales will remain in the rack until processed; there is no individual bale handling at the plant. This is a very important aspect of any multi-bale handling system. This reduction in bale handling not only reduces cost, but also reduces damage to the bales. The integrity of the bales will be maintained, and bales will have additional protection from the rain since the rack has a top cover.

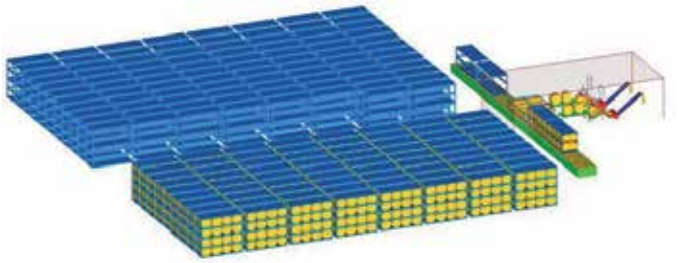

Figure 10. Illustration of at-plant storage for the rack system. At-plant storage shown with 144 racks with bales, 5 racks on conveyor (2 full, 1 being emptied, 2 empty), and 176 empty racks.

8.2. Functionality analysis for rack system concept

In this example, the analysis begins with round bales in ambient storage in SSLs and ends with a stream of size-reduced material entering the bioenergy plant for 24/7 operation.

8.2.1. List of baseline constraints

The constraints for this example are:

1. The rack design, and the design of the trailer to haul the rack, must conform to the standards for highway transport.
2. The rack used for this example holds sixteen 5×4 round bales. This round bale has a diameter of 1.52-m (5 ft) and a length of 1.22-m (4 ft). With two racks on a truck, a truckload is 32 this kind of round bales.
3. The bales will be pre-loaded into the racks while the trailer is parked at the SSL. When a truck arrives, a trailer with two empty racks is dropped, and the trailer with two full racks is towed away. The goal for the load time (trailer exchange) is 10 min, or less.
4. Hauling of the trailer and racks will be done 24 hours/day. Truck drivers will work five, 8-hour shifts per week and operations will be continuous for a 6-day work week. The rack loading crew at an SSL will be organized such that each worker will work a 40-h week. SSL operations will load bales into racks six 10-h workdays per week.
5. At the Receiving Facility, the racks are lifted from the trailer and placed on a conveyor to be conveyed into the plant for immediate use, or stacked two high in at-plant storage. The goal at the Receiving Facility is for the forklift to lift two full racks from the trailer and replace them with empty racks within of 10 min, or less.
6. The plant will operate with a maximum of 3 days of at-plant storage.
7. The example assumes that the plant processes one bale per minute. Assuming a bale weighs 900 lbs (0.408 Mg) in average (15% M.C.). The dry mass (DM) per bale is,

$$0.408\ Mg/bale \times \left(1-0.15\right) = 0.3468\ Mg\ DM/bale$$

Assuming one bale is processed per minute, the processing rate is,

$$0.33468\ Mg\ DM/bale \times 1\ bale/min \times 60\ min/h = 20.8\ Mg\ DM/h$$

For comparison, 20.8 Mg DM/h is 500 Mg DM/d. This size is in the 100 to 1000 Mg/d range recommended for Regional Biomass Processing Depots by Eranki et al. [54].

The processing time for racks is:

$$\frac{60 \text{ bale/h}}{16 \text{ bale/rack}} = 3.75 \text{ rack/h}$$

Thus, the number of truckloads consumed by the plant for a 24/7 operation is,

$$3.75\,rack/h \times 24\,h/d \times 7\,d/wk = 630\,rack/wk = 315\,truck\ loads/wk$$

8.2.2. Operation Plan for 24-h Hauling

The plant will operate 24/7, but the Receiving Facility will be open 24 hours per day for 6 days. At 6:00 Sunday morning, there will be enough feedstock accumulated in at-plant storage for the maximum 3-day buffer (72 hours). Hauling operations begin again at 6:00 Monday morning. At-plant inventory is decreased to a 2-day buffer (48 hours) to supply the material for operation from 0600 Sunday morning to 6:00 Monday morning.

To discuss the 24-h-hauling concept, it is convenient to consider SSL operations as: 1) "day-haul" operation, and 2) "night-haul" operations. For the day-haul operation, the racks are transported as they are filled during the day. For the night-haul operation, the required number of empty racks, enough for one day's operation, are pre-positioned at the SSL. Cost of pre-positioning the racks is not considered in this example. The SSL crew fills these racks with bales during their 10-h workday and they are hauled during the night. Each truck arriving during the night unhooks a trailer with two empty racks and hooks up a trailer with two full racks. The next morning, the SSL crew will fill the empty racks delivered during the night and fill them during their workday.

8.2.3. Operational plan for receiving facility

A forklift (10-ton or 9.07-Mg capacity) will operate continuously at the receiving facility. This forklift will lift two full racks from the trailer and place them onto a conveyor into the plant for direct processing, or stack these racks in at-plant storage. Then the forklift will lift two empty racks onto the trailer and then the truck returns to the SSL. Empty racks will be stacked in the storage yard until they are lifted onto trailers.

The operational plan calls for two forklifts at the receiving facility, identified as a "work horse" and a "backup." The workhorse will operate continuously and the backup will operate during the day when trucks are waiting in the queue. Key point – the system must have a backup forklift because, if a forklift is not available to lift racks off of and onto trailers, all operations cease.

The handling of the racks emulates the handling of bins at a sugar mill in South Florida. In the bin system, a truck has three bins, two on the first trailer and one on a "pup" trailer. The bins are side-dumped if material is processed directly (Figure 11), or the bins are off-loaded and

stacked two-high in the storage yard for nighttime operation (Figure 12). When the bins are dumped directly, it takes 3 min to dump the bins. For normal operation, one truck hauls 10 loads (30 bins) a day. At 37 tons/load (33.6 Mg/load), each truck hauls 370 ton/d (336 Mg/d). Sugar cane is 80% moisture content, so 370 ton = 74 dry ton/d/truck (67.2 Mg DM/d/truck).

Figure 11. Bins being side-dumped at sugar mill in South Florida, USA.

The conveyor for moving the racks into the plant is an adaptation of a piece of commercial technology. Conveyor design and function is similar to the conveyor used to move cotton modules into a cotton gin. Cotton modules and the 16-bale racks have approximately the same dimensions. The conveyor cost used in the analysis was obtained from a cotton equipment manufacturer. The receiving facility operates 6 d/wk, thus, on average, the daily delivery will be:

$$\frac{630 \text{ racks/wk}}{6 \text{ d/wk}} = 105 \text{ racks/d} = 53 \text{ trucks/d}$$

For this example, plant size (23 dry ton/h or 20.9 Mg DM/h) was chosen based on the expected capacity of the two forklifts at the plant. One forklift is expected to lift two full racks and lift and load two empty racks on a trailer at the rate of one truck every 27 minutes averaged over the 24-h a day. The design of the receiving facility and at-plant storage area has to facilitate this operation. A larger at-plant storage area will lower the forklift productivity (ton/h) because the average cycle time to move an individual rack is greater.

8.2.4. Size of at-plant storage yard

For example, maximum at-plant storage for a 3-day supply is, 3.75 racks/h times 72 h, or 270 racks. Racks will be stacked two high in "units" with two rows of 24 spaces each (Figure 12), thus there are 48 storage spaces in each unit. Each unit stores 96 racks. Three units are required for 270-racks storage.

$$3.75 \textit{ racks / h} \quad x \quad 72 \textit{ h} = 270 \textit{ racks}$$

If 7-day at-plant storage is required, the total number of racks increases to 630 racks, or seven 96-rack units as shown in Figure 14. This implementation of a 7-day supply is not

believed to be a cost effective choice, not only because of the capital investment in the racks, but also because of the forklift operating time required to cycle back and forth over a storage area this large. This is a key issue---the larger the at-plant storage, the lower the forklift productivity (ton/h), and thus the higher the forklift cost ($/ton).

The rack system competes best when the racks are filled and emptied as many times as possible in a given time period, not when they act as storage units. Other multi-bale handling units are more suitable for a larger at-plant storage like the one shown in Figure 14.

Figure 12. Bins being stacked in at-plant storage at sugar mill in South Florida, USA.

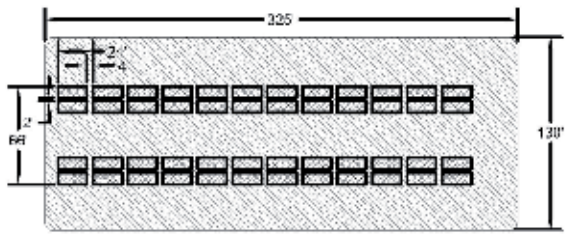

Figure 13. Sample layout of at-plant storage showing a unit (48 storage spaces). Racks are stacked two high for a total capacity of 96 racks (Total area required is approximately 4047 m^2).

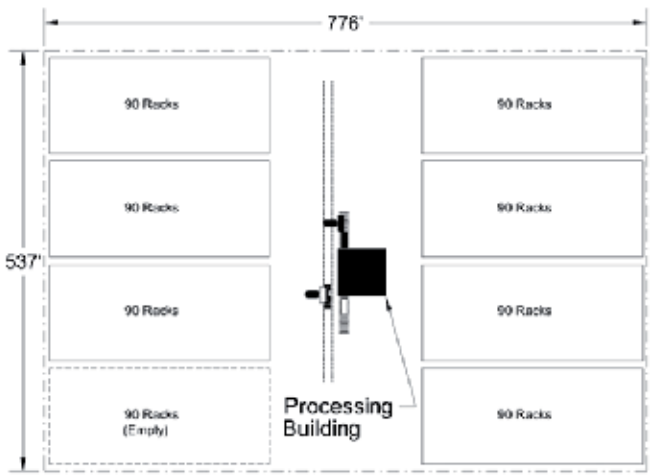

Figure 14. Sample layout of at-plant storage showing a 7-day supply of full racks and space for storing one unit of empty racks. (Total area required is approximately 38445 m^2).

8.2.5. Bale loading into racks at the SSL

Ideally, the bale- loading- into- racks operation at the SSL should be able to load 16 bales in a rack in 20 minutes. This design criterion has not been attained with actual equipment.

For a workday with 10 productive hours (600 min), a 20-min-per-rack operation can theoretically load 30 racks, or 15 truckloads. Given the reality of SSL conditions, an actual operation could not sustain this productivity. In this analysis, it is reasonable to assume that a mature operation can average 70% of the theoretical productivity. The number of loads-day-operation used for this analysis will be reduced to 15×0.7 = 10.5 loads/d. The number of individual SSL operations required is 315 loads per week, divided by 6-day SSL operation per week, which is 52.5 or 53 loads per day average operation. This is five operations averaging 10.5 loads per day.

Thus, loading operations will occur at five different SSLs with a different set of equipment for each operation. Each SSL operation will load an average of 21 racks per day. Time to move the crew, equipment and reposition the empty trailer/rack from one SSL to the next is not dealt with in this analysis. Thus, the 21 racks/d productivity may be optimistic.

There are several options to load the rack. The option which found to be the most cost effective is the "side-load" option [58]. A telehandler with special attachment can pick up two bales per cycle (Figure 15) and load these bales into the side of the rack. Assuming that the average productivity to be achieved under production conditions is 34 min per rack, then the time needed to load two racks on a trailer is 68 min. Remember, this is the assumed average load time for year-round operation.

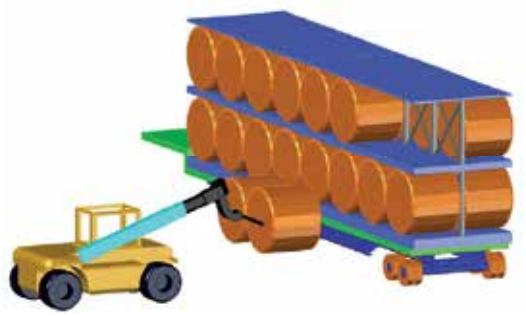

Figure 15. Concept for side loading bales into rack on trailer.

At the SSL, loading of the racks is the most challenging design for a cost-effective biomass logistics system. It is difficult to reduce the cost of these operations because the labor productivity (tons handled per worker-hour) might be low. By uncoupling the SSL loading and highway hauling, the truck does not have to wait for racks to be loaded in order to pick up the trailer. Also, the SSL crew does not have to wait for a truck to arrive with empty racks. However, the hooking/unhooking of trailers may be problematic for some drivers.

The day-haul operations are uncoupled by providing two extra trailers at the "day haul" SSL, and the night-haul operations are uncoupled by providing 9 extra trailers for the "night

haul' SSL. Each truck tractor then has 11 extra trailers (22 racks) in the system. This may not be a best (least cost) approach, but it does provide a reasonable starting point.

8.2.6. Influence of SSL Size on Rack Loading Operations

If the yield of switchgrass in a commercial-scale operation averages 4 ton/ac (8.96 Mg/ha), this equates to approximately 9 bales/ac (22 bales/ha). It is suggested that the minimum size of a SSL contain the bales from 60 ac (24.5 ha) is 540 bales, and the maximum size SSL approximately stores bales from 1,200 ac (487 ha) 10,800. If 70% of the theoretical loading (30 racks/d) is achievable, the contactor will load 21 racks times 16 bales/rack is equal to 336 bales per day. The minimum size SSL will remove all of the bales in about 540/336, which is 1.6 days. The maximum size SSL will be loaded out in 10,800/336 = 32 days.

Cost of SSL operations at the smaller SSLs will be higher because of the mobilization charge to move equipment and crew to the next SSL for a fewer days of operation. It is probable that the load-haul contract offered by the biorefinery for each individual SSL will consider the haul distance and SSL size and thus the per-ton price will be different for each SSL.

8.2.7. Total Trucks Required – 24-h Hauling

To achieve 24-h hauling, the truck drivers will work 8-h shifts and the trucks will run continuously from 0600 Monday to 0600 Sunday, a total of 144 h/wk. The total racks processed each week are 630, equal to 315 truckloads. If a uniform delivery is assumed, the average truck unload time is,

$$315\ trucks\ /\ 144\ h\ =\ 2.2\ trucks/h\ =\ 1\ truck/27\ min.$$

This productivity is well within the Rack System design goal of a 10 min unload time. As previously stated, the 24-h hauling concept envisions that the SSL crew will leave a supply of loaded racks on trailers at the SSL when they finish their 10-h workday. These racks will be hauled during the night. The next morning, the loading crew will load empty racks delivered during the night and load them during their workday.

The key variable in hauling is the truck cycle time. To calculate cycle time, for example, an average haul distance is needed. An actual database was developed for a proposed bioenergy plant location at Gretna, Virginia, USA to calculate the average haul distance.

An analysis was done for a 30-mi (48 km) radius around Gretna to identify potential production fields based on current land use determined from current aerial photography and GIS methods. It is conservatively assumed that 5% of the total land was assigned into switchgrass production. SSLs were established at 199 locations (Figure 5), and the existing road network was used to determine the travel distance from each SSL to the proposed plant location at Gretna. Some loads were hauled 2 miles (3.2 km) and some were hauled over 40 miles (64 km). A weighted ton-mi parameter was computed and found to be 25.4 miles (40.6 km). This means that, across all 199 SSLs, each ton travels an average of 25.4 miles (40.6 km) to get to the plant.

Truck cycle time is calculated using the 25.4-mile average haul distance, a 45 mph average speed, 10-min to hook/unhook trailers a SSL, and 10-min to lift full and empty racks from the trailers. Theoretical cycle time is 1.46 h. In 24 hours of operation, one truck can haul:

$$24\ h/\left(1.46\ h/load\right)\ =\ 16.4\ loads\ per\ truck\ per\ day$$

Assuming that a truck fleet can average 70% of the theoretical capacity, then (0.7×16.4 =11.5 loads per truck per day can be achieved. Remember, since the trucks run continuously, a decimal number of loads can be used as the average achieved per-day of productivity.

It is not practical to use the each-haul contractor-runs-their-own-trucks assumption for 24-h hauling. The way to maximize truck fleet productivity is to have the Feedstock Manager have the control to send any truck to any SSL where a trailer with full racks is available. This greatly facilitates the hauling at both day-haul and night-haul SSLs.

Total trucks being controlled by the Feedstock Manager is:

$$53\ loads/day\ required\ at\ the\ plant/11.5\ loads\ per\ truck\ =\ 4.6\ trucks\ \left(5\ trucks\right)$$

Since five SSL crews, one per SSL, are loading trailers/racks. More realistic is that eight to ten trucks will be available and some would also have responsible for bring other supplies to the biorefinery or hauling waste and other value-added products from the biorefinery.

8.2.8. Total Racks and Assets Required for 24-h Hauling

Since the only time deliveries are not being made is the 24-h period from 0600 Sunday to 0600 Monday, the amount in at-plant storage can be reduced. Using 1.5 days as the minimum at-plant storage, so the total capacity hours required in at-plant storage at 0600 Sunday, when deliveries are ended for the week, is

$$24\ h\ \left(actual\right)\ +\ 1.5\ d\ \times\ 24\ h/d\ \left(at-plant\ storage\right)\ =\ 60\ hours$$

$$3.75\ racks/h\ \times\ 60\ h\ =\ 225\ racks$$

Total trailers are calculated as follows. Each truck has one trailer connected, two parked at a "day-haul" SSL and nine parked at a "night-haul" SSL for a total of 12 trailers. The total racks on trailers are calculated as:

$$5\ trucks\ \times\ 12\ trailers\ per\ truck\ \times\ 2\ racks/trailer\ =\ 120\ racks$$

Total racks required are:

At-plant	+	On 60 trailers	+	Reserve	=	Total
225	+	120	+	5	=	350

The actual number of racks required is calculated by subtracting the racks on parked trailers from the rack total (empty + loaded) at the plant. Potentially, 60 loaded trailers can

be parked at the receiving facility when hauling ends for the week at 0600 Sunday. In order for this procedure to work, the racks on most of these 60 trailers have to be returned to SSLs during the period 0600 Sunday to 0600 Monday so they will be in position for operations to begin at each SSL at 0600 Monday. This requires some empty back hauls. Cost for these empty back hauls is a level of detail that must wait for a more sophisticated analysis.

When racks on trailers are counted as part of the at-plant storage, the minimum number of racks is:

At-plant	+	On 60 trailers	+	Reserve	=	Total
(225-120)	+	120	+	5	=	230

Average number of cycles per rack is 29,610 racks processed per year divided by 230, that is 129 cycles per year; or about 2.7 cycles per week for 47 weeks of annual operation.

8.3. Cost analysis for 24-h hauling using rack system

The costs given in this section are presented without supporting detail. They were calculated using the procedures given in [69]. They are "best estimates" given current cost parameters. All costs are given on a $/Mg DM basis for operation of a bioenergy plant consuming 23 dry ton/h (20.9 Mg DM/h). The challenge is to find a way that machine productivity (Mg/h) can be increased.

8.3.1. Total truck cost

The assumed truck cost (tractor and trailer for hauling the two racks) is $630/d for a 24-h workday, which includes ownership plus operating cost, plus labor, but excluding fuel.

Truck cost, excluding fuel, is:

$$\frac{\$630/\mathrm{d}}{11.5\,\mathrm{loads/d}\times 12.2\,\mathrm{dry\ ton/load}} = \$\,4.49/\mathrm{dry\ ton} = \$4.95/\mathrm{Mg\ DM}$$

Truck fuel cost for the 25.4 miles (40.6 km) average haul distance is:

$$\left(25.4\ mi\ \times 2\right)/4\ mi/gal\ =\ 12.7\ gal\ \times\ \$3.50/gal\ =\ \$44.45/load$$

$$\$44.45/load/\ 12.2\ dry\ ton/load\ =\ \$3.64/dry\ ton\ \left(\$4.01/Mg\ DM\right)$$

Total truck cost is ownership and operating plus fuel, that is 4.95 plus 4.01 = $8.96/Mg DM.

8.3.2. Load, unload operations

1. Handling racks at plant: $1.93 (forklift) + $1.02 (backup forklift) = $2.95/dry ton
2. SSL operation: $3.66 (telehandler) + $0.98 (extra trailers) = $4.64/dry ton
3. Rack cost: cost for 230 racks = $1.80/dry ton

4. Storage yard at processing plant: $0.13/dry ton
5. Conveyor entering plant: $0.28/dry ton

(Note: 1 dry ton = 0.907 Mg DM)

8.3.3. Size Reduction

1. Unroller-chopper cost: $5.76/dry ton.
2. Rack cost: all costs associated with the ownership and maintenance of the racks.
3. Loading cost: all costs associated with the loading of bales into racks. These costs are referred to as "SSL operation costs".
4. Truck cost: all costs associated with the ownership and operation of the trucks.
5. Receiving Facility cost: all costs associated with the unloading of racks from trucks, placement of racks onto conveyor (or placement in at-plant storage), conveyor operation, operation of at-plant storage, and removal of racks from at-plant storage and placement on trucks for return to SSL.
6. Size reduction: all costs associated with the unloading of bales from the rack, operation of conveyor for single file bales delivered to size reduction machine, and operation of machine for initial size reduction.

Truck cost is 34% of the total cost, SSL operations are 20%, Receiving Facility operations are 14%, size reduction is 24%, and the racks are 8%. It is clear why the Rack System Concept was organized to maximize truck productivity; truck cost is the largest cost component. Truck cost plus SSL operations are $12.77/dry ton, or 54% of total cost. The Receiving Facility cost is $3.37/dry ton, only 14% of total cost. As with all other multi-bale handling system concepts, the Rack System provides an opportunity for minimizing cost between the plant gate and the size reduction unit operation.

The total cost shown in Table 2 does not include the farmgate contract cost (production, harvesting, in-field transport, storage in SSL, and profit to producer). The farmgate contract cost can be estimated from local data for production, harvest, and ambient storage of round bales of hay. In the Southeast the key issue relative to the hay cost comparison is the difference in yield; switchgrass will yield about 9 Mg/ha as compared to traditional hay species that yield about 4.5 Mg/ha.

Operation	**Cost ($/dry ton)***
Racks	1.80
Loading at SSL	
Telehandler	3.66
Extra Drop-deck Trailers	0.98
Truck cost	8.13
Unloading at plant	
Workhorse forklift	1.93
Backup forklift	1.02
At-plant storage (Gravel lot with lighting)	0.13

Operation	Cost ($/dry ton)*
Conveyor into plant	0.28
Unroller-chopper (Initial size reduction)	5.76
Total	$23.69

* $1/dry ton = $1.103/Mg DM

Table 2. Total cost for hauling, receiving facility operations, and size reduction for rack system example – side-load option, 24-h hauling.

9. Conclusions

The key decision points for the design of a logistics system for a bioenergy plant operating 24/7 year-round are summarized as follows.

1. A complete logistics system is defined as one that begins with the biomass standing in the field and ends with a stream of size-reduced material entering a bioenergy plant for 24/7 operation. Optimizing one unit operation in isolation may increase the cost of an "upstream" or "downstream" operation such that total delivered cost is increased.
2. Most feedstock is harvested only part of the year, thus storage is a part of the logistics system. A cost effective logistics system provides for efficient flow of material in and out of storage.
3. Just-in-time (JIT) delivery of feedstock provides for a minimum at-plant storage cost. Since JIT delivery is not practical for typical biomass logistics systems, there is always a cost trade-off between the size of at-plant storage and the other design constraints needed to insure a continuous feedstock supply. Knowledge of quantities of biomass in Satellite Storage Locations (SSLs) provides the Feedstock Manager at a bioenergy plant an opportunity to minimize the at-plant storage cost.
4. Farmgate contracts that require a summer-early fall harvest must compensate for the removal of nutrients, and contracts that require a winter harvest must compensate for loss of yield incurred by the delayed harvest.
5. Assigning different unit operations to different entities in the business plan can lower average delivered cost. For example, it is more efficient to pool all farmgate activities into a farmgate contract and have a hauling contractor handle all load-haul activities. This division is defined as a division between "agricultural" and "industrial" operations. The key benefit achieved is in the capitalization of the equipment. Load-haul contractors can afford to invest in industrial-grade, high-capacity equipment designed for year-round operation as compared to farmgate contractors who will use their equipment 400 hours (or less) per year.
6. Uncoupling of the unit operations in the logistics chain can provide an advantage.
   a. In the agricultural operations, baling uncouples the harvesting and in-field hauling operations. When the harvesting operation is not constrained by in-field hauling---both unit operations can proceed at maximum productivity.

   b. In the industrial operations, it is important to uncouple truck loading from hauling. Maximum loads-per-day-per-truck are achieved when the loading crew never has to wait for a truck to arrive and the truck never has to wait to be loaded.
7. Truck cost is the largest component of total cost in most logistics systems, thus it is essential to maximize truck productivity (Mg hauled per unit time) by increasing both Mg-per-load and loads-per-day. A 10-min load time and a 10-min unload time is a desired goal for design of most logistic systems.
8. Multi-bale handling units are in need to solve the rapid loading/unloading challenge.
9. Twenty-four-hour hauling can minimize truck cost ($/Mg). The challenge is to design a logistics system with a practical procedure for loading trucks at night at a remote location.
10. The design of the Receiving Facility, because of the need to unload trucks quickly, is critical in the design of a complete logistics system. Typically, this design specifies that each load have the same configuration, and requires a delivery schedule where approximately the same number of loads is received each workday.
11. The most cost-effective logistics system will be structured such that information technologies (GPS, bar codes, entry of data over cell phone network) and optimization routines developed for other logistics systems can be used to optimize asset utilization in real time.

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Bio-Reactors

Chapter 7

Biological Activated Carbon Treatment Process for Advanced Water and Wastewater Treatment

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Yongning Feng and Xiaochang C. Wang

Additional information is available at the end of the chapter

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1. Introduction

The development of biological activated carbon (BAC) technology is on the basis of activated carbon technology development. Activated carbon which is used as a kind of absorption medium plays an important role in perfecting the conventional treatment process. Furthermore, activated carbon technology becomes one of the most mature and effective processes to remove organic contaminants in water. Removal of the odor in raw water can be regarded as the first attempt of activated carbon which can play a part in water treatment. The first water treatment plant in which granular activated carbon adsorption tank used was built in 1930 in Philadelphia, United States[1]. In the 1960-1970s, developed western countries started to use activated carbon technology in potable water treatment to enhance the removal of organic contaminants. By then, prechlorination was commonly used as the first step of activated carbon treatment. As the inflow of carbon layer contained free chlorine, the growth of microorganism was inhibited and no obvious biological activity showed in the carbon layer.

In order to improve the removal efficiency of refractory organics, especially the removal of precursors of DBPs, ozonation is commonly used in preoxidation before activated carbon process. The process which combines ozonation and activated carbon treatment was firstly put into practice in the year of 1961 at Amstaad Water Plant in Dusseldorf Germany. The successful trial in Dusseldorf soon arose great attentions from the engineering field in Germany as well as the Western Europe[2]. The advantages of microorganisms growing in the activated carbon layer was first affirmed by Parkhrust and his partners in 1967[3,4], this demonstration enabled the lengthening of the GAC's (Granular Activated Carbon) operation

life to a great extent and Ozonation-Biological Activated Carbon technology was finally established. Since early 1970s, the study and application of Ozonation-Biological Activated Carbon treatment were conducted in large scales, among which the major ones are as the followings: the application in water plant of Auf dem Weule, Bremen Germany on a half productive scale[5] and the application in Dohne water plant of Muelheim Germany on productive scale[6]. The successful application of Ozonation-Biological Activated Carbon technology in Germany is widely spread and used in neighboring countries, and the treatment itself was perfected gradually. In late 1970s, the treatment was popularized in Germany. In the year of 1976, the United States Environmental Protection Agency (US EPA) legislated that the activated carbon process must be adopted in potable water treatment process in urban areas with a population over 150,000. Among the water plants using activated carbon treatment, the most representative ones are: Lengg Water Plant in Switzerland[7] and Rouen La Chapella Water Plant in France[8,9], see Fig. 1. the flow diagram. The BAC process was firstly proposed in 1978 by G.W.Miller from the US and R.G.Rice from Switzerland[9]. In 1988, the quality requirements for potable water were improved in Japan and during the years 1988-1992, Kanamachi, Asaka, Kunijima and Toyono water treatment plants using the Ozonation-Biological Activated Carbon process were built[10].

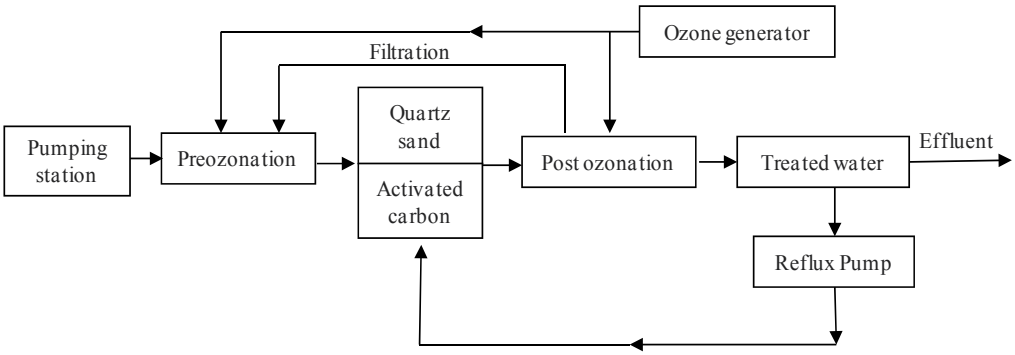

Figure 1. Flow diagram of the water plant in Rouen La Chapella

By now, BAC process has become the major process in advanced water treatment, which is commonly used in developed countries such as America, Japan, Holland, Switzerland, etc[1]. Meanwhile, the process is also widely used in industrial wastewater treatment as well as waste water reclamation. According to the prediction of experts, because of the increasing seriousness of the pollution in potable water and the strictness of requirements for potable water quality, the BAC process which combines the functions of physical-chemical absorption and biological-oxidation degradation, will become the conventional process widely used in potable water treatment plant[9].

2. Composition of biological activated carbon process

2.1. Composition and application

2.1.1. Basic principles of biological activated carbon technology

Biological Activated Carbon process is developed on the basis of activated carbon technology, which uses the synergistic effect of adsorption on activated carbon and biodegradation to purify raw water. Activated carbon has a high specific surface area and a highly developed pore structure, so it is characterized by its great effect on absorbing dissolved oxygen and organics in raw water. For Biological Activated Carbon technology, activated carbon is used as a carrier, by accumulating or artificially immobilizing microorganisms under proper temperature and nutrition conditions, the microorganisms will reproduce on the surface of the activated carbon and finally form BAC, which can exert the adsorption and biodegradable roles simultaneously[11]. The Biological Activated Carbon technology consists of the interaction of activated carbon particles, microorganisms, contaminants and the dissolved oxygen, in water solution. Fig. 2. shows the simplified model that how the 4 factors interact with each other[12]. The relationship between the activated carbon and contaminants is simply the effect of adsorption of activated carbon, and the reaction depends on the properties of the activated carbon and contaminants. Meanwhile, the activated carbon can adsorb DO and microorganisms which were adsorbed on the surface of activated carbon, feed on DO will biodegrade contaminants. In brief, by the interaction of these 4 factors, the purpose for removing contaminant from raw water can be achieved by adopting the biological activated carbon.

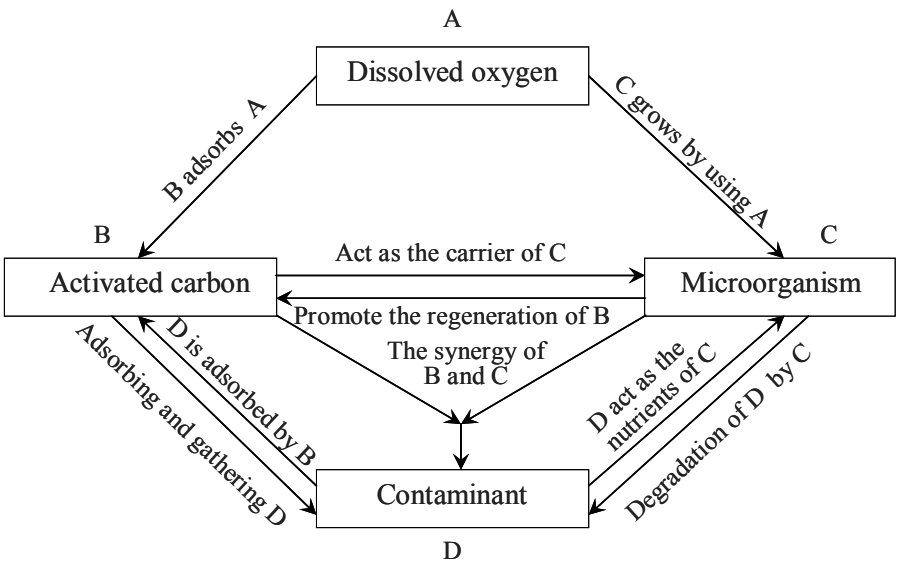

Figure 2. Simplified interaction model of factors in BAC process

2.1.2. Application fields and the typical process flow of biological activated carbon technology

At present, the application of BAC technology is mainly focused on 3 aspects: the advanced treatment for potable water and the industrial waste water treatment. The typical process of the advanced treatment for drinking water and sewage reuse is shown in Fig. 3. All of the three processes are based on conventional coagulation-sedimentation-filtration way. They are distinct from the different positions, the two processing points, to import ozone and the activated carbon. In process a, the activated carbon procedure is between sedimentation and filtration. The outflow from the activated carbon layer will bring some tiny carbon particles and fallen microorganisms, which will be removed by a sand filter in the end. To improve the filtration efficiency, chlorination and enhanced coagulation were done firstly before this procedure. In this process, the quality of the outflow is guaranteed with a relatively higher ozone dosage. While in Process b, the ozonation and the activated carbon procedure is done after the filtration, by which, the ozone depleting substances will be removed therefore a lower ozone dosage than Process a. However, micro carbon particles and microorganisms which leap out of the activated carbon layer will have an undesirable impact on the quality of the outflow, so the frequent backwash on the activated carbon layer is required. Process c is characterized by a two-level ozone procedure, which means to put ozone separately before and after the sand filtration, the remaining procedures are same with Process b. A lower ozone dosage before the sand filtration is used to improve the filtration efficiency[13].

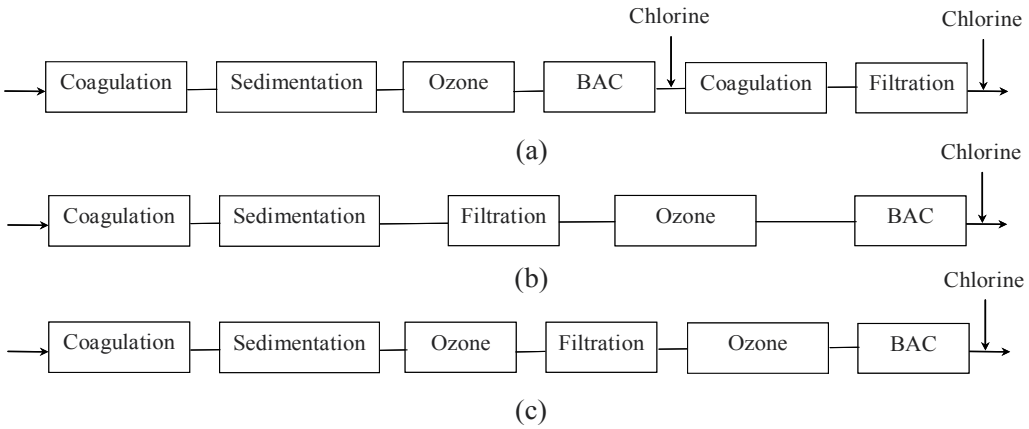

Figure 3. Typical processes of BAC

Also, it is widely used in industrial waste water treatment, such as printing and dyeing wastewater, food processing wastewater, pharmaceutical wastewater, etc. Throughout the typical process of BAC treatment, it is obvious that these three technological processes are related to oxidation-BAC technology. Compared with conventional bio-chemical technology, contact oxidation-BAC process has its unique characteristics. Firstly, contact oxidation can remove organics and ammonia-nitrogen, reduce odor and the amount of DBPs precursor, as well as to reduce the regrowth possibility of bacteria in pipeline, so as to increase the biological stability. Secondly, contact oxidation can reduce the processing load

of BAC treatment, and, to some extent, increase the working life and capacity of remaining filtration and BAC, which ensure a safer, reliable outflow[14-17].

2.1.3. Basic operational parameters of BAC process[18]

To design a BAC system, it is necessary to comprehend the characteristics of water quality, water amount and some certain index of water treatment. First of all, the experiments on the adsorption performance and biodegradability of the waste water are indispensable. Then, according to the result of static adsorption isotherms experiment on the raw water, the appropriate kind of the activated carbon can be chosen, and on the basis of dynamic adsorption isotherms experiment, the basic parameters can be determined. Ultimately, according to the process scale and condition of the field, BAC adsorption devices and its structure as well as supplementary equipment can be determined.

The activated carbon used in BAC process, should be highly developed in the pore structure, especially for the filter pores. Quality of the outflow is directly influenced by the filtering velocity, height of the carbon layer, the retention period and the gas-water ratio. In practice, the general filtering rate is 8-15 km/h. Retention period: According to the different pollutants, the general retention period should be 6-30 min; when the process is mainly removing smelly odor from the raw water, the period should be 8-10 min; when the process is mainly dealing with COD_{Mn}, the period should be 12-15 min. Gas-water ratio: As to aerobic microorganism, sufficient DO in the activated carbon layer is needed. Generally, DO>1 mg/L is proper in the outflow, therefore the design is based on a (4-6):1 gas-water ratio, specific details are based on the height of the carbon layer and concentration of organic contaminants. Generally, the thickness of the activated carbon layer is 1.5-3 m, which is determined by the leaping curve of the activated carbon. The growth of microorganisms and suspended solids brought by the inflow on the long-term operating biological carbon bed may block the carbon layer, and the biofilm on the surface of the activated carbon is unlikely to be discovered by naked eyes. Once the thickness is out of limits, the adsorption ability of the activated carbon will be affected undesirably. Therefore, the carbon layer should be washed periodically. Related parameters for backwash are shown in table 1.

Backwash type		Granular activated carbon grade	
		2.38~0.59 mm	1.68~0.42 mm
Air-water backwash	Water wash intensity [L/(m²·s)]	11.1	6.7
	Water wash interval (min)	8~10	15~20
	Air wash intensity [L/(m²·s)]	13.9	13.9
	Air wash interval (min)	5	5
Water wash and surface wash	Water wash intensity [L/(m²·s)]	11.1	6.7
	Water wash interval (min)	8~10	15~20
	Air wash intensity [L/(m²·s)]	1.67	1.67
	Air wash interval (min)	5	5

Table 1. Backwash parameters for activated carbon

2.2. O_3-BAC process and the evaluation of ozonation

2.2.1. Mechanism and characteristics of O_3-BAC process

In practice, there are still some problems when BAC technology is used alone, for example, some difficult biodegradable materials can not be removed effectively and the working life of BAC would be reduced. Meanwhile, in order to ensure the safety of water distribution system, disinfection is indispensable after biological treatment. When chlorine treating potable water is used, large amounts of halogenated organic byproducts will be produced during the reaction between the organics and the chlorine. Among those byproducts, THMs, HAA_s, etc., are carcinogenic. Therefore, before BAC treatment, pre-ozonation, the O_3-BAC Process, is widely used, which concludes 3 procedures: ozonation, adsorption effect of activated carbon and biodegradation[19]. When O_3-BAC Process is used, organic will be firstly oxidized into small degradable molecules by strong oxidation of ozone, then the small degradable molecules will be adsorbed onto the activated carbon and degraded by microorganism, simultaneously the oxygen discomposed from ozone will enhance the level of DO, which makes DO in raw water be saturated or approximately saturated, which in turn, provides necessary condition for biodegradation[20-25]. Fig. 4. shows a simplified model of mutual effects among the main factors during O_3-BAC Process [26].

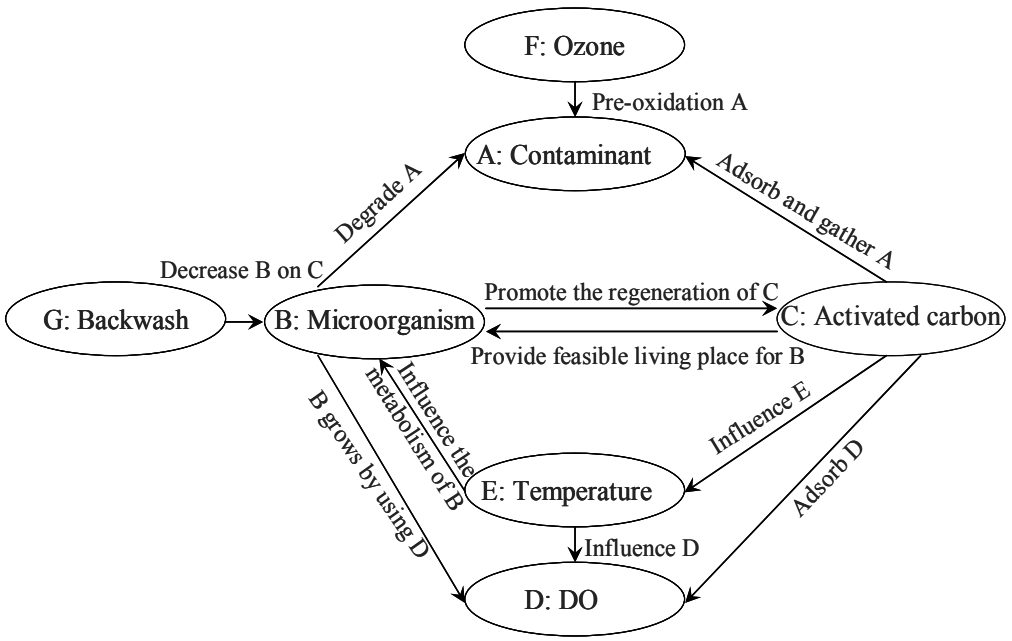

Figure 4. Model scheme of O_3-BAC

2.2.2. Effect of ozonation on molecule weight distribution and the molecule structure of organic matters[27-30]

2.2.2.1. Effect of ozonation on molecule weight distribution of organic matters

Pre-ozonation can change the properties and structures of organics in raw water. By using high performance liquid chromatography (HPLC), the variations of molecule weight before and after ozonation is studied, the result is shown in Fig. 5. As shown, the molecule weight mostly distributed within a range of 2000-6000, and 2000-3000 after the ozonation, the amount of organics with a relative molecule weight under 500 is increased dramatically. This indicates that macromolecules are in a high proportion in raw water, but after the ozonation, the proportion of macromolecular organic matter decreases while small molecule weight organics increases, which implies that part of the intermediate material from ozonation is namely the increased small molecular weight organic matter.

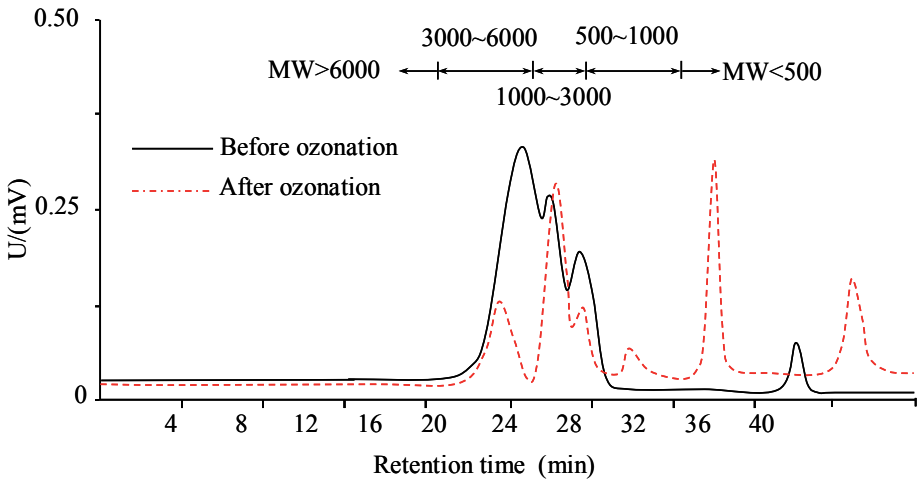

Figure 5. Molecular weight distribution before and after ozonation

2.2.2.2. Effect of ozonation on the structure of organic matters

In this part, Gas Chromatography-Mass Spectrometer (GC-MS) is used to analyze the structure of organics in raw water before and after ozonation, results are shown in Fig. 6. As shown, the organics in raw water are mainly aromatic hydrocarbons, chain hydrocarbon and aliphatic organics; after ozonation, the amount of aliphatic organic matters increases significantly and the amount of esters tend to increase too, which indicate that part of the aromatic organics are oxidized into organics of oxygen-containing groups, such as fatty acids, carboxylic acids and esters.

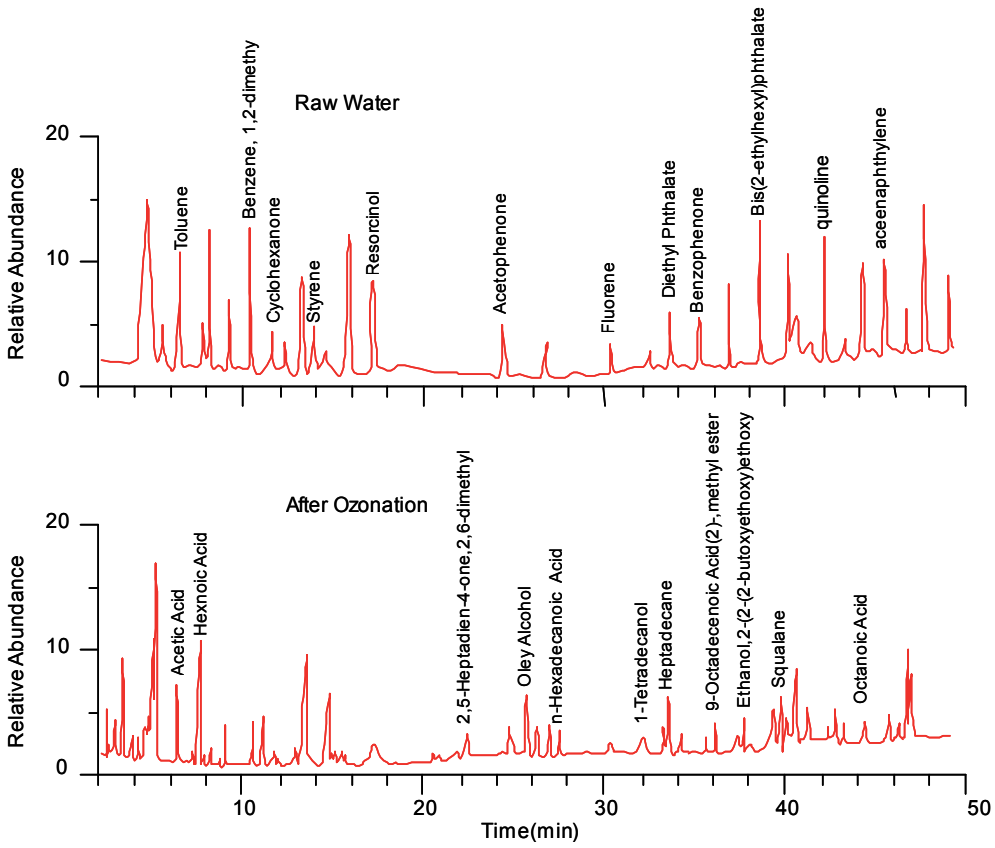

Figure 6. GC-MS chromatogram of raw water before and after ozonation

2.2.3. Improvement of biochemical properties of organics by ozonation

During the process of ozonation, complex chemical reactions occurred between ozone and organics. Pre-ozonation can change the biodegradability of organics in water, so generally BDOC is used as an index to analyze the water after ozonation. Fig. 7. shows the variation tendency of DOC, BDOC and BDOC/DOC in raw water after ozonation. The result indicates that after ozonation, DOC in raw water is seldom removed while BDOC increases obviously, among which the ratio of BDOC/DOC increases from 11.6 % to 26.4 %. After ozonation, the biodegradability of organics in water is highly improved, which enhance the biodegradation of BAC filter bed in the following procedure.

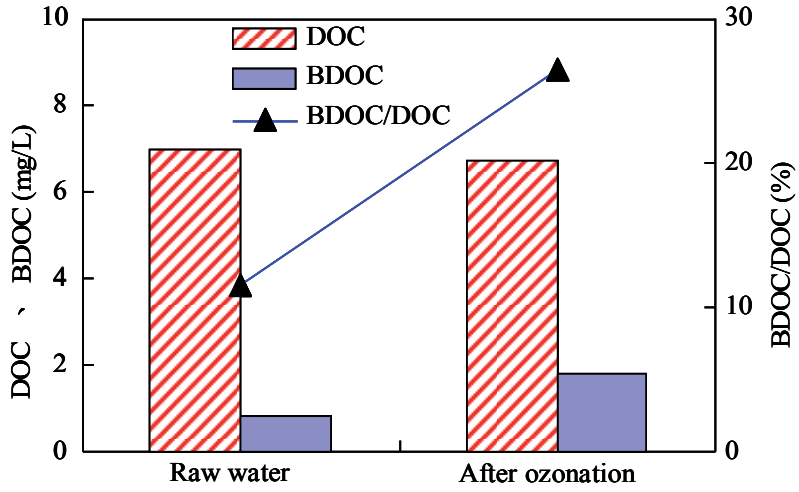

Figure 7. Variations of DOC, BDOC, BDOC/DOC before and after ozonation

2.2.4. Improvement of ozonation on biodegradability of organic matters

Relevant researches indicate that ozonation can change the molecule weight distribution, structures and the biodegradability of the organics in water. To thoroughly understand the role organics degradation and ozonation played in the whole process, degradation of the organics without dosage of ozone shall be taken for comparison. Variation of DOC, BDOC, BDOC/DOC on each layer of BAC bed with ozone or without ozone are both reviewed, the result is shown in Fig. 8 and 9.

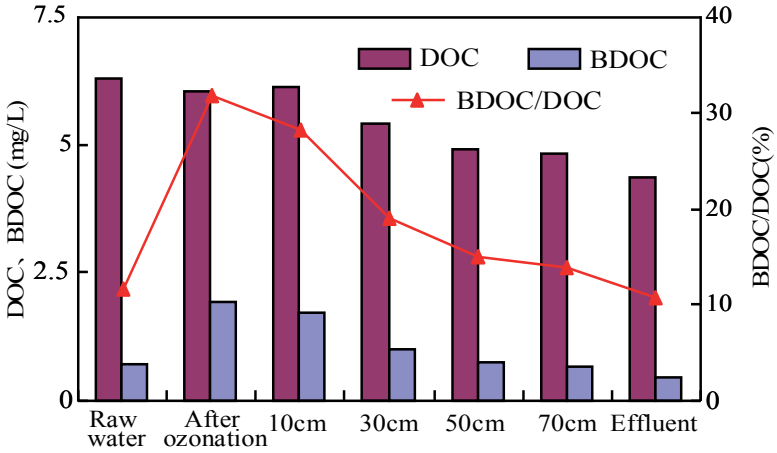

Figure 8. Degradation effects of different layers in BAC bed with ozone pretreatment

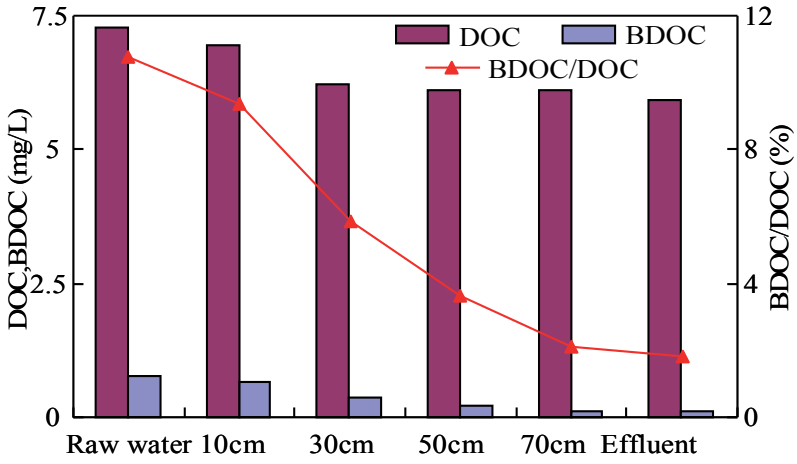

Figure 9. Degradation effects of different layer in BAC bed without ozone pretreatment

As shown in the figure, no matter the ozone is added or not, the tendencies of organics degradation in BAC bed are similar. With the addition of ozone, the properties of organics after ozonation will change, and the amount of BDOC increased. Viewed from aspect of general efficiency of removal, with the addition of ozone, the removal rate of DOC is 31 %, while 18 % removal rate without ozone.

According to further analysis of the data in Fig. 9, results show that, degradation efficiency of DOC without ozone is 18.4 %, DOC of outflow from the BAC bed is 5.92 mg/L and the removal quantity of DOC is 1.351 mg/L, in which the removal quantity of BDOC is 0.676 mg/L. In this case, the removal of NBDOC takes a proportion of 49 % in the total removal of DOC. Bio-degradation mainly removes BDOC in organics, while adsorption mainly removes NDOC and part of BDOC. That is to say, for BAC process absorption takes a proportion of 49 % during the entire removal process without dosage of ozone. Similarly, to analyze the data shown in Fig. 8, the variation of adsorption and bio-degradation effects during the organic degradation process whether added with ozone or not are shown in Table 2.

BAC/DOC Degradation	With Ozone Pretreatment	Without Ozone Pretreatment
Adsorption	35 %	>49 %
Biodegradation	65 %	<51 %

Table 2. Effect of ozonation on adsorption and biodegradation during BAC process

According to Fig. 3, on the aspect of the adsorption and bio-degradation in DOC removal process, ozonation can enhance the bio-degradation effect greatly, while exert a negative effect on adsorption of activated carbon.

3. Characteristics of microorganisms and mechanisms of pollutants degradation in BAC filtration

3.1. Immobilization of microorganisms on the carriers

At present, most of the BAC process is based on ordinary BAC which is naturally formed during the long-term operation. Due to the complexity of biofacies on its surface, the greatest deficiency of conventional BAC is that dominant microflora are hard to be formed, which has been improved by the rapid development of modern biology technology and the technology known as immobilization biological activated carbon (IBAC). The basic principle of IBAC is screening and acclimation dominant biocommunity from nature, followed by immobilizing the community on activated carbon, to enhance the efficiency and rate of degradation. Meanwhile, dominant biocommunity should be nonpathogenic and has strong antioxidant capacity and enzyme activity which enable the growing and reproducing under poor nutrition environment. Therefore, the effect of biodegradation after activated carbon adsorption saturation is highly improved by IBAC[31-32].

3.1.1. Intensification and immobilization of dominant biocommunity

Dominant microflora is selected by artificial screening and domesticating or directive breeding through biological engineering technology. Separation is the first step for getting dominant biocommunity. However, the filtered biocommunity may contain pathogenic bacteria, or with low-rated growth or high requirements for nutrition, which make it inadaptable for practical application, therefore, preliminary screening is indispensable for guaranteeing the security of selected bacterial strains. Due to the high complexity of biofacies, various species but less nutrition matrix in water, low-activated bacterial strain screened. This situation makes a relatively high disparity from the effective biological treatment. Therefore, intensification on selected bacterial strain is necessary. The process of intensification is actually the process of induction and variation of biocommunity. By changing nutritional conditions repeatedly, the bacterial strain will gradually adapt to the poor nutrition environment in fluctuation, so that the bacterial strain immobilized on activated carbon can preserve a strong ability for biodegradation[33].

During IBAC treatment, the process of microorganisms immobilizing is of great complexity, which is not only related to various acting forces, but also involves microorganism growth as well as the ability for producing extracellular and external appendages[34]. DLVO theory can give a better explanation that how bacteria are attached, when bacteria are regarded as colloidal particles[35-37]. Although there is a relatively high repulsive force during the process when the bacteria approaching to the activated carbon, the bacteria will ultimately contact with the activated carbon under the bridging effect of EPSs[38]. The bacteria may take advantage of the bridging effect with surficial particles, which enables the attachment of itself with filter materials at secondary extreme point in accordance with the theoretical level diagram of DLVO theory, rather than by the certain distance or overcoming the necessary energy peak as that of non-biological particle[39], see Fig. 10. for details. Although there are

various ways to immobilize microorganisms, and any method with a limitation for free-flowing of microorganisms can be used to produce and immobilize microorganisms, an ideal and universal application way for immobilizing microorganisms is still not available. There are 4 common methods of immobilization, see Table 3 [40-42] for details.

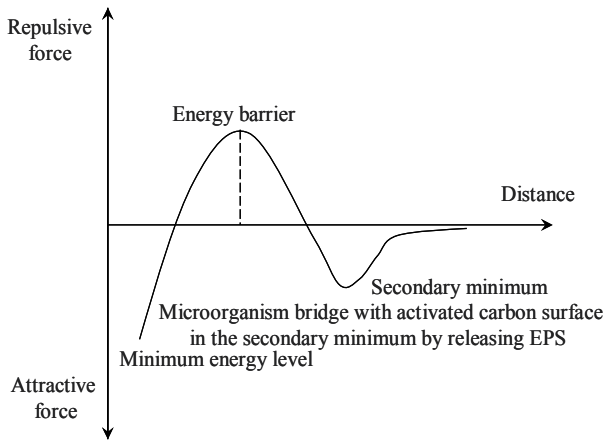

Figure 10. Total potential energy for express microbial immobility on activated carbon based on DLVO theory and short-range force

Property	Covalention	Adsorption	Covalent	Embedding
Implementing difficulty	Moderate	Easy	Difficult	Moderate
Binding force	Strong	Weak	Strong	Moderate
Active surface	Low	High	Low	Moderate
Immobilized cost	Moderate	Low	High	Low
Viability	No	Yes	No	Yes
Applicability	Bad	Moderate	Bad	Good
Stability	High	Low	High	High
Carrier regeneration	Unable	Able	Unable	Unable
Steric hindrance	Larger	Small	Larger	Large

Table 3. Comparison of the methods for immobilization of common microorganisms

3.1.2. Growth characteristics of dominant microflora on the surface of activated carbon[43]

In the early period of IBAC operation, the results for the biomass and biological activity on the surface of activated carbon monitored continuously are shown in Fig. 11. and Fig. 12.. At initiating stage, there is a rapidly dropping period for the dominant micoflora biomass on activated carbon. As time goes by, dominant biocommunity becomes steady gradually. At initial stage of BAC process, the biomass on activated carbon surface is in little, different from IBAC process, therefore, the microbial action can be ignored basically. During this period, variation of the biological activity for dominant biocommunity is similar to the change of biomass.

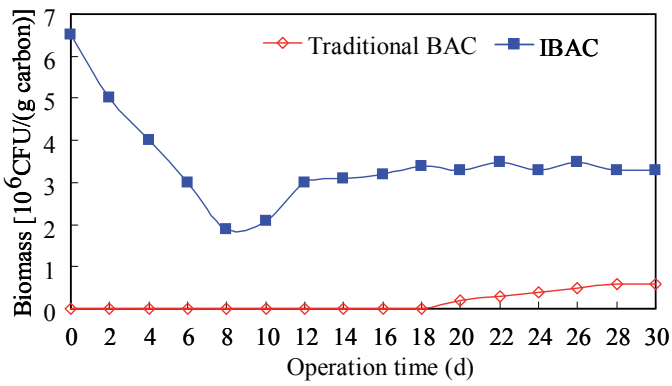

Figure 11. Variations of biomass on activated carbon

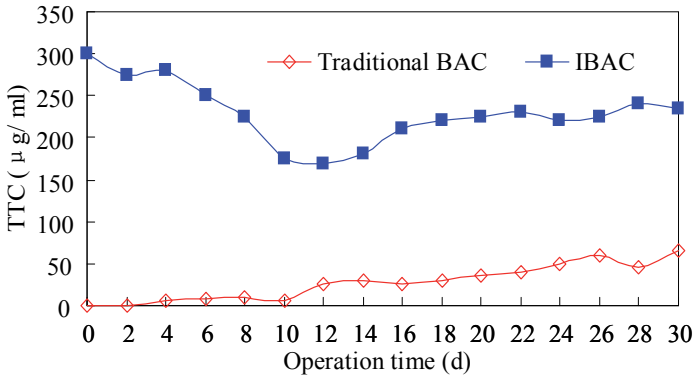

Figure 12. Variations of biological activity in two activated carbon processes

The variations of biomass and biological activity of microbe on activated carbon in long-term operation process are shown in Fig. 13. As shown, the biomass on carbon bed remains constant which mainly because of a gradual adaptation process of dominant biocommunity to the water, during which the adhesive ability of the dominant biocommunity is relatively low. Therefore microbe on the upper layer will be washed into the lower layer of the activated carbon, which has a beneficial effect on the reasonable distribution of the dominant biocommunity. With the extension of operating time, the dominant biocommunity will be adapted to the environment gradually. Meanwhile, due to the higher concentration of nutrient media in the upper layer, the biomass on the upper layer will be greatly increased and remains constant for a long period. Being different from the constancy of biomass on activated carbon, the biological activities in the upper and the lower layers of IBAC bed have a decreasing tendency as operation time goes. Shown from the result of PCR-DGGE, this reduction is mainly caused by the continuous incursion of the native bacteria with low capacity.

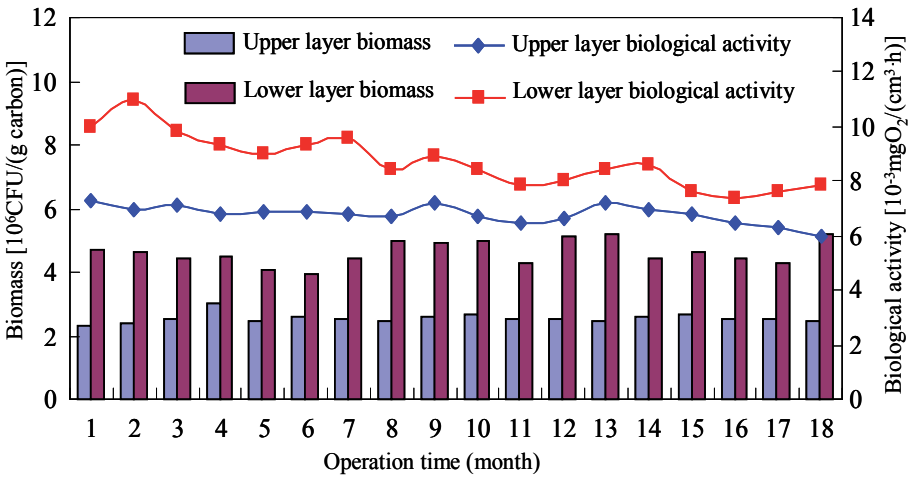

Figure 13. Variation of biomass and biological activity on activated carbon

3.1.3. Influencing factors of dominant microflora biological stability

During the IBAC process, the biological stability of the dominant microflora is the key factor to ensure an effective operation. However, the factors including the property of the activated carbon, the dosage of ozone, hydraulic retention time and the condition of backwash all have impacts on the dominant biocommunity at different levels. When all combined influences considered, the main factors of the activated carbon effects on the stability of dominant biocommunity are the distribution of pores in activated carbon, the physical and mechanical properties as well as its chemical property, among which molasses value is the primary controlling index [44-46]. Although ozonation may improve the biodegradability of water, provide an oxygen-rich condition and reduce the incursion of undesirable bacteria, as well as enhance the biological activity of dominant biocommunity, however, relevant research shows that when the residual ozone reaches even over 0.1 mg/L in the inflow of IBAC system, a restrain on dominant biocommunity will be shown[47]. By increasing the contact time of the dominant microflora with organics, the adsorption and mass transfer of organics can be enhanced, thus the biological activity of the dominant biocommunity is improved. Generally, the most appropriate retention time is about 20 min[48]. The backwash step has an impact on the biological activity of the dominant biocommunity, which makes biological activity of the community in each layer rapidly decreases from the normal level to the minimum, but still remains in a certain range. After the sharp cutoff, the biological activity of the community will recover at normal level rapidly. Meanwhile, the intensity of backwash and its time also have a great impact on biological stability[49].

3.2. Characteristics of the biocommunity structure and distribution

The huge superficial area and rich porosity of the activated carbon provide a preferable habitat for microbe and prompt the formation of biological membrane. According to the research, the synergy effects of activated carbon adsorption and biodegradation enable O_3-BAC to realize the removal of organics in water (see 3.4 for details). Meanwhile, as the duration of BAC use gets longer, the action of organisms plays a more and more predominant role[50,51]. Quite a few researchers point out that the range of bacteria using the activated carbon as a carrier to habitat includes aerobic, anaerobic and facultative bacteria[52,53]. In this chapter the variation of bacteria communities and the characteristic of the community structure will be discussed, and the system chosen here is down-flow O_3-BAC system shown in Fig. 14.

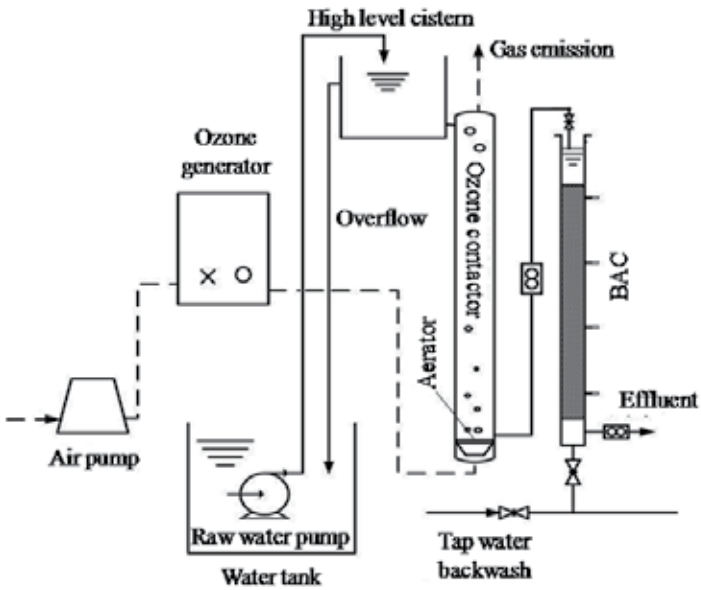

Figure 14. Pilot test setup

3.2.1. Characteristics of strain distribution

Various kinds of strains exist in BAC column (see Fig. 15). The picture demonstrates, (L-R), 4 sections (1, 2, 3 and 4) from top to bottom of the down-flow BAC layers respectively. The shadows in the finger prints represent different strains, and the numbers of the shadow zones represents the numbers of strains, while every shade represents a kind of strain, so the different shades represent the number of species indicatively. Fig. 15. shows that the number of shadow zones is 16 that can be discovered obviously, which indicates that the number of strains exist in BAC column is 16 according to detection. Meanwhile, along the carbon layer from top to bottom of the picture (L-R), there are 7 shadow zone shades turning weak gradually, which indicates that with the increasing of the carbon layer depth, these strain kinds are reduced gradually. There are 5 shadow zone shades turning darker,

which indicates that with the increasing of carbon layer depth, these strain kinds increase. Another 4 zones remained do not exist in every section, among which the quantity of one kind of strain is not changing, 2 kinds of strains only exist in the upper side of the activated carbon, while only 1 strain exists in the bottom of the activated carbon bed.

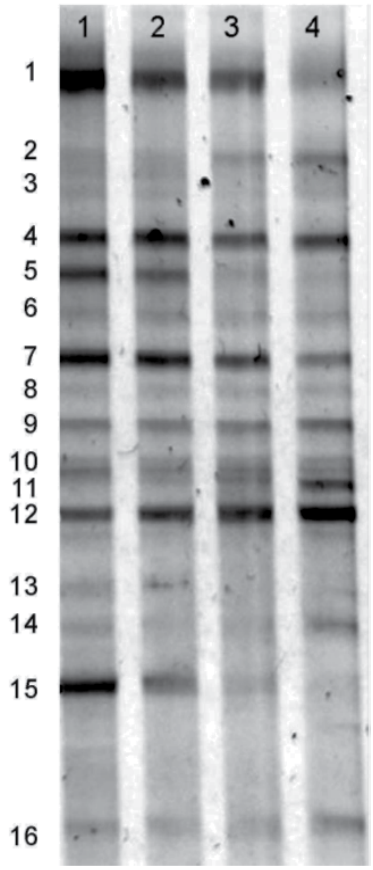

Figure 15. Analysis results of PCR-DGGE

3.2.2. Characteristics of biomass distribution

The growth of microorganism in BAC bed can be divided into three phases: logistic phase, stable phase and decayed phase (see Fig. 16). In the first period, microbe multiplies. In the initial period of biofilm formation, microbe quantity is rather low, and the highest biomass only reaches 70nmolP/gC. When the water temperature is proper and the inflow contains a lot organics, biological multiples rapidly. After one and half a month of the reduction period, it reaches the stable phase. Generally, in this phase, the concentration of the substrate declines, which means the degradation rate is rather high; DO consumption is also in large amount. In the second phase, microbe grows stably. After a period of cultivating when the water temperature is proper, the microbe basically reaches a state of stability, in which the microbe quantity maintains in the range of 450-520nmolP/gC and the microbe

grows normally. Meanwhile, the removal effect on pollutants in water is also steady, and at this point, the eco-system of BAC bed is relatively steady. This phase is characterized by equilibrium between biological membranes of new cells and the loss of membranes causing by physical force. In the decay phase, when water temperature as well as other conditions varies fiercely, the microbe quantity decreased rapidly and entering the final phase, in which degradable rate decreased, quality of outflow will be worsen.

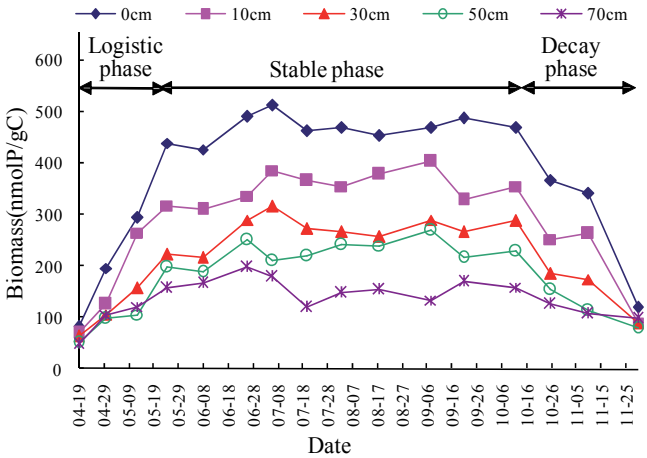

Figure 16. Variation of biomass in BAC Bed at different time

As the substrate is oxidized and degraded by the microbe membrane from top to bottom, the concentration decreased gradually, lead to the decline of available organic concentration and the poor nutrition condition of the microbe. Besides, it's also related to DO concentration. DO distribution in BAC column is gradually decreased from top to bottom, the lower it goes on the carbon bed, the fewer the available DO for microbe to reproduce, so does the biomass. Thus, the biomass distribution in BAC bed shows a feature of gradually decreasing from top to bottom (see Fig. 17).

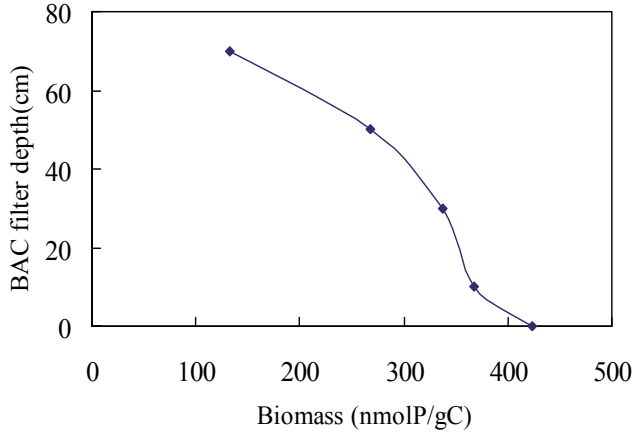

Figure 17. Variation of biomass along at different time BAC filter depth

3.2.3. Characteristics of biocommunity distribution

For down-flow BAC bed, though there was a decreasing tendency of bacteria adsorbed on activated carbon from top of the carbon layer to the bottom , the variation tendencies of ammonifying bacteria, anti-vulcanization bacteria and denitrifying bacteria are not exactly the same, among which ammonifying bacteria showed a decreasing tendency from top of the carbon layer to the bottom; anti-vulcanization bacteria showed an increasing tendency from top of the carbon layer to the bottom reaching a steady quantity; while denitrifying bacteria which located higher than 30cm of the carbon layer showed a gradual increasing tendency, however, after reaching the critical value of 30cm, it would decrease rapidly (shown in Fig. 18). As whole, aerobic bacteria is dominant in BAC bed.

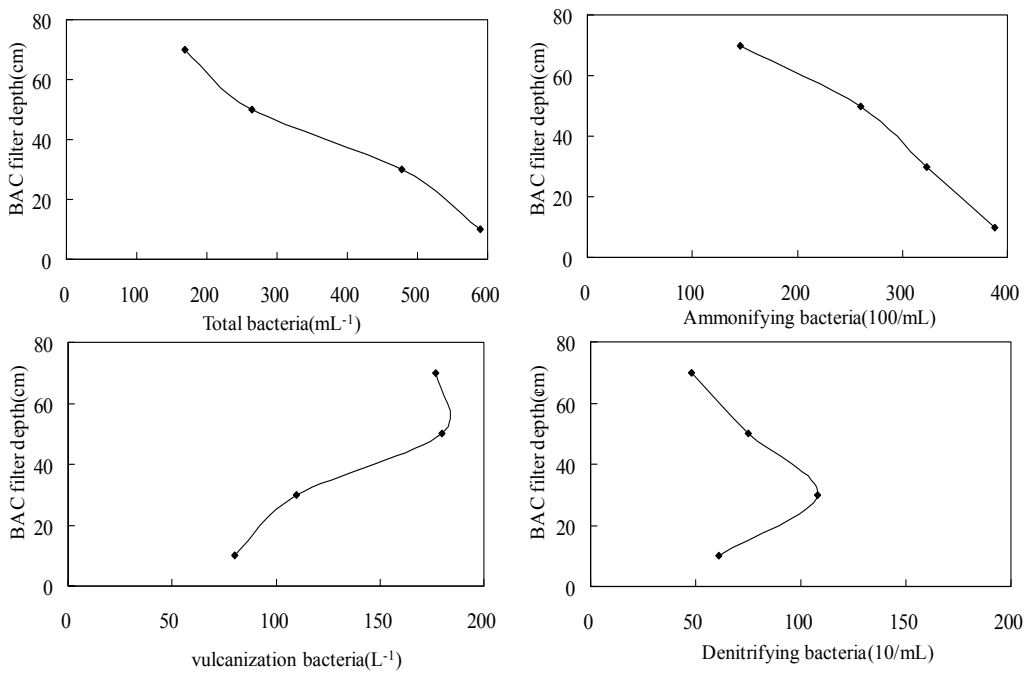

Figure 18. Distribution of biocommunity along BAC filter depth

As ammonifying bacteria is strictly aerobic, and denitrifying bacteria is amphitrophic, while anti-vulcanization bacteria is strictly anaerobic, the result shown in Fig. 18 is conducted by effects of two sides. Firstly, BAC is a kind of biofilm in essence, the thicker it gets, the more likely to create an anaerobic or hypoxia environment internal, which directly induces the existence of strictly anaerobic bacteria and amphitrophic bacteria. Secondly, variation of DO in activated carbon bed changes the distribution of biocommunity. Because aerobic bacteria is dominant in BAC bed, in the down-flow system, there is a decreasing tendency along the carbon layer (shown in Fig. 19), although, the concentration of DO in bottom of the layer is about 2-3mg/L, the flow inside BAC bed is laminar flow, which is not easy for the transmission of oxygen, hence, the bottom of activated bed shows anaerobic or anoxic characteristic, resulting in existence of amphitrophic bacteria and anaerobic bacteria.

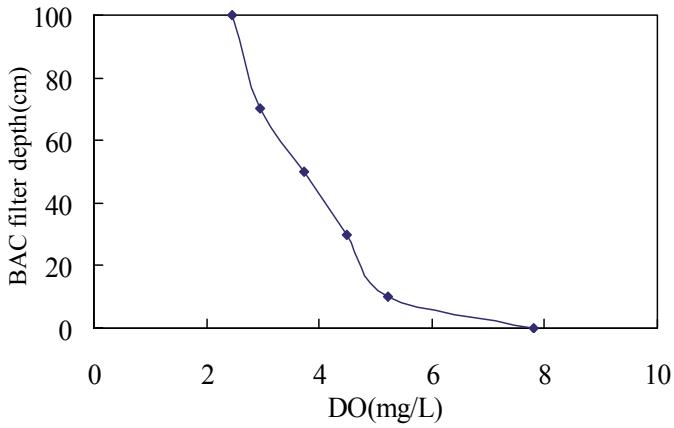

Figure 19. Variation of DO along BAC filter depth

During a steady operation of BAC process, a complex ecological system consists of bacteria, fungi, and algae which is from protozoa to metazoa is formed, which indicates the biofacies in BAC bed is of great abundance, and the ecological system of great diversity is fully developed which has a strong ability of avoiding the load impact. Part of the typical organisms inside BAC bed examined by microscope is shown in Fig. 20[54].

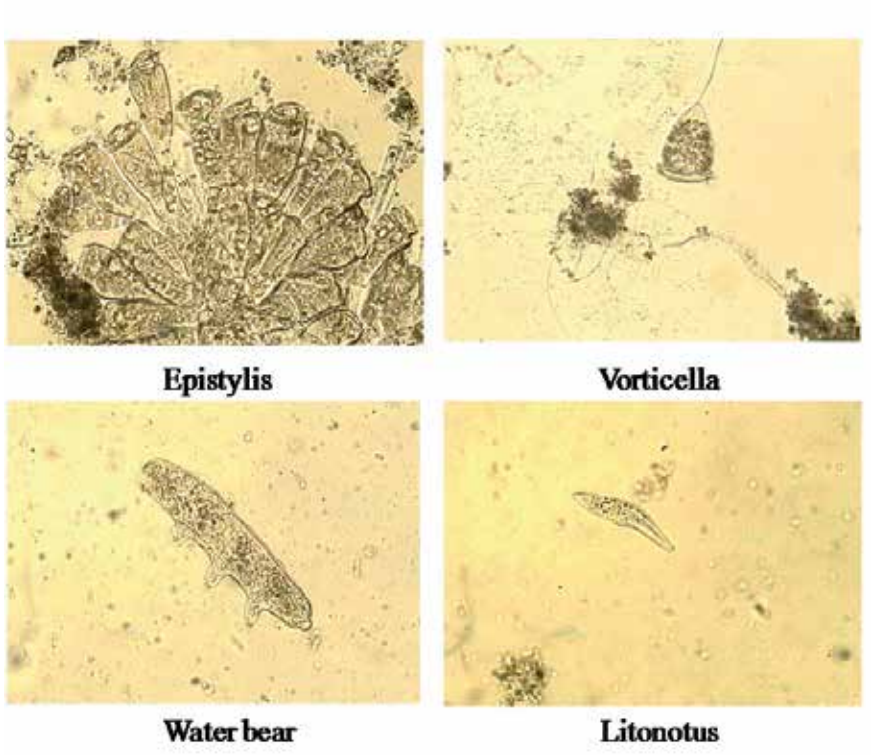

Figure 20. Microscope examination of microorganisms in BAC filter

3.2.4. Biocommunity diversity [55]

By collecting the biofilm on the surface of BAC, taking the total DNA of microbe and building a cloning library of 16S rDNA bacteria, as well as choosing a random one to clone with and conducting a DNA sequencing, the most similar bacteria to the cloned one can be determined after comparing with the community recorded in the database. Without relying on the domesticating method, the biocommunity's structure can be directly analyzed.

As shown in Table 4, in the biocommunity, α-Proteobacteria is dominant, β-Proteobacteria is the second category of this ecological system and δ-Proteobacteria is the third. Meanwhile, Planctomycetes bacteria also take a big proportion in this gene library.

Classification	Proportion (%)
α-Proteobacteria	26.5
β-Proteobacteria	16.3
γ-Proteobacteria	2.0
δ-Proteobacteria	16.3
Nitrospira	2.0
Planctomycetes	12.2
Bacteroidetes	2.0
Gemmatimonadetes	6.1
Acidobacteria	4.1
Unclassified	—
Proteobacteria	—
Unclassified Bacteria	10.2
Actinobacteria	2.0

Table 4. Fraction of different bacteria in gene clone library

After entering the sequence of genotype into NCBI website and comparing it by BLAST procedure with the existing sequence, it can be concluded that many of the bacteria's 16S rDNA sequencing has a rarely similarity with the existing ones in database, among which many have a similarity below 95%, reaching 88% the minimum, and most of the sequencings are from environment like soil, activated sludge, underground water, rivers, lakes and the urban water supply system.

Proteobacteria and other phylogenetic trees (shown in Fig. 21 and Fig. 22) were built to further understand the status of bacterial system development and assure the species of the cloned bacteria. As shown in Fig. 21 and 22 in samples, most of the cloned ones are similar to the bacteria which are not cultivated, only cloned 1-22 shares the same species with Chitinimonas taiwanensis in the phulogenetic tree.

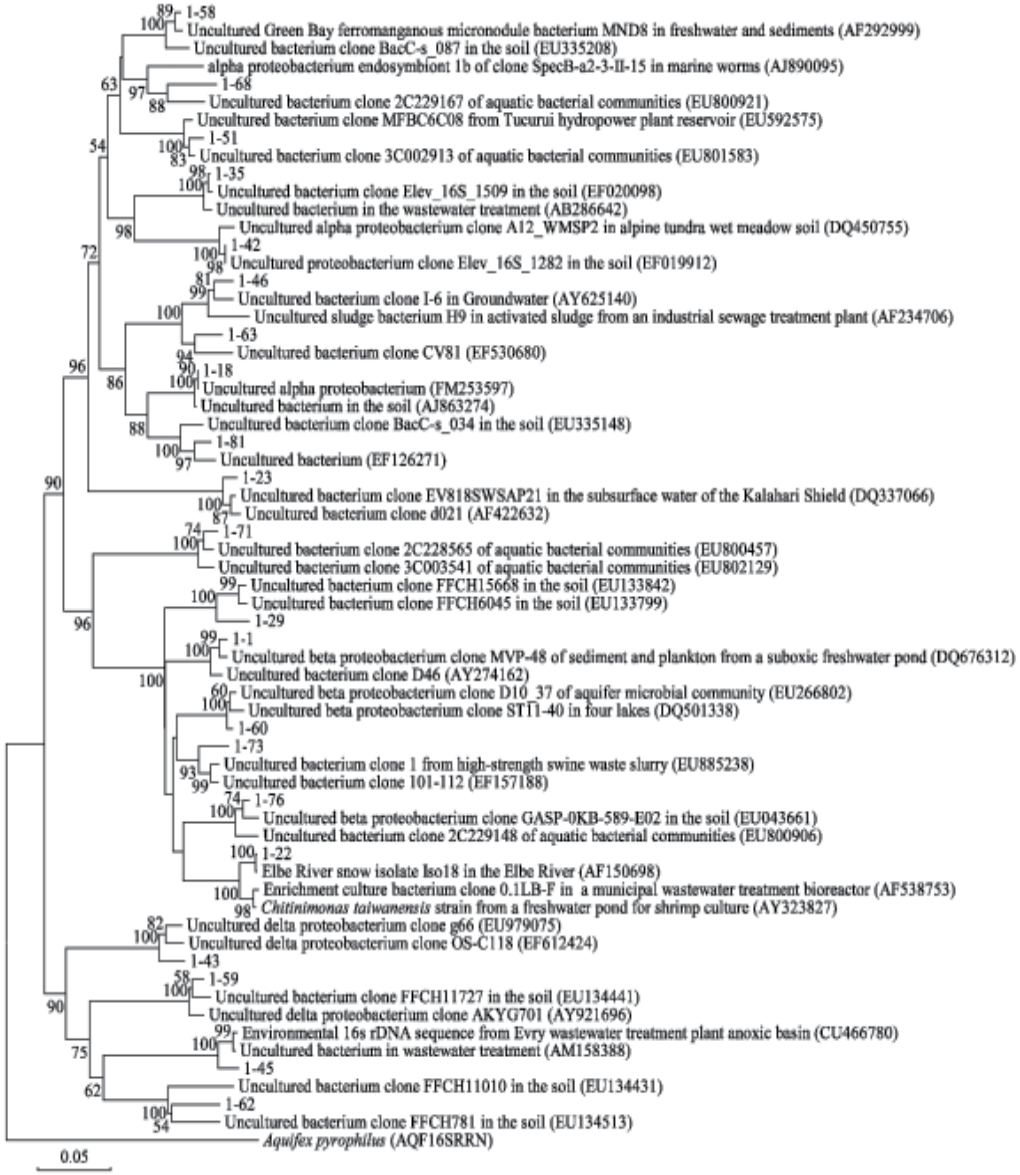

Figure 21. The Phulogenetic tree of Proteobacteria

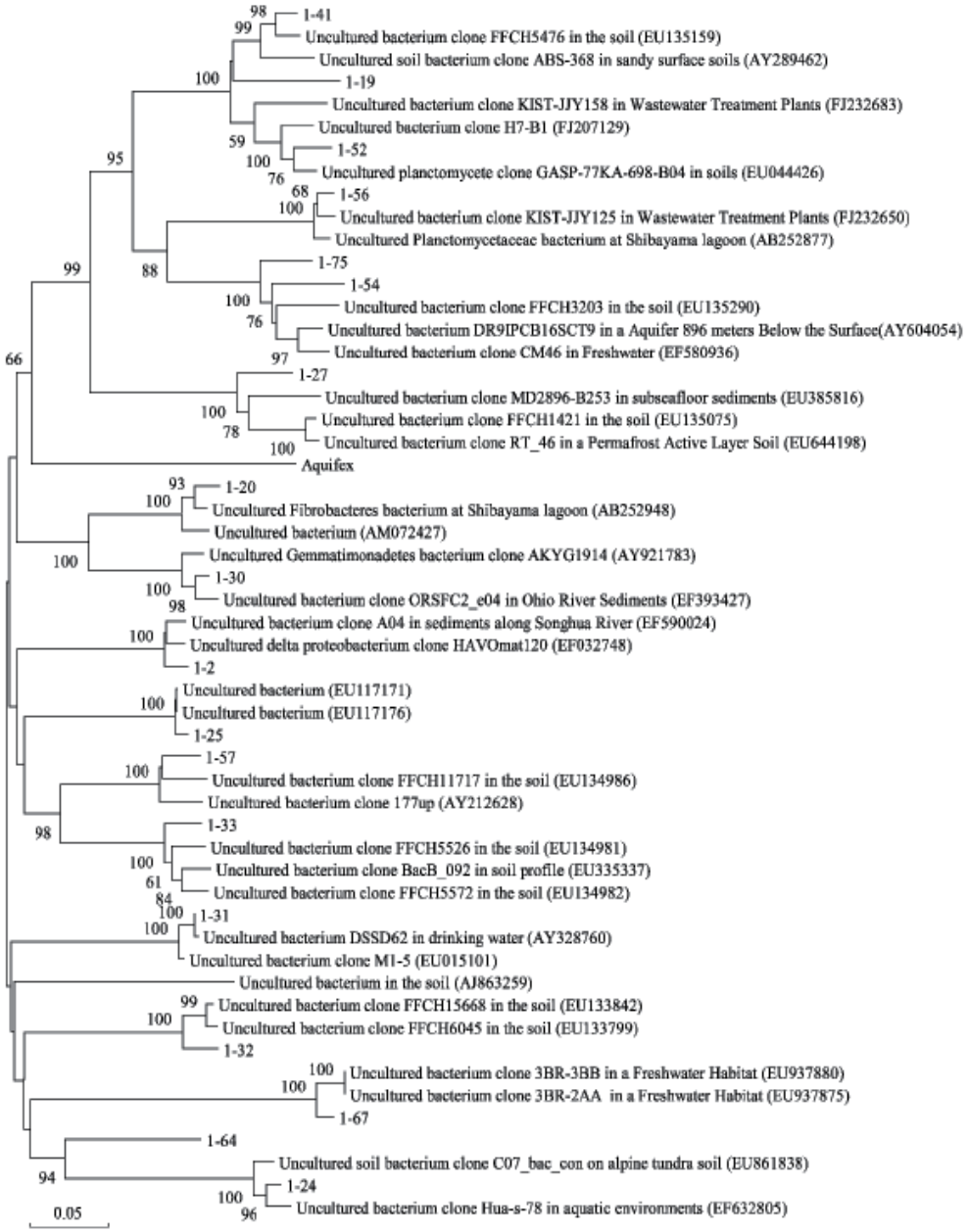

Figure 22. Phulogenetic trees of other bacterial system in carbon samples

3.3. Synergy of activated carbon adsorption and biodegradation

3.3.1. System construction and research method

The adopted system device is shown in Fig. 23., ozone is dosed via titanium microporous aerator from the bottom column of contact reaction to realize the contact of gas and water in a reversal way. Generally the dosage of ozone is 1.5mg/mgTOC, and the retention time is 11 min. After ozonation, the outflow will equivalently enter the bio-ceramsite filter column (the diameter of ceramic particles is 2-3mm) and bio-activated carbon filter column through the high level cistern and the control valve respectively. Two filter columns are both made of organic glass with 70mm inner diameter and 1650mm height, and the effective height of each filer is 1050mm. For each column, there are sample outlets at height of 100mm, 300mm, 500mm and 700mm along the top of each filter. The empty bed contact time for each column is 15min, and the cycle period of backwash is 3d. Under the same condition, by analyzing the removal effect of BAC and BCF towards DOC and BDOC to conduct quantitative analysis of organics removed by BAC adsorption and biodegradation, the theory on removal of pollutants in water by BAC will be discussed and the removal induced by the synergy effect of BAC adsorption and biodegradation[56,57] will be determined accordingly in this part.

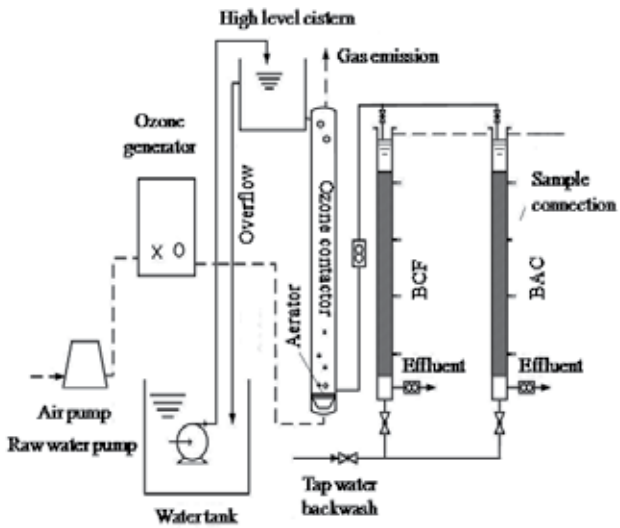

Figure 23. Pilot test arrangement

3.3.2. Quantitative calculation method of relationship between adsorption and biodegradation in BAC filter bed

3.3.2.1. Quantitative calculation method

This part compares O_3-GAC with O_3-BCF in which the biodegradation is dominant. By determining the iodide adsorption by BCF, only to find out the iodide adsorption is nearly

zero, which may conclude that for BCF filter bed the removal of organics in water is mainly realized by biodegradation, and the BCF adsorption is extremely faint. Assuming the quantity of biodegradation is in proportion to biological activity, the more active the microbe is, the more organics degraded will be. Therefore, determining the quantity of organics being degraded by BAC can be conducted by calculating the ratio of BAC biological activity with BCF biological activity, which can also determine the roles played by BAC filter bed adsorption and biodegradation in the process of organic removal. The calculation in details is shown in Fig. 24. Shown from the simplified calculation diagram in Fig. 24, two kinds of indexes need to be assessed to quantitatively determine the relationship between BAC adsorption and biodegradation within the process of organics removal:

1. to evaluate the comprehensive effect of BAC and BCF on organics removal, the system is characterized by DOC and BDOC;
2. to compare and analyze the biological activity of carriers from each filter bed.

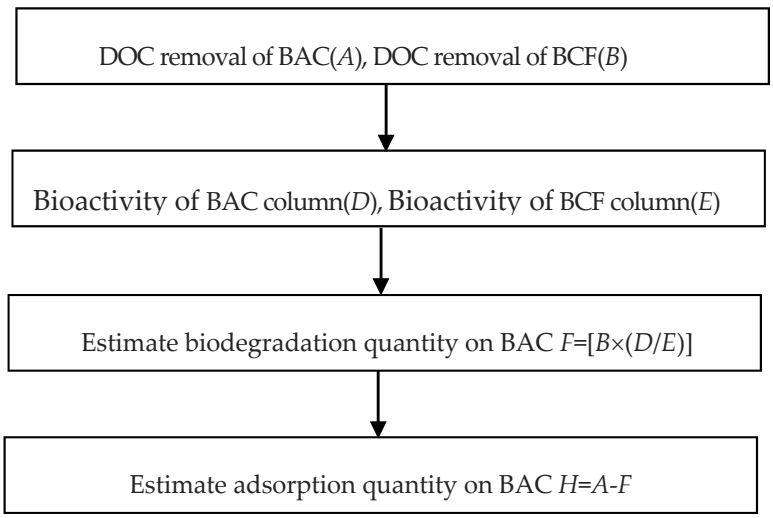

Figure 24. Illustration of quantification of the removal of organic matters by adsorption and biodegradation

3.3.2.2. Analysis of biological activity

The basic idea of in situ substrate uptake rate detection method[58,59] is to converse time-variant variables into space-variant variables. First assumption is to consider matrix concentration as a differential function of filter bed height; the second assumption is that under proper conditions, any of the micro-unit taken from the inner-filter bed can achieve stability. Through the total differential method or the law of conservation of mass, the final expression of matrix degradation velocity can be determined, at this point, the law of conservation of mass is applied, for its intuitionism.

As shown in Fig. 25, under a constant living environment for microbe, in situ oxygen uptake rate (ISOUR) can be described as the oxygen consumption of microbe grown on filter materials per unit time and volume (written as R, mg/(L·h)). Consider the filter porosity in filter column as e, mass concentration of DO in inflow and outflow are $\rho(DO_i)$ and $\rho(DO_e)$ respectively, taking ωdh as the micro-unit taken from depth of h in bio-filter bed (ω is section area of filer column, m^2), and the variation in DO caused by the effect of microbe inside the micro-unit can be described as $R(1\text{-}e)\omega dh$, according to the law of conservation of mass:

$$(\rho(\mathrm{DO_i}) - \rho(\mathrm{DO_e}))Q - R(1-e)\omega dh = 0 \tag{1}$$

ISOUR can be described as

$$\frac{dC}{dt} \tag{2}$$

In this equation: Q stands for the quantity of water through the filter column, m^3/h; v stands for the filter velocity, m/h;

$$\frac{d\rho(DO)}{dh}$$

stands for the gradient of DO at a height of h, along the filter variation height curve. As far as the aerobic biological treatment concerned, the biodegradation velocity on organic matrix is in proportion to its ISOUR. With abundant DO in BAC and BCF filter columns, the breeding microbe is mostly aerobic bacteria. Therefore, by calculating the oxygen consumption rate in the process of treating organics by BAC and BCF filter columns, the respective biological activity can be determined to analyze and evaluate the effect of biodegradation of BAC column.

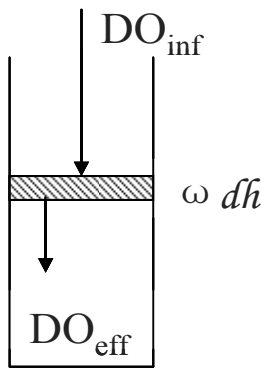

Figure 25. Calculation model of ISOUR

3.3.3. Quantitative comparison between relationship of adsorption and degradation in BAC filter bed

3.3.3.1. Analysis of biological activity (ISOUR)

Variation of DO in each section of BAC and BCF is shown in Fig. 26, according to equation (3-2), the biological activity of each section can be determined, and the result is shown in Figure 27. As can be seen from Fig. 26 and 27, an obvious change in biological activity along with the various depth of carbon layer exists in both BAC filter bed and BCF filter bed, showing a gradual decreasing tendency along with the depth, which is closely related to the distribution of biomass on carriers. Biological activity ratio of BAC to BCF can be determined based on Fig. 27, shown as D/E in Fig. 24, and the result is shown in Fig. 28.

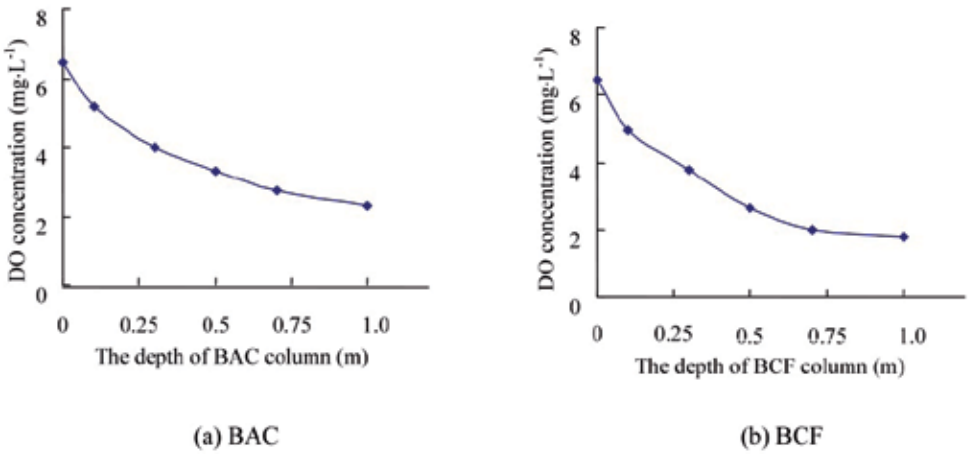

Figure 26. Variation of DO along BAC and BCF filter

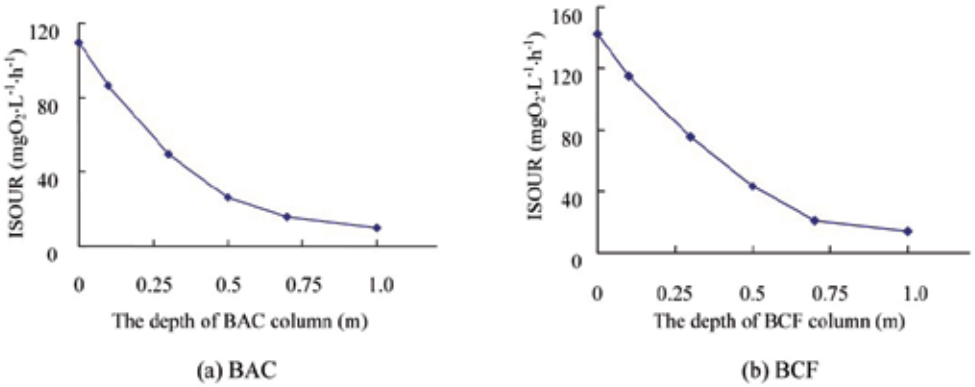

Figure 27. Variation of microbial activity along BAC and BCF filter

Fig. 28 indicates that the microbial activity of BCF filter bed is higher than that of BAC bed, and D/E for each section is ranging from 0.60 to 0.77. Among those sections, four of them have a ratio higher than 0.7, hence D/E=0.72 is set in quantitative calculation.

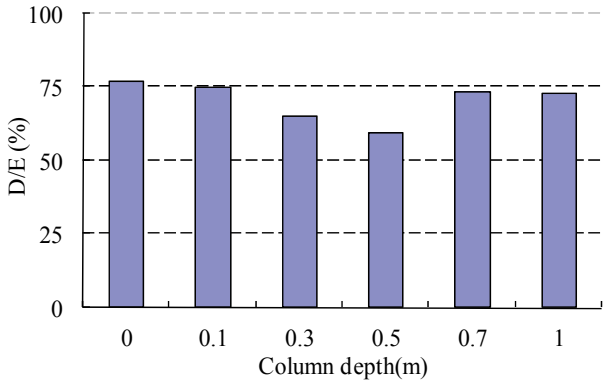

Figure 28. Ratio of microbial activity in BAC and BCF section (D/E)

3.3.3.2. Analysis of the organic removal effect of BAC and BCF

The respective removal effect of BAC filter bed and BCF filter bed on DOC and BDOC after ozonation is shown respectively in Fig. 29 and Fig. 30. Only a few DOC, which accounts for 4% of raw water is removed by ozonation. For calculation, the removal of DOC and BDOC shown in Fig. 29 and Fig. 30 is in relative terms with ozonation. Analysis of the statistics of Fig. 29 and Fig. 30 is shown in Table 5, in which NBDOC stands for non-biodegradable dissolved organic carbon. As shown in Table 5, the removal effect of BAC on DOC is mainly focused on removing BDOC, among which removal of BDOC accounts for 85% of the degradation DOC, while for BCF filter bed, this proportion can be as high as 98%.

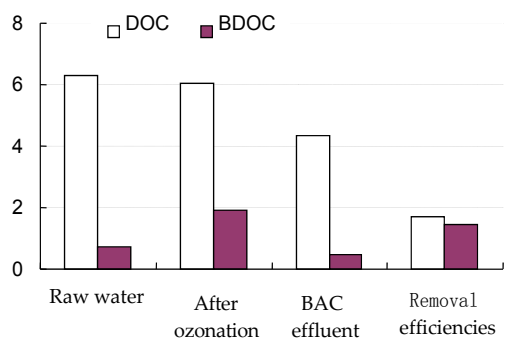

Figure 29. Results of DOC and BDOC removal in BAC filter

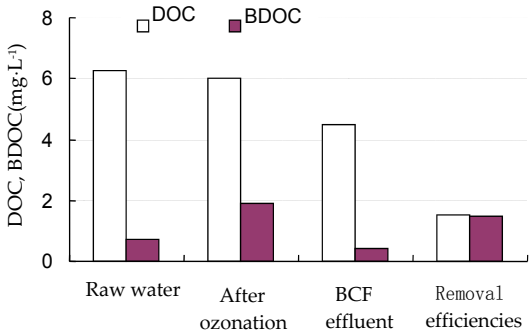

Figure 30. Results of DOC and BDOC removal in BCF filter

Column	DOC removal	BDOC removal		BDOC/DOC (%)
		NBDOC	BDOC	
BAC	1.685	0.234	1.451	85
BCF	1.525	0.03	1.495	98

Table 5. Calculation results of DOC and BDOC removal in BAC and BCF filter

3.3.3.3. Quantitative analysis of O_3-BAC adsorption and biodegradation

According to the calculation method in Fig. 24, and considering the analysis of BAC, BCF biological activity and their effects on removing organics, Table 6 shows quantitative calculation results of organic matter removal by adsorption and biodegradation in BAC filter. To intuitively understand how O_3-BAC biodegradation and adsorption work, Fig. 31 along with the analysis of Table 6 and Fig. 29, shows a simplified model of O_3-BAC removes organics.

Column	DOC removal	BDOC removal	
		NBDOC	BDOC
Adsorption	0.595	0.234	0.361
Biodegradation	1.09	0	1.09
Total	1.685	0.234	1.451

Table 6. Calculation results of organic matter removal by adsorption and biodegradation in BAC filter

As shown in Fig. 31, it is conducted by the synergy effect of activated carbon adsorption and biodegradation of O_3-BAC. Under the system research conditions, BAC bed biodegradation is dominant which accounts for 65% removal of total organics, furthermore, this 65% is mainly organics of BDOC, which indicates that the main organics removed by biodegradation is readily degradable dissolved organics, which can be concluded that biodegradation has a remarkable selectivity. In contrast, activated carbon adsorption plays a supporting role in the system, while via adsorption the quantity of removed difficult biodegradable material is roughly similar to the quantity of the readily one[60].

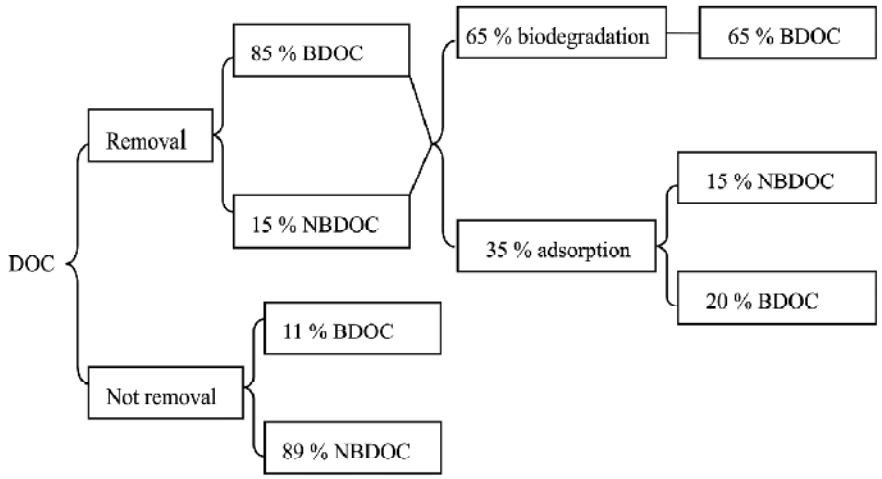

Figure 31. Illustration of organic matter removal by BAC filter after ozonation

3.4. Degradation kinetics of pollutants in BAC bed

3.4.1. Kinetics model establishment

Adopted system device is shown in Fig. 32. The relationship among microbial growth, the initial concentration of microbe and that of the substrate is the key factor to establish the degradation kinetics model. The relationship can be reflected by various models and the Monod equation is universally acknowledged as the practical model[61]. For water treatment field, the specific degradation velocity of the substrate is more practical than the specific growth velocity of microbe. Considering the specific degradation velocity of the substrate according to the physical law, the equation below is founded[62]:

$$-\frac{dC}{dt} = v_{\max}\frac{XC}{K_s + C} \tag{3}$$

Wherein, $\frac{dC}{dt}$ stands for the degradation velocity of organic substrate; C stands for residual organic substrate concentration in mixed liquor after a reaction time t; K_s stands for saturated constant; $v_{\max}$ stands for the max specific degradation velocity of organic substrate.

In the equation, C is the key factor for calculating kinetic equation. When $K_s > C$, the equation below is founded:

$$-\frac{dc}{dt} = k_1 XC \tag{4}$$

When $K_s < C$, the equation below is founded:

$$-\frac{dc}{dt} = k_2 X \tag{5}$$

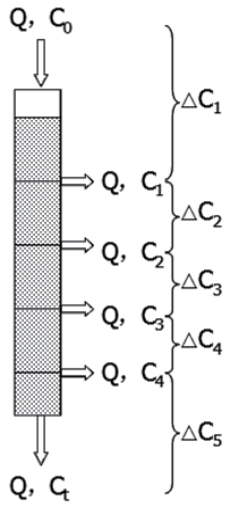

Figure 32. Illustration of material balance

Taken *X* (biomass) as horizontal coordinate and $\frac{dC}{dt}$ as vertical coordinate, if the diagram obtained is linear, the model belongs to high matrix organic degradation, while if the diagram is in exponential form, the model belongs to low matrix organic degradation.

As both BAC and BCF have a homologous microbial characteristic, by drawing an analogy with the degradation model of BCF, the BAC biodegradation effect can be described. According to the experimental system, material balance can be established which is shown in Fig. 32.

As shown in Fig.32, according to material balance law, along the section, the equation below is founded:

$$QC_0 = Q\left(C_1 + \sum_{i=1}^{1}\Delta C_i\right) = Q\left(C_2 + \sum_{i=1}^{2}\Delta C_i\right) = Q\left(C_3 + \sum_{i=1}^{3}\Delta C_i\right) = Q\left(C_4 + \sum_{i=1}^{4}\Delta C_i\right) \quad (6)$$

In which ΔC can be calculated by each C of all the sections, Δt can be calculated by the height of filter through each section and the filter velocity; the biomass of each BCF section represented by X can be measured, hence, taking biomass as the horizontal coordinate, $\Delta Ci/\Delta t$ of each section as the vertical coordinate to conduct linear regression analysis so as to determine the type of biodegradation model.

3.4.2. Characteristic of BAC degradation towards organics

3.4.2.1. Characteristics of BCF bed degradation

Fig. 33 shows the monitoring result of biomass in different BCF bed sections during a 7-month operation period. It can be seen from Fig. 33 that less biomass initially, till 20th May, the biomass of each section increased obviously and being steady gradually, which

symbolically means it is mature for biofilm colonization in bio-ceramsite filter bed. However, from the middle of October, biomass of each section is decreasing rapidly, which is partially induced by the gradual reduction for water temperature during operational period. Therefore, it is appropriate for choosing the data recorded from 20th May to the middle of October to be compared with. Fig. 34 shows the BDOC variation of each BCF bed section in this period. Fig. 34 suggests that organic concentration of each section is relatively steady and decreases along the direction of water flow, as well as gradual reduction between the differences of organic concentration from each section.

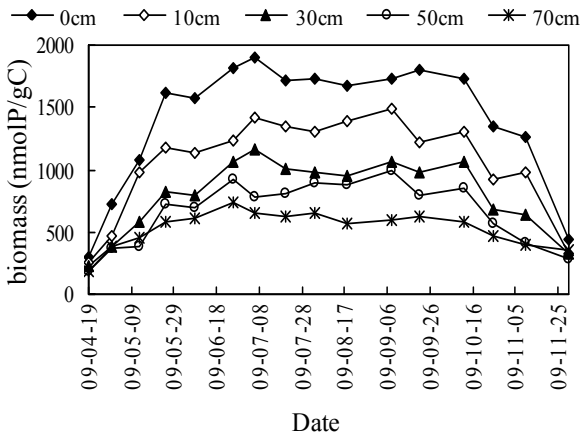

Figure 33. Variation of biomass along BCF bed

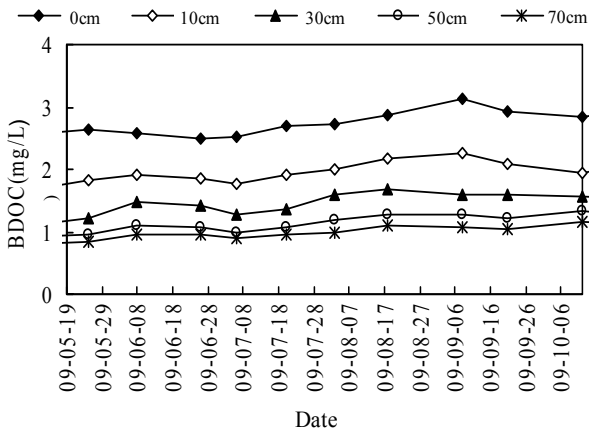

Figure 34. Variation of BDOC along BCF bed

By choosing the biomass of each section in the duration from 20th May to the middle of October and the mean value of BDOC variations to analyze the relationship between the biomass and $\Delta Ci/\Delta t$ variation according to the built model, the result is shown in Fig. 35. From Fig. 35, BCF bed biodegradation is in compliance with high matrix featured degradation model.

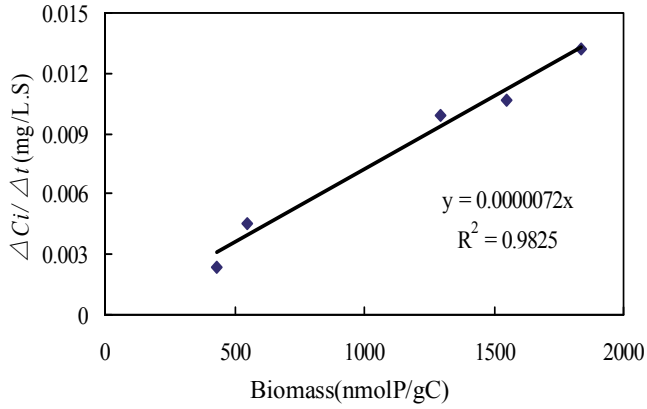

Figure 35. Calculation of BDOC degradation model in BCF column

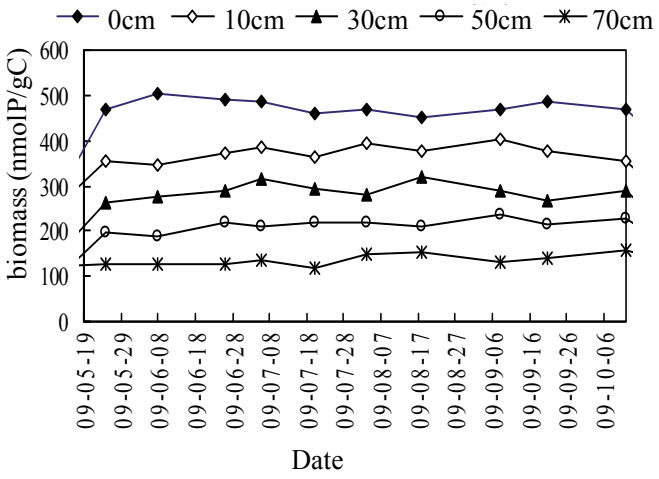

Figure 36. Variation of biomass along BAC column

3.4.2.2. Characteristic of BAC bed degradation

The variation of biomass in BAC bed at the same period is shown in Fig. 36. Fig. 36 shows that the biomass of BCF is much more than that of BAC, considering from the calculation above, we can find that for BCF system, biodegradation is in compliance with high matrix kind. Hence, it is the same with BAC system. Considering the two matrixes share the same homologous microbial, we can establish the BAC biodegradation equation by employing the calculated K:

$$\Delta C_i/\Delta t = 0.0000072 X_i \tag{7}$$

Wherein ΔC_i stands for the difference between the organic substrate concentration in mixed liquor residual of section i and that of section i-1; Δt stands for hydraulic retention time from section i to section i-1; X_i stands for biomass of section i.

3.4.2.3. Analysis of BAC bed organics degradation

Fig. 37 shows the analysis of the monitored variation of organics in each section of BAC bed at the same period, from which, organics concentration along the direction of water flow is decreasing gradually. However, as discussed previously, variation of organics is not only conducted by biodegradation, among which activated carbon adsorption is of the same importance. According to the biodegradation model mentioned previously, via calculation, BDOC removal conducted by microorganism biodegradation of each section as well as organics quantity adsorbed by activated carbon can be defined as ΔBDOC.

It can be seen from Fig. 37 that as the carbon layer goes deeper, the biomass become less gradually, also a decreasing in biodegradation and the BDOC quantity which is degraded by microbe, while at this point, the adsorption increases gradually.

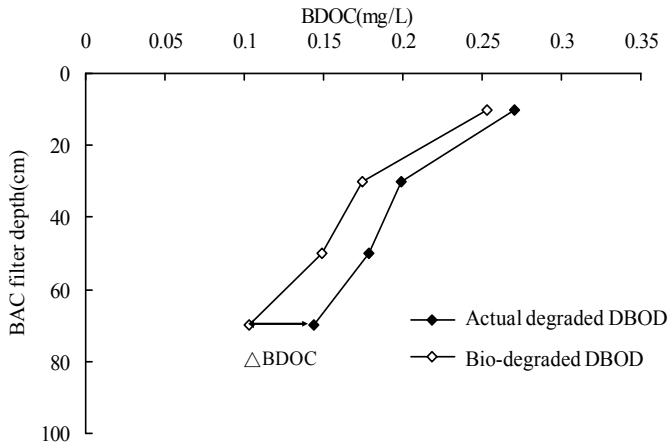

Figure 37. Calculation results of organic matter removal by degradation in BAC bed

4. Study on the safety of water quality by O_3-BAC process

4.1. Safety of microorganism

Because of ecclasis of microorganism and hydraulic erosion, microorganism may release from activated carbon bed, which will cause a series of water quality safety problems. Therefore, the microorganism safety of O_3-BAC process is very important for its application.

Microorganism safety of O_3-BAC technology should include the following four aspects[63], which characterize microorganism safety from different aspects respectively. Firstly, pathogenic microorganisms and toxic substances produced by their metabolism are the core problems concerning biological safety. Secondly, biocommunity mainly including bacteria, protozoa and metazoan is the expansion of the first aspect, which is based on some kinds of correlativity relationships between the biocommunity and pathogenic microorganisms. Thirdly, there is a certain connection between water quality parameters such as turbidity,

biological particle number and the risk of pathogenic microorganisms. Fourthly, Assimilable Organic Carbon (AOC) is one of the indexes characterizing bacteria regrowth potential. Low AOC indicates a small possibility for bacteria regrowth and low pathogenic microorganisms risk[64].

Correlations exist among those four aspects. Among them, the first and second aspects are relatively intuitive, and have intimate connections with microorganism safety, but not easy to be detected. The third and the fourth aspects are indirect indexes that can be detected quickly and easy to be automatically controlled, which is particularly important to the operation management of water plant.

According to the research and operation practice, O_3-BAC process produced abundant biocommunity, but pathogenic microorganisms were not found in the activated carbon and so were significant pathogenic microorganisms in effluent as well[64]. The effluent turbidity maintained under 0.1NTU on the whole, but it may beyond the standard during the preliminary stage (0.5~1h), the later period or the whole procedure of activated carbon bed filtration. Particle number was generally less than 50/ml in outflow, when the system worked stably, thus the microorganism safety can be guaranteed. However, it would reach up to 6000 per milliliter in primary filtration water. On the other hand, the effluent AOC basically maintained under 100 μg/L. According to relevant research result, AOC concentration in 50~100 μg/L could restrain the growth of colibacillus.

On the whole, microorganism safety problems have not been found in O_3-BAC process until now, while it must arise enough attention. This problem should be controlled by optimizing design parameters, strengthening the operation management and developing new treatment technology.

Ozonation contact reactor should be set up with more ozone dosing points to guarantee the removal effect of cryptosporidium and giardia insect, and CT value should be greater than 4 generally. The thickness of the activated carbon bed should be greater than or equal to 1.2m in general. In order to guarantee microorganism safety, a sand layer with certain thickness shall be considered to be added under the carbon layer. Besides, setting up reasonable operation period for biological activated bed and strengthening the management of initial filtrated water. If possible, water quality monitoring of every single biological activated carbon processing unit should be taken into account, for example, setting online monitoring equipments for turbidity and grain number. Furthermore, it can be effective by using other processing technologies or combining O_3-BAC with other technologies to solve this problem. For instance, reversed O_3-BAC and sand filter or O3-BAC and membrane filtration hybrid process (UF and MF) can be applied[65].

4.2. Safety of water quality

In addition to microorganism safety, toxic and hazardous compounds may be produced in actual operation of O_3-BAC process. Those toxic and hazardous compounds can be divided into two parts in general, namely ozonation byproducts and biodegradable byproducts respectively.

4.2.1. Ozonation byproducts

Ozonation byproducts can be divided into two kinds according to its origin, which are produced under the condition of humic substances and bromide existing in water. The former is caused by the reaction of O_3 and hydroxyl radicals, and the latter is caused by hypobromous acid. Composition of byproducts would become more complicated when ammonia nitrogen and amino acid exist at the same time[66].

4.2.1.1. Byproducts caused by humic substances

HA is the main composition of NOM in water, the molecular weight of material after ozonation is lower than that of NOM, and contains more oxygen during the reaction. The main byproducts are shown in Table 7. Among these byproducts, carbonyl compounds especially aldehydes should be paid most attention. Animal experiments indicated that formaldehyde, acetaldehyde, glyoxal and methylglyoxal have acute toxicity as well as and chronic toxicity, and furthermore, tube test also implied that these substances have genotoxicity, carcinogenicity, mutagenicity in different levels.

Carbonyl compound	Aldehydes	Aliphatics	Formaldehyde、Acetaldehyde、Propionaldehyde
		Aromatic	Benzaldehyde
	Dialdehyde	Aliphatics	Glyoxal 、 Methylglyoxal 、Maleic aldehyde 、Furaldehyde
	Ketones		Acetone
Oxygenous carboxylic acid			Aldehydoformic acid、 Methylglyoxal、Diethyl Ketomalonate、
Carboxylic acid	Monocarboxylic acid		Formic acid、Acetic acid ~$C_{29}H_{59}COOH$
	Dicarboxylic acid		Oxalic acid、Maleic acid、Galactaric acid、Fumaric acid
	Acromatic carboxylic acid		Benzoic acid、Phthalic acid
Bioxide			Hydroquinone、Catechol
Other			Heptane、Octane、Toluene

Table 7. Main ozone byproducts owing to NOM

4.2.1.2. Byproducts caused by bromine

When bromine exists in water, bromine will be oxidized into hypobromous acid by ozone, and then hypobromous acid will be oxidized into bromate, which has carcinogenicity to human body[67]. When ammonia nitrogen and amino acid exist at the same time in water, the reaction speed of bromate and ammonia nitrogen is faster, thus, dosing little amounts of ammonia would often restrain the production of organobromine compounds. Moreover, when bromine concentration is higher in the water, cyanogen bromide becomes the main ozonation byproduct[68]. Byproducts caused by bromide are shown in Table 8.

By-products caused by bromides	Bromoform	$CHBr_3$
	Bromoacetic acid	Bromacetic acid Dibromoaceticacid Tribromoacetic acid
	Bromohydrin	Bromine sec-butanol
	Acetonyl bromide	Bromopropanone Dibromoacetone
	Hypobromous acid Hypobromite	HBrO、MeBrO
	Bromic acid、 Bromate	$HBrO_3$、$MeBrO_3$
By-products under the condition of ammonia nitrogen coexist	Bromopicrin	CBr_3NO_2
	Dibromoacetonitril	$CHBr_2CN$
	Cyanogen bromide	BrCN
	Bromine amine	NH_2Br、$NHBr_2$、NBr_3

Table 8. Main ozone by-products owing to Br^-

Ozonation byproducts could be removed by adsorption of activated carbon. Meanwhile, ozonation and hypermanganate hybrid method will reduce the amount of byproducts. Taking more ozone dosing points can shorten the mean contact time and reduce the residual ozone concentration, thus bromate production will be decreased accordingly. Moreover, dosing acid to lower pH, ammonia, excessive H_2O_2 and OH scavenger to water may reduce BrO_3^- production[69] as well.

4.2.2. Bio-degradation byproducts

The microorganisms in the BAC will produce some microbial products such as SMP (soluble microbial products), EPS (extracellular polymeric substances), etc., when microorganisms degrade organics in wastewater.

Most of such substances are difficult to be degraded and hazardous to human. When drinking water is treated by O_3-BAC advanced treatment process, the degradation byproducts in a certain concentration will be produced, which causes health risks by drinking water. The disinfection byproducts will be formed through difficult biodegradation byproducts during chlorination, which are complex, various and more harmful to human body. The microbial degradation byproducts are organic, thus these substances may lead to the growth of bacteria in the pipeline. Moreover, the microbial degradation products will result in membrane clogging, and reduce the membrane flux as well as the membrane life during the O_3-BAC and membrane filtration hybrid process to produce high-quality water[10].

Although the effluent of BAC may contain some microbial byproducts, the content is limited and is not harmful for human health. Therefore, controlling the ozonation byproducts is more important and meaningful.

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Chapter 8

Biomass Digestion to Produce Organic Fertilizers: A Case-Study on Digested Livestock Manure

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Additional information is available at the end of the chapter

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1. Introduction

Biogas production by anaerobic digestion of organic wastes coming from agricultural practices is one of most promising approach to generate renewable energy, giving as end-product a digested organic biomass with specific characteristics useful for soil fertilization. This last aspect represents an opportunity in relation to the need to close the nutrient cycles within the agricultural and natural ecosystems, particularly in specific systems underwent to a constant resources depletion, as those of Mediterranean area, where the C-sink loss represents one of the main causes of desertification [1], [2]. The composting process was yet identified as one of the promising answers to the need of soil organic matter conservation, such as the addition to the soil of different organic materials of different origins [3], but the anaerobic digestion could represent an effective further step able to guarantee the recycle of nutrients, coupled with an environmental-friendly energy production [4].

Particularly, anaerobic digestion of livestock manures allows us to achieve several purposes: i) renewable energy generation; ii) reduction of nitrate leaching in livestock exploitations, iii) production of an organic biomass as by-product employable as organic fertilizer [5]. Actually, digestates coming from livestock manure give biomasses characterized by biologically stable organic matter and relevant nitrogen content; these traits suppose that these biomasses may be usefully utilized as N-fertilizers and soil organic amendments in agriculture, but also as a component of growing media in pot horticultural cultivation.

It should be remarked that production of greenhouse horticultural crops requires the use of growing media of high quality, with specific physical-chemical characteristics. Being the peat, organic component traditionally used in substrates formulation, a non-renewable resource (its extraction involving many environmental issues), it becomes ever more urgent the need to individuate alternative organic materials with the same functioning, such as composts or products coming from biogestion processes [6]. Nevertheless, the use of

biodigestate as amendment, still now not allowed in Italy, should provide the declaration of stability parameters for organic matter, since the utilization of matrices not properly stabilized could lead to the risk of high fermentescibility of organic components and, thus, consequent phytotoxicity phenomena [6],[7]. The stabilization of organic matter actually involves the mineralization of the most labile organic fraction, with the following decrease of C/N ratio; this means physical, chemical and biological changes of the starting material and, thus, the decrease of porosity, increase of pH, CEC, bulk density and salinity, due to a concentration of organic compounds which, generally, are characterized by lower molecular weight respect to the starting ones, more resistant to microbial degradation [8].

The amendment properties of composts and biodigestates could be assessed by different analytical methods, such as the isoelectrophoretic techniques (IEF) [9],[10],[11]. Results obtained after IEF characterization of the extractable fraction in alkaline environment of dried vine vinasse (an anaerobically digested solid residues, constituted by exhausted stems, skins and grape seeds, obtained after distillation of the "grappa", the Italian "acquavite"), showed an increase of the extractable organic components in alkaline environment, with a higher content in less acidic organic fraction, probably due to a "concentration effect" of the more complex organic components not, or only partially, degraded during the anaerobic digestion process.

Other works demonstrated also that the same dried vine vinasse, applied together with other mineral components in growing media, was able to increase nutrient availability [12] and express a sort of biostimulant activity on plant roots [13]. Study performed by optical microscopy demonstrated that digested vine vinasse in combination with clinoptilolite addition, promoted maize roots development, by increasing mucigel production by root tip and thus favoring the following solubilization and uptake of nutrients by plant from the added organic biomass (Figure 1).

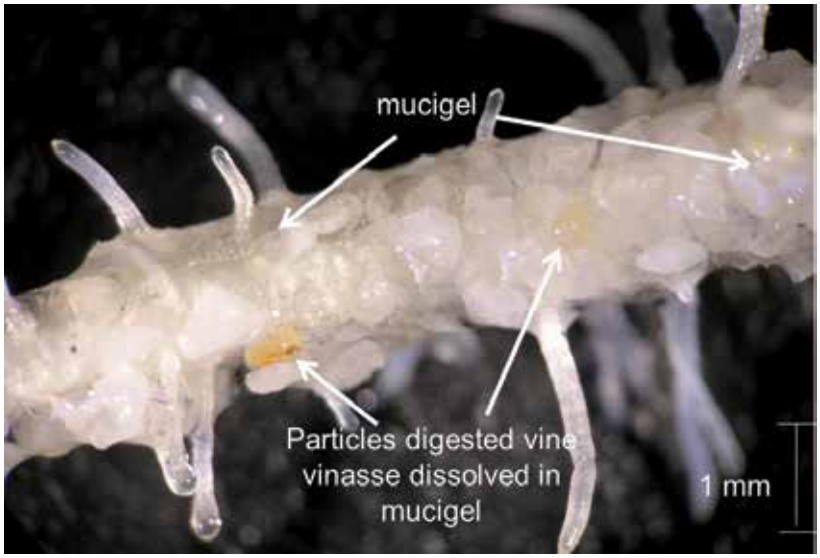


Figure 1. Root of Zea mais L., treated with micronized clinoptilolite and digested vine vinasse.

In relation to the N-fertilizer attitude of a biodigestate, it has a particular relevance in the case of the biodigestion of animal manure. The anaerobic process allows to maintain constant the total N amount of the original material, even if the organic N is mainly transformed into ammonia, the mineral form most easily available to the crops. The separation between the liquid and the solid fractions after biodigestion allows to recover the ammonium-N in the fluid fraction and the residual organic matter in the solid fraction, so to emphasize the different characteristics of the two fractions: the first one, usable as a typical N-fertilizers, able to furnish nutrient supply to plant; the second one as organic amendment, able to supply organic matter to soil and then improve its chemical, physical and biological characteristics. A proper composting process applied to the solid fraction of this digestate could lead to a further improvement of the biomass, by promoting its biochemical stability and giving those amendment properties yet described, which constitute the adding value of the final product.

What is relevant is that the risk of nitrate leaching in water represents the main limitation to the direct application of not pre-treated livestock manure to soil: effectively, its amendment properties, linked to soil organic matter addition, often go in conflict with the Council Directive 91/676/EEC on the "Water protection from nitrates" [14], especially in vulnerable zones, such as those of Italian northern regions. In this sense, the biodigestion process represents a great opportunity to utilize livestock manure digestates as N fertilizer, potentially allowing to overcome the limit of 170 Kg N ha^{-1} $year^{-1}$ superimposed by EU Nitrates Directive, since the high N-efficiency coefficient of these digestates, similar to that of soluble mineral fertilizers [15]. The solid fraction, containing organic matter already partially stabilized, could also permit its application during the winter season, provided the obtained amendment has a constant composition and a fraction of slow release-N, eventually increased by a following composting process [16],[10],[17]. On the other hand, the anaerobic conditions ensure the formation of high amount of ammonium during the organic matter degradation process, without incurring in the subsequent oxidation into nitrate [18],[4]. Being the ammonium the N-form more rapidly assimilated by the crops, this could be a further element in favor of the utilization of biodigestates as components of growing media, effectively conjugating the physical amendment properties with those chemical, connected with fertilization.

In 2007, the Italian ministry of agriculture financed a Research Project on the "Anaerobic digestion of livestock manure and EU Nitrates Directive – Effect due to the anaerobic digestion on N availability in livestock manure for overcoming the limit of 170 Kg N ha^{-1} $year^{-1}$ superimposed by imposto EU Nitrates Directive". The main aim of the reported study was to verify the N-fertilizer properties of a digestate coming from a swine livestock manure, taking into account the possibility to utilize this processed agricultural waste of animal origin for limiting the risk of environmental pollution. Hereafter, results related to the effect of the digested and not-digested solid fraction of this bovine livestock manure applied as N-organic fertilizers on lettuce growth in a greenhouse experiment are reported.

2. Materials and methods

2.1. Soils characterization

Two different soils of Northern Italy were chosen in a specific area located in Pedemont Region (Vercelli, Italy) characterized by cold and humid climate, in relation to their recognized vulnerability for nitrate leaching. The two soils, Tetto Frati (A) and Poirino (B), sampled at 0-30 cm, were characterized for main chemical, physical and biochemical parameters: pH, CEC (meq 100 g^{-1}), total organic C % [19] and total N (mg Kg^{-1}) [20], texture (sand %, loam %, clay %), bulk density (g cm^{-3}), basal respiration [($mg_{C\text{-}CO2} \times Kg_{soil}^{-1}$)×$day^{-1}$], C microbial biomass content (mg kg^{-1}) [21], metabolic quotient qCO_2 [($mg_{C\text{-}CO2}$×mg C_{biom}^{-1}×h)×10^{-3}], mineralization quotient qM, [22] and C microbial biomass/C organic ratio (%) [23].

2.2. Biomasses characterization

The organic biomasses, not-processed and processed, were supplied by the anaerobic digestion plant of the "Research Centre for Animal Production - Foundation Centre for Studies and Research" (C.R.P.A. – F.C.S.R. S.p.A., Reggio Emilia, Italy). Their main chemical characteristics of solid fractions of swine livestock manure are below reported:

pH:	not-digested = 7.0	digested = 8.0
N content (as received):	not-digested = 0.47%	digested = 0.39%
N content (dry matter)	not-digested = 2.9%	digested = 3.0%
C organic (dry matter):	not-digested = 43,5%	digested = 35,9%

In order to evaluate the organic matter stability of the two biomasses, both the digested and not-digested solid fractions of swine manure were previously analyzed by isoelectrofocusing (IEF) technique. Organic matter extraction was carried out on 2 g of each biomass with 100 mL of a solution NaOH/$Na_4P_2O_7$ 0.1N for 48 hours at 65°C. After centrifugation and filtration, the extracts were stored at 4°C under nitrogen atmosphere. Ten millilitres of NaOH/$Na_4P_2O_7$ extracts were dialysed in 6,000-8,000 Dalton membranes, lyophilised and then electrofocused in a pH range 3.5-8.0, on a polyacrylamide (acrylamide/bis-acrylamide: 37.5/1) slab gel, using 1 mL of a mixture of carrier ampholytes (Pharmacia Biotech) constituted by: 25 units of Ampholine pH 3.5-5.0; 10 units of Ampholine pH 5.0-7.0; 5 units of Ampholine pH 6.0-8.0. A prerun (2h 30', 1200V; 21mA; 8W; 1°C) was performed and the pH gradient formed in the slab gel was checked by a specific surface electrode. The electrophoretic run (2h; 1200V; 21mA; 8W; 1°C) was carried out loading the same C amount of water-resolubilised extracts (1 mg C×50 L^{-1} × $sample^{-1}$). The bands obtained were stained with an aqueous solution of Basic Blue 3 (30%) for 18 h and then scanned by an Ultrascan-XL Densitometer (Amersham – Pharmacia) [10].

2.3. Pot trial on lettuce (experimental plan, plant biometric survey and elemental analysis)

In a 300 m^2 greenhouse, lettuce (Lactuca sativa L., var. Romana) were transplanted into 2 L and 16 cm diameters pots, containing the A and B soils; growing density was 16 pots m^2. The experiment was performed from April to June, 2009, at temperatures range of 15-28°C.

Treatments consisted in a factorial combination of two increasing N doses (200 and 400 $kg_N \times ha^{-1}$), applied as solid fraction of digested swine livestock manure, not-digested solid fraction of swine livestock manure and granular urea [$CO(NH_2)_2$], taken as conventional mineral fertilization; not-fertilized soils were also considered as control treatments. Even if the N recommended dose for lettuce growth is about 90-100 $kg_N \times ha^{-1}$, the choice of such high N supply was made on the basis of the need to overcome the limit of 170 Kg_N ha^{-1} by substituting these organic biomasses rich in N to the mineral fertilization, without incurring in undesired effects on plant and environment: the dose of 400 $kg_N \times ha^{-1}$ was just applied for evaluating its potential phyto-toxicity on lettuce in relation to the different fertilization treatments. The corresponding fertilizers'doses per pot were: 128.6 g and 257.2 g for ND, 153.2 g and 306.3 g for D and 1.4 g and 2.6 g for urea.

Treatments were arranged in a randomized complete-block design with six replicates. Drip irrigation was managed in relation to plant water-demand, as reported in Figure 2.

Figure 2. Example of pot cultivation of lettuce in greenhouse; drip irrigation was used for guaranteeing daily water supply to the plants .

Lettuce plants were harvested 70 days from sown; biomass dry weight (g), dry matter (%), leaf area (cm^2) and leaf number were determined for each plants. In order to evaluate the effect of alternative fertilization treatments on micro and micronutrient uptake by lettuce, N, P, K, Mg, Cu, B, Fe, Mn leaf contents, plant material was incinerated at 400°C for 24 hours; ashes were then redissolved in HCl 0.1N and the supernatant filtrated to obtain a limpid solution; the nutrient content was determined by simultaneous plasma emission spectrophotometer (ICP-OES) on obtained solution and calculated in relation to dry matter.

2.4. N use efficiency

After analysis of N leaf tissue content (%) by Kijeldhal method, N-use efficiency (NUE %) was calculated, as the percentage of the N uptaken by the lettuce plant respect to N supplied by the fertilizer.

In order to study the long-term effect of the alternative fertilization approaches, the soil residual N at the end of experiment was obtained after Kjeldhal digestion and titrimetric determination [20]. Then, the available $N\text{-}NO_3$ and exchangeable $N\text{-}NH_4$ in the soils were determined after extraction of 4 g of each soil in 40 mL of KCl 0.2 N solution and subsequent colorimetric analysis of the supernatant by Automatic Analyzer Technicon II.

2.5. Statistical analysis

Plant biometric and soil N data were evaluated by ANOVA to verify the statistical differences of the tested parameters in relation to the different fertilization treatments.

Elemental data were analysed using vector analysis, which allows the simultaneous evaluation of plant dry weight and nutrients content in an integrated graphic format [24],[25]. Elemental data in relation to the different treatments in soils A and B were normalized with respect to urea al 200 kg_N ha^{-1}, taken as reference treatment.

3. Results and discussion

3.1. Soils characterization

In Table 1, the chemical-physical and biochemical parameters of the two considered A and B soils are reported.

The comparison between soils characteristics showed that A was a typical sandy-loam soil, with a lower organic C, lower C microbial biomass content, lower C microbial biomass/C organic ratio and higher mineralization quotient respect to B, which was a loamy soil, with higher organic C, higher C microbial biomass content, higher C microbial biomass/C organic ratio and lower mineralization quotient (Figure 1). On the basis of these results, the two soils were defined as a low biological fertility soil (Soil A) and a medium biological fertility soil (Soil B) [[23],[26].

The choice of these two different soils was performed in order to evaluate the effect played by the different soil characteristics on the behaviour of the digested and not-digested materials utilized in the experiment: it is well known that in all biologically mediated processes, such as the mineralization/immobilization of N coming from organic fertilizers, the microbial biomass has a key-role in addressing the degradation of organic substrate and making available N to the plant [27],[28],[29].

On the basis of what above discussed, the results were elaborated by considering the two soils as two independent experimental units, where the same experimental design was applied.

Parameter	Soil A	Soil B
Sand (%)	48,4	15,8
Loam (%)	43,1	75,6
Clay (%)	8,5	8,6
pH	8,2	6,1
C organic (g kg^{-1})	12	17
N total (mg kg^{-1})	0,83	0,81
CEC (meq $100g^{-1}$)	8,2	12,5
Bulk density (g cm^{-3})	1,34	1,45
Basal respiration (mg $_{C\text{-}CO2}$ Kg_{soil}^{-1}) day^{-1}	7,6	5,7
C microbial biomass (mg kg^{-1})	172	281
C microbial biomass/C organic (%)	1,43	1,65
qCO_2 (mg $_{C\text{-}CO2}$ mg $_{Cbiom}^{-1}$ h) 10^{-3}	1,85	0,85
q*M* (%)	4,3	3

Table 1. Main chemical, physical and biochemical parameters of A and B soils.

3.2. Biomasses characterization

In Figure 3, isoelectric focusing of the extracted organic matter from not-digested and digested solid fraction of swine manure utilized in the experimental activity is reported.

As reported in literature [30],[31], the more humified is the organic matter, the less acidic and higher in molecular weight are the organic compounds which compose it. This information, which is valid for soil humic matter but also for humic-like compounds in biomasses, allows to use IEF technique in order to separate organic matter extracted from different materials. Taking into account that, generally, organic matter focused in a pH range between 3.0 and 5.5, obtained results indicated that the organic matter from digested solid fraction was better stabilized respect to the not-digested one: in fact, the increasing peaks at pH >4.7 indicated a good stabilization of extracted organic matter from digested material, that means increased amendment properties of this material [16].

Effectively, the increase of the peaks'area in the pH region higher than 4.5 indicates firstly, that during the biodigestion changes in chemical composition of the organic material took place and, secondly, that these transformations led to the constitution of a final biomass

made by less acidic compounds, higher average molecular weight molecules, that means more chemically stable material [10].

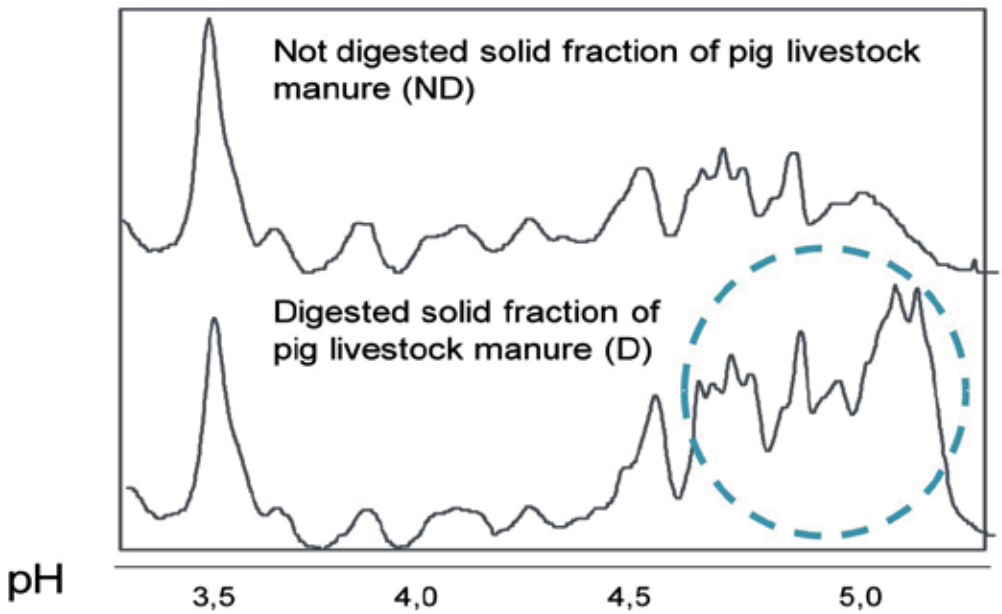


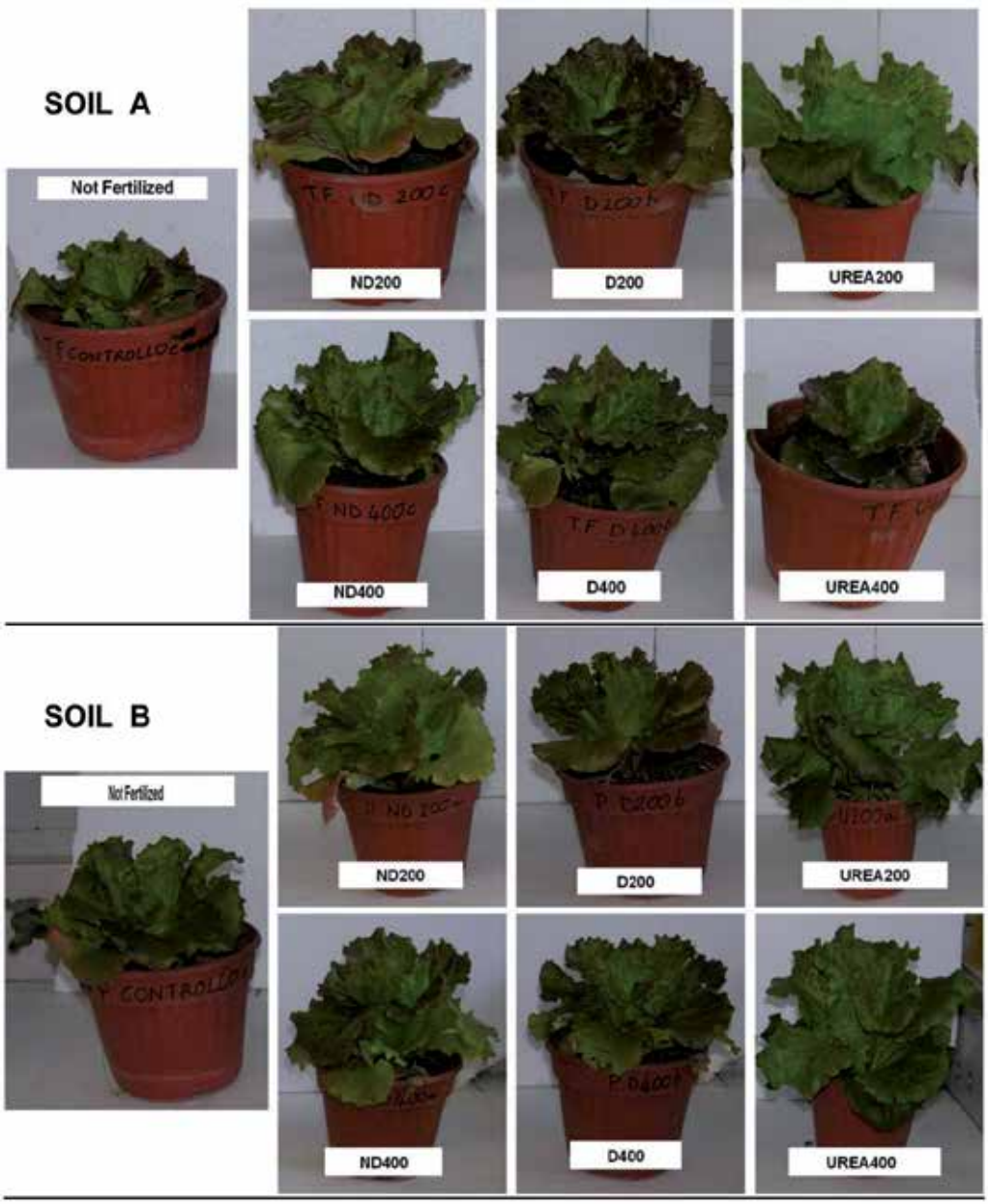
Figure 3. Isoelectrofocusing of extracted organic matter in alkaline environment from not-digested and digested solid fraction of pig livestock manure.

3.3. Pot trial on lettuce (plant biometric survey and elemental analysis)

In Figure 4, lettuce plants differently fertilized in the two soils at the end of the experiment (60 days) are shown.

The effect of the different treatments and soils is evident when comparing the lettuce growth. The phytotoxic effect of urea at highest dose was dramatically shown in Soil A, while at the same urea dose, in soil B the lettuce was able to increase its development although the excess of N mineral supply: this result attested clearly the role of soil in influencing the actual availability of nutrients, and in particular of N, for plant uptake. It was promising the good performances of both the not-digested and the digested solid fraction of livestock manure at the highest N dose, particularly in Soil A: even if the 400 $kg_N \times ha^{-1}$ supplied by urea gave the worst result on lettuce, apparently the excess of N added with the organic biomasses did not determine any decrease of lettuce growth, but on the contrary, a very good development of lettuce foliage (Figure 5).

This aspect is a positive point in order to propose the increase of the limit rate of 170 $kg_N \times ha^{-1}$ $year^{-1}$ for digestate application to soil, especially because these results were obtained in a soil with a sandy texture, low organic C content and, consequently, particularly vulnerable for nitrates.



(ND = not-digested solid fraction of livestock manure; D = digested solid fraction of livestock manure; 200 = 200 $kg_N \times ha^{-1}$; 400 = 400 $kg_N \times ha^{-1}$).

Figure 4. Lettuce grown in relation to the different fertilization treatments in soil A and B.

(400 = 400 $kg_N \times ha^{-1}$).

Figure 5. Example of lettuce leaves development in relation to the different fertilization treatments in Soil A.

The quantitative results related to biomass production and quality of plants are reported in Table 2.

Soil A	Dry weight (g $plant^{-1}$)	Dry matter (%)	Total leaf area (cm^{-2})	Number of leaves
Not fertilized	1,4 b	5,2 b	450 b	15 a
Urea 200 Kg ha^{-1}	2,9 d	4,8 b	1450 d	24 c
Urea 400 Kg ha^{-1}	0,4 a	5,4 c	180 a	13 a
Not digested 200 Kg ha^{-1}	1,2 b	4,4 a	730 c	17 b
Not digested 400 Kg ha^{-1}	2,5 c	7,3 d	750 c	18 b
Digested 200 Kg ha^{-1}	1,9 c	5,0 b	760 c	17 b
Digested 400 Kg ha^{-1}	2,4 c	5,0 b	850 c	18 b
Soil B	**Dry weight (g $plant^{-1}$)**	**Dry matter (%)**	**Total leaf area (cm^{-2})**	**Number of leaves**
Not fertilized	0,7 a	1,9 a	900 b	18 ab
Urea 200 Kg ha^{-1}	3,5 c	3,7 c	2000 c	23 c
Urea 400 Kg ha^{-1}	0,8 a	5,2 cd	1300 bc	22 c
Not digested 200 Kg ha^{-1}	1,7 b	3,7 c	850 b	18 ab
Not digested 400 Kg ha^{-1}	1,6 b	3,2 b	1250 bc	20 b
Digested 200 Kg ha^{-1}	0,7 a	2,1 a	700 a	17 a
Digested 400 Kg ha^{-1}	1,6 b	3,5 c	1000 b	20 b

Table 2. Lettuce dry weight, dry matter, total leaf area and number of leaves obtained after fertilization treatments in A and B soils (average value; different letters means significant differences at P-level<0.05).

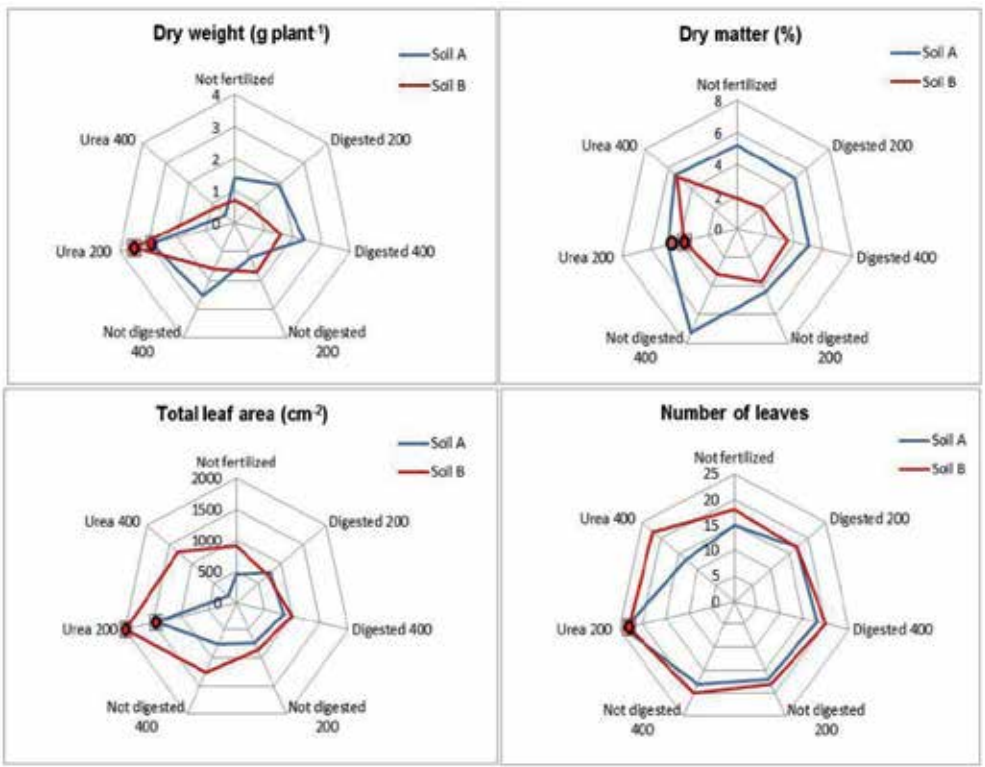
Firstly, again a strong "soil effect" was recorded in relation to plant growth parameters for all the treatments, due to the different chemical-physical characteristics and biological fertility of the two soils. Not fertilized plants showed a limited vegetative development

while, as expected, lowest urea dose (200 $kg_N \times ha^{-1}$) gave the best plant growth (6.7 g $plant^{-1}$), as confirmed by recorded parameters as shoot dry weight, percentage of dry matter, leaf area and leaf number, especially in B Soil. On the contrary, urea at 400 $kg_N \times ha^{-1}$ dramatically depressed plant growth in Soil A (0.8 g $plant^{-1}$), due to evident toxicity phenomena.

Digested and not digested biomasses gave best results when applied at the higher dose respect to the lowest one; actually, in treatments with both the biomasses at 400 $kg_N \times ha^{-1}$, plant parameters were closer to those obtained with urea at 200 $kg_N \times ha^{-1}$. It is relevant that in Soil A the application of both the digested and the not-digested solid fractions of livestock manure at 400 $kg_N \times ha^{-1}$ gave weight parameters higher than those observed at 200 $kg_N \times ha^{-1}$. Otherwise, in Soil B, only fertilization with digested solid fraction of livestock manure at 400 $kg_N \times ha^{-1}$ gave an increase of all the tested parameters respect to the 200 $kg_N \times ha^{-1}$ dose, while the not digested biomass did not show any differences among the N rates.

No toxicity phenomena were detected also at the highest doses of added biomasses and this is an important and positive result in the scope of utilizing these materials in substitution of mineral fertilizer also with high doses of N supply without collateral effect on plant.

For better clarify the effect of the alternative fertilization treatments on lettuce plant parameters in relation of the two soils, radar graphs are reported in Figure 6.



(200 = 200 $kg_N \times ha^{-1}$; 400 = 400 $kg_N \times ha^{-1}$).

Figure 6. Radar graphs of plant parameters related to the fertilization treatments in soil A and B.

Lettuce number of leaves was little affected by soil characteristics, while dry weight, dry matter and total leaf area were evidently influenced by both the soil and the fertilization. On general terms, in the Soil A the percentages of dry matter of lettuce were higher respect to the corresponding values recorded in Soil B; on the contrary, the total leaf areas were lower in Soil A than in Soil B. The water uptake seems to have had a great importance in the two considered systems: probably, it was strongly affected by physical characteristics of the two soils. In Soil A, with about 50% of sand content, the water was less available to plant because of its lower water retention capacity respect to Soil B, so to determine the tendency to reduce the lettuce total leaf area and increase leaves dry matter. On the contrary, in the loamy Soil B, the higher water availability determined a decrease in percentages of lettuce dry matter and the increase of leaf areas, so to give an indication of the dependence of lettuce quality mainly from soil characteristics rather than the fertilization treatments. Anyway, taking into account 200 $kg_N \times ha^{-1}$ of urea as the reference dose for lettuce production, it is relevant that the not-digested solid fraction of livestock manure at 400 $kg_N \times ha^{-1}$ gave the highest value of lettuce dry matter among all the treatments.

For evaluating macro (N, P, K, Mg) and micronutrients (Cu, B, Fe, Mn) use efficiency, biomass dry weight and elemental concentrations were plotted, including curved content isoclines [32],[33]. Each point on the bidimensional plot represent a vectors, taken as control equal to 100% both the concentration and the related dry weight obtained after addition of 200 $kg_N \times ha^{-1}$ urea (intersection point) in graphs (Figure 7 and Figure 8).

Plant tissue composition was significantly affected by the different treatments, depending on the added materials and rate. In relation to both the macro and the micronutrients, it should be remarked that all results were shifted to the limiting vector space (left side of the plot, under 100% lettuce dry matter, corresponding to urea at 200 $kg_N \times ha^{-1}$), representing the reducing growth treatments.

In relation to N (Figure 7), the phytotoxic effect of mineral fertilizer at 400 $kg_N \times ha^{-1}$ is particularly evident in Soil A, where the increase of concentration of N corresponded to the greatest decrease of lettuce dry matter respect to the control; the same severe phytotoxicity was not recovered after treatments with organic biomasses. The most promising results were obtained after addition of not-digested and digested solid fraction of livestock manure at highest dose, giving a dry matter similar to those obtained with the control mineral fertilization, but with a net decrease in N uptake: this finding attests that the N use efficiency was particularly high when 400 $kg_N \times ha^{-1}$ of both organic materials were added to Soil A, since the lack in N prompt availability was not so heavy in limiting plant growth. Such a positive result was not so evident in Soil B, because of the clear reduction of lettuce dry matter (about -50%) after treatments with digested and not-digested materials respect to urea at 200 $kg_N \times ha^{-1}$, even if the 400 $kg_N \times ha^{-1}$ urea application gave a tendency to an excess of N consumption by lettuce, which did not correspond to an increase of dry matter production.

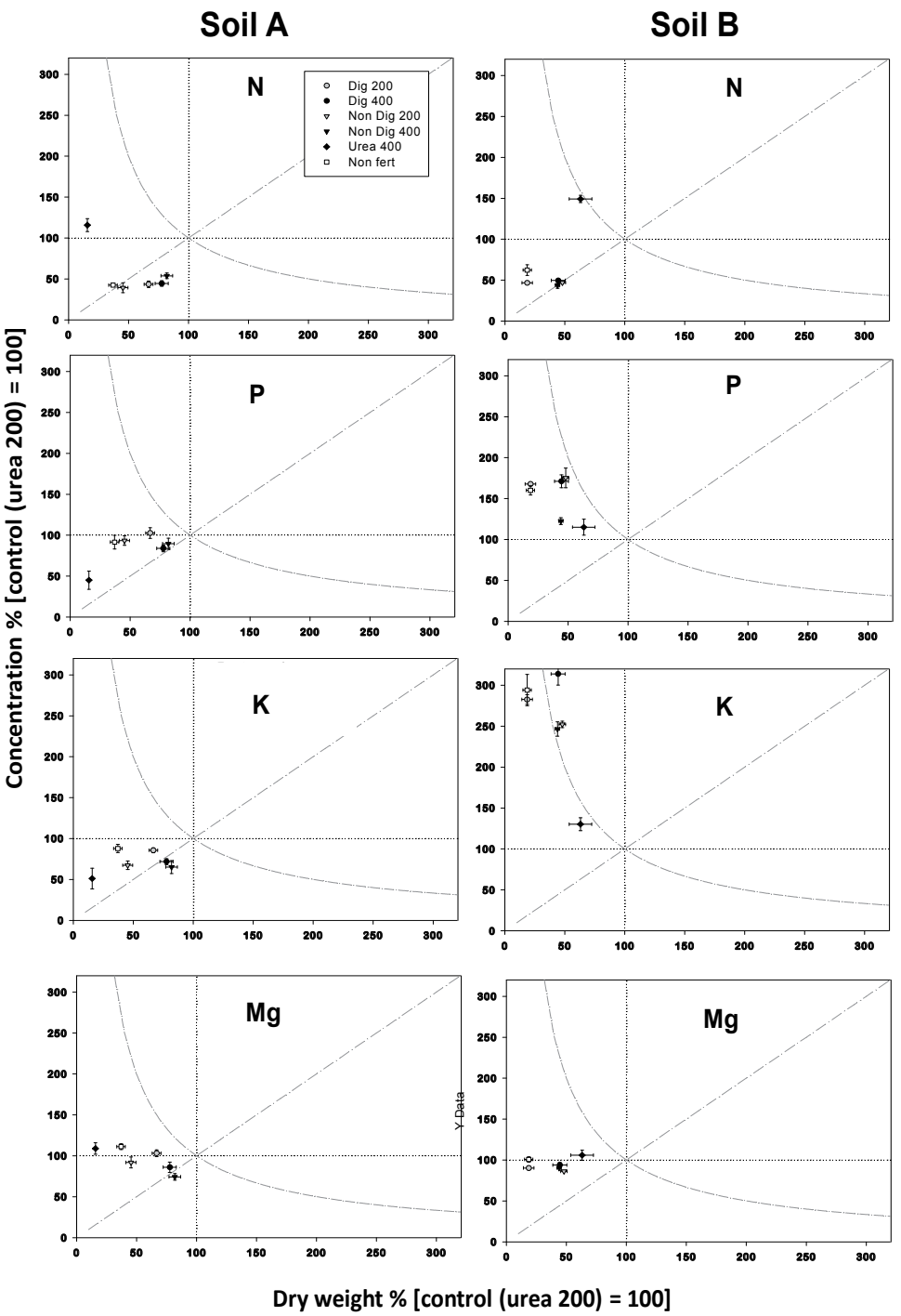


Figure 7. Comparison of relative macronutrients concentration and relative dry weight of aerial part of lettuce plant at the end of growing cycle. Control values of dry weight and concentration used as reference (point of isolines intersection).

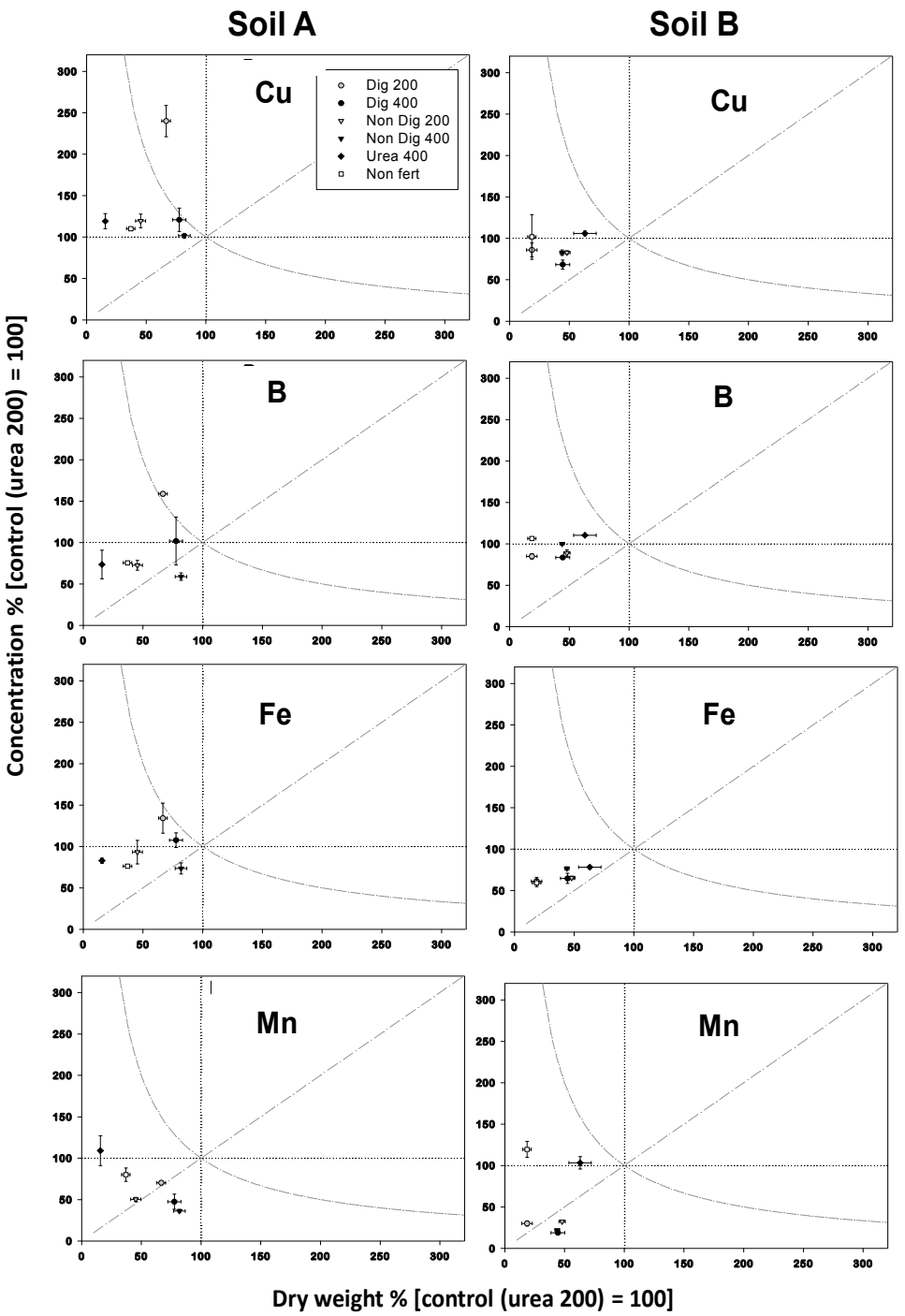


Figure 8. Comparison of relative micronutrients concentration and relative dry weight of aerial part of lettuce plant at the end of growing cycle. Control values of dry weight and concentration used as reference (point of isolines intersection).

Similar results were obtained also for Mg (Figure 7), since again this nutrient concentration seemed to be another limiting factor for lettuce growth, in both the soils.

Different behaviour was recorded for P and K (Figure 7): their related uptakes were strongly affected by both the soils characteristics and the fertilization treatments: while in Soil A the limiting factor for lettuce growth was clearly the soil P and K availability (left space of the graph, under 100% of nutrient concentration for the urea control), in Soil B the effect was opposite (especially for K), since the excess of nutrients appeared to be the main cause of plant growth decrease (left space of the graph, above 100% of nutrient concentration for control 200 $kg_N \times ha^{-1}$), determining a typically defined "nutrient luxury consumption".

In relation to micronutrients Cu, B, Fe and Mn (Figure 8), sometimes their deficiency represented the main limiting factor for lettuce growth (as Fe in Soil B for all the treatments), sometimes the excess of their concentration could have again determined a luxury consumption (as Cu and B in Soil A, after addition of 200 $kg_N \times ha^{-1}$ of digested livestock manure). It is interesting the effect played by soils on Mn lettuce uptake: in Soil A, after addition of 200 $kg_N \times ha^{-1}$ of digested livestock manure, both the Mn uptake and the lettuce dry weight were reduced only of about 30% respect to the values posed to 100% for urea control; on the contrary, in Soil B, the same parameters strongly decreased of about 80%, so confirming the role of soil chemical and physical characteristics on nutrient availability in relation to the different treatments.

3.4. N use efficiency

In Table 3, N use efficiency (calculated as percentage of $N_{uptaken}/N_{fert}$), the residual soil N, the available soil $N\text{-}NO_3$ and $N\text{-}NH_4$ in relation to the different fertilization treatments in soil A and B are reported.

As far as the lettuce N use efficiency is concerned, in both the soils the highest NUE % was obtained after urea application, with the exception of urea at the rate of 400 $kg_N \times ha^{-1}$ in Soil A, which caused an evident phytotoxicity, already manifested by the reduction of lettuce plants growth. In the same soil, higher NUEs were obtained after application of the digested and not-digested solid fraction of livestock manure at the higher 400 $kg_N \times ha^{-1}$ rate. In B soil, while application of the digested biomass at both the doses gave similar NUE values, a quite doubled NUE was obtained after addition of not digested livestock manure at 200 $kg_N \times ha^{-1}$: these positive results could be explained by the presence of higher amount of promptly available N in the not-processed livestock manure respect to the digested one. Anyway, the recorded N use efficiency obtained after the application of both the biomasses was, as expected, lower respect to the urea application at the same rates.

Residual soil N at the end of the cropping cycle after the treatments with digested and not digested biomasses was greater in B soil than in A soil, particularly after digestate application: we suppose that, in this medium-high fertility soil, N-immobilization process took place at higher extent, probably exerted by the soil microflora which was more abundant in B soil, as confirmed by biochemical parameters reported in Table 1. Also the texture could have played a key-role in this process: the textural characteristics of Soil B (~

84%) probably favoured the ammonium fixation, particularly on the loamy-clay components of that soil.

Soil A	NUE ($N_{uptaken}/N_{fert}$ %)	Residual soil N (g pot^{-1})	Assimilable N-NO_3 (mg pot^{-1})	Exchangeable N-NH_4 (mg pot^{-1})
Not fertilized	0	0,82 a	5,1 c	21,9 b
Urea 200 Kg ha^{-1}	27,1 d	1,49 b	60,2 d	18,3 a
Urea 400 Kg ha^{-1}	2,3 a	1,75 c	146,1 e	25,8 c
Not digested 200 Kg ha^{-1}	4,6 b	1,61 c	4,8 b	26,9 c
Not digested 400 Kg ha^{-1}	6,1 bc	1,89 c	4,1 a	21,9 b
Digested 200 Kg ha^{-1}	4,1 b	1,99 c	4,3 a	23,6 c
Digested 400 Kg ha^{-1}	8,0 c	1,97 c	4,7 b	22,3 bc
Soil B	**NUE ($N_{uptaken}/N_{fert}$ %)**	**Residual soil N (g pot^{-1})**	**Assimilable N-NO_3 (mg pot^{-1})**	**Exchangeable N-NH_4 (mg pot^{-1})**
Not fertilized	0	0,89 a	3,1 a	23,8 ab
Urea 200 Kg ha^{-1}	27,3 d	1,10 b	5,8 c	26,1 b
Urea 400 Kg ha^{-1}	12,8 c	1,23 b	67,1 d	32,5 c
Not digested 200 Kg ha^{-1}	6,8 b	1,43 c	4,2 b	22,5 a
Not digested 400 Kg ha^{-1}	2,4 a	2,44 d	5,8 c	22,1 a
Digested 200 Kg ha^{-1}	2,3 a	2,11 cd	6,5 c	27,4 c
Digested 400 Kg ha^{-1}	2,6 a	2,87 d	6,1 c	25,3 b

Table 3. Nitrogen use efficiency NUE ($N_{uptaken}/N_{fert}$ %), residual soil N (g pot^{-1}), N-NO_3 and N-NH_4 in relation to the different fertilization treatments in soil A and B (average value; different letters means significant differences at P-level<0.05).

In relation to the N forms available in the soils at the end of the experiment, after the treatments with the organic biomasses, the main mineral N fraction is represented by ammonium form, with the exception of urea application, which dramatically promoted the increase of soil nitrate: this finding is extremely positive, since both the digested and the not-digested solid fraction of livestock manure allowed to limit the excess of nitrate ions in the soil solution. Moreover, it is noticeable that the use of these organic biomasses addressed the N immobilization by the microbial biomass activity, guaranteeing an increase of soil residual fertility.

4. Conclusion

Digested, but also the not digested solid fraction of swine livestock manure, showed N availability compatible with those requested from lettuce, so to be used to fertilize short-term horticultural crops. Obtained lettuce plants had a good quality at the end of the experiment, in both the soils and for both the used organic biomasses. Even if the plant dry matter was partially reduced respect to that obtained after mineral fertilization, the macro and micronutrient uptake was lower after organic biomasses addition, indicating an optimization of nutrient use efficiency. Besides, N uptake was well balanced, so to be the N supply able to guarantee a correct development of lettuce.

Since the main aim of our work was to demonstrate the possibility to use these processed biomasses as N source for plant cultivation by limiting the potential nitrate leaching in soil, as normally happened when livestock manure are directly applied to soil, the obtained

results confirmed the significant environmental benefits due to the use of anaerobically digested biomasses as organic fertilizers. They showed to be able to promote the development of lettuce crop, probably through the slow release of available N into the soil system, without incurring in toxicity phenomena also when applied at high N supply, being this positive effect particularly effective in a sandy soil, poor in organic matter.

Thus, the biodigestion process represents a great opportunity not only for generating renewable energy, but also to obtain an organic biomass with good fertilizer/amendment characteristics to be applied to the soil, giving the possibility of use alternative nitrogen sources, contemporary promoting plant growth and limiting the environmental impact.

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Chapter 9

Removal of Carbon and Nitrogen Compounds in Hybrid Bioreactors

Małgorzata Makowska, Marcin Spychała and Robert Mazur

Additional information is available at the end of the chapter

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1. Introduction

Biological wastewater treatment methods allow to remove pollutants at high efficiency but they require application of modern knowledge and technology. In bioreactors used for carbon and nutrients removal from wastewater two forms of biomass are utilized: a suspended biomass (dispersed flocs) and an attached biomass (biofilm). The latter needs a carrier on surface which it can grow.

Both types of biomass, despite some similarities, show also many differences. Probably as a result of complex relations (competition, migration, physical factors like flow velocity and biochemical factors like oxygen supply) the flocs and attached biomass can demonstrate many differences, e.g. texture, active surface, heterotrophs and autotrophs ratio, and especially biomass age. A compilation of these two technologies in one hybrid reactor allows to utilize advantages of these technologies and to achieve high carbon and nitrogen removal efficiency. The additional advantages of this new technology (moving bed biological reactor – MBBR; other similar terms: Integrated Fixed Film/Activated Sludge - IFAS, Mixed-Culture Biofilm - MCB) are cost savings and reactor volume reduction. Simultaneous processes maintenance (SND reactor) and specific parameters preservation enable treatment of specific wastewater.

2. Bioreactors' characteristics

2.1. Suspended biomass reactors

Reactors with suspended biomass (activated sludge), commonly used in wastewater treatment, utilize a biocenose of various heterotrophs and autotrophs which are able in certain conditions to remove efficiently pollutants from wastewater. They use dissolved and suspended matter (after hydrolyzing) for biosynthesis and assimilation. One of the basic activated sludge process

parameter is the biomass age. This parameter indicates the time of biomass retention in the system and is calculated from biomass balance. The biomass age (sludge retention time, SRT) has an impact on the substrates removal and can be maintained using recirculation, independently on the hydraulic retention time (HRT). On the other hand the pollutions' loads on biomass have a direct impact on the nitrogen and phosphorus removal.

The basic kinetic equation of substrate removal, used in many mathematical models of biological wastewater treatment, is that of Monod type: it describes the substrate utilization rate as a function of specific rate of microorganisms growth [1]:

$$\frac{dS}{dt} = \frac{\mu_{max}}{Y_{max}} \cdot \frac{S}{S + K_S} \cdot X \tag{1}$$

where:
μ_{max} – maximum growth rate, 1/d,
Y_{max} – substrate utilization yield, g_{sm}/g_{sub},
K_S – saturation coefficient, g/m^3,
S – substrate concentration, g/m^3.

In the activated sludge technology three types of reactors are used: continuous stirred tank reactor (fully mixed flow reactor), plug flow reactor and sequencing batch reactor (SBR). The other differentiating factor is aeration of fluid in the reactor, so there are in general three types of reactors: aerated, non-aerated, and intermittently aerated.

Activated sludge flocs have an irregular structure. The disperse rate is related to accessibility of substrate and oxygen for the inert layers of flocs. Li and Bishop [2] prepared microprofiles of the oxygen and substrates concentration in the floc using clark-type microelectrode (figure 1). Redox potential (ORP) changes and oxygen concentrations indicate conditions inside the floc and concentrations of different nitrogen forms describe nitrification process performance (mainly in the top layers of floc) and denitrification (inert layers of floc). The diameter of flocs was in range from 1.0 to 1.4 mm, and oxygen uptake rate was equal to approximately 1.25 mg O_2/dm^3 min.

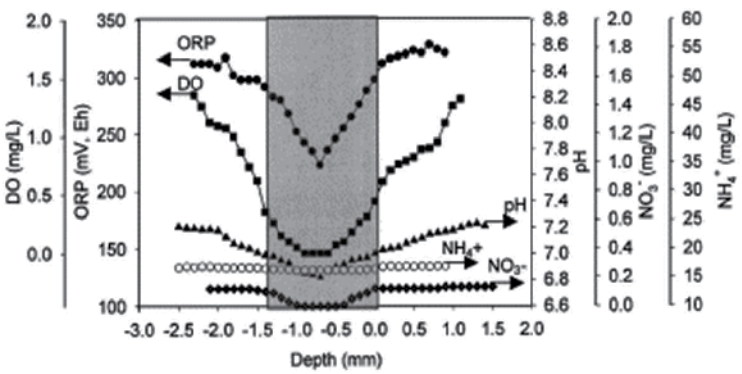

Figure 1. Microprofiles of dissolved oxygen redox potential, pH, nitrates and ammonium concentrations in a floc of activated sludge [2]

2.2. Bioreactors with attached biomass

Attached biomass reactors operate as moving beds, packed (fixed) beds and membranes. The attached biomass (biofilm) has a thickness up to 1.5 mm. The substratum can be fixed (trickling filters and submerged beds) or moving (moving bed biofilm reactors). A main factor affecting access of biomass to the substrate is the effective surface area. The biofilm volume concentration can be even 10 times higher than concentration of activated sludge floc biomass and commonly is in range of 10 to 60 kg/m^3. The biomass age is much longer in biofilm than in flocs and ranges from several to even more than 100 days. The other important factors are: organic substrate loading on substratum surface area and (what is often correlated) the organic substrate loading of the biomass.

The substrate utilization rate in biofilm can be expressed by equation:

$$\frac{dS}{dt} = \frac{\mu_{max}}{Y_{max}} \cdot \frac{S}{K_S + S} \cdot d \cdot X_b \cdot A_b \tag{2}$$

where: d – biofilm thickness, m,
X_b – biofilm density, g/m^3,
A_b – biofilm specific surface area, m^2/m^3.

The substrate penetration into the biofilm depth is affected by SUR (substrate utilization rate) and diffusion coefficient. The relative depth of substrate penetration into the biofilm can be expressed by penetration coefficient (β) assuming that the rate of reaction is zero-order [1]:

$$\beta = \sqrt{\frac{2 \cdot D \cdot S}{k_0 \cdot z^2}} \tag{3}$$

where: D – substrate diffusion coefficient, m^2/s,
k_o – zero-order reaction constant for biofilm, kg/m^{3}s,
z – biofilm thickness, m.

When $\beta > 1$, the substrate penetrates trough the whole depth of biofilm, when $\beta < 1$ – substrate penetrates the biofilm only up to the certain depth. When two substrates are considered e.g. oxygen and organic compounds, one of them can be limiting. If the condition: $\beta_{O2} < \beta_{BZT5} < 1$ is satisfied, the conditions in the biofilm will be anaerobic. Conditions and processes in biofilm can be indicated by microprofiles [3]. A dramatic peak of redox potential (figure 2) indicates a change in oxygen conditions – from aerobic near the biofilm surface – to anaerobic – near the substratum. Nitrogen compounds changes indicate the nitrification process caused by oxygen penetration into the subsurface layers of biofilm depth.

2.3. Hybrid bioreactors

A practically useful solution is compilation of the described above two technologies in one reactor named a hybrid reactor. Both activated sludge and biofilm technologies advantages are utilized in this system.

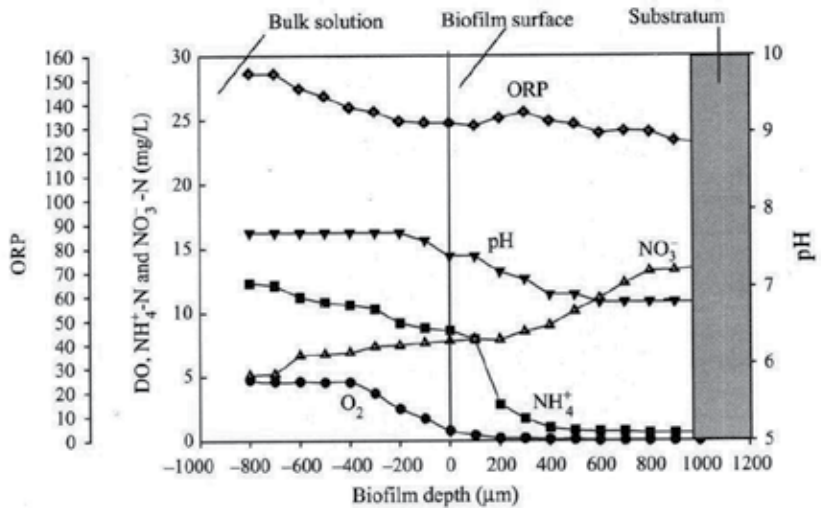

Figure 2. Microprofiles of biofilm [3]

In this type of reactors (Integrated Fixed Film/Activated Sludge - IFAS, Mixed-Culture Biofilm – MCB, hybrid bioreactors) a secondary settler is used and suspended biomass is returned to the bioreactor, so certain suspended biomass concentration can be maintained. However, when suspended biomass flocs are relatively large (up to 1500 μm of diameter) they can clog the small pores of carriers [4] resulting in attached biomass growth interruptions and oxygen access limitations.

Additional modifications can reduce energy consumption. The biofilm substratum may consist of various plastic carriers with effective surface area up to several hundred square meters per cubic meter. The volumetric density of carriers with biomass in fluidized beds should be similar to the wastewater density or slightly higher. There are many market-available types of carriers.

Hybrid reactors with moving carriers were firstly developed in Norway in nineties of XXth century. Characteristics of this technology were given firstly by Odegaard et al. [6]. They proposed the carriers filling rate of 70% of volume and obtained the removal efficiency of 91-94% for organic compounds and of 73-85% for nitrogen compounds. The impact of the substrate loading of reactor on the treatment performance was studied by Orantes and Gonzales-Martinez [7] and Andreottola et al. [8]. These researchers applied this technology to the specific conditions – for resorts in Alps. Andreottola et al. [9] and Daude and Stephenson [10] designed such reactor as a small WWTP for 85 p.e.

The hybrid reactor can be designed basing on organic loading of biomass and knowing the geometry of carriers. The number of carriers (N) can be calculated as [11]:

$$N = \frac{L_S}{A_{k1} \cdot A_b^* \cdot G_b} \tag{4}$$

where: L_S – removed organic load, kg/d,
A_{k1} - one carrier effective surface area , m^2,
A_b^* - organic loading of biomass, $g/g_{dm}d$,
G_b – biofilm surface density, g_{dm}/m^2.

The simultaneous application of activated sludge and moving bed technologies has a positive influence on the nitrification process. Paul et al. [12] found that 90% of autotrophs in hybrid bioreactor is a component of biofilm (autotrophs are 40% of total number of microorganisms). Despite relatively low kinetic constants of autotrophs growth and substrate utilisation rate comparing to the heterotrophs (Y_H = 0.61 g_{dm}/g_{COD}, Y_A = 0.24 g_{dm}/g_{COD}, μ_{Hmax} = 4.55 d^{-1}, μ_{Amax} = 0.31 d^{-1}), the high (over 90%) nitrification efficiency in hybrid reactor can be achieved, even in terms of high hydraulic loading rates.

The nitrifying bacteria in the biofilm on the carriers are able to reach the nitrification rate up to 0.8 gN/m^2d at 10°C [13] and even up to 1.0 gN/m^2d at 15°C [14].

2.4. Spatial and ecological forms of biomass

There is a little research related to the interactions between activated sludge flocs and biofilm e.g. migration of the organisms. These interactions are complex, and both relations: between flocs and biofilm, and between heterotrophs and autotrophs in the biofilm should be considered in modeling [15] and operation. Albizuri et al. [15] assumed that these interactions could act with mediation of colloidal components. It is well know fact that there are many grazing species (e.g. *Ciliata*) which creep on the flocs/biofilm surface or swim near the flocs and biofilm surface. On the other hand there is some number of species existing in deeper layers of biofilm, which probably can not migrate. It is worth to note that the structure of activated sludge flocs is heterogeneous and deep layers of biomass in flocs are anaerobic, what results in different species composition (anaerobic bacteria).

The biological composition of flocs in hybrid bioreactors is similar to typical biological content of activated sludge flocs. Similarly the size of hybrid bioreactor flocs in hybrid reactors is close to typical activated sludge flocs - diameter in the range of 150-500 μm [16].

In hybrid bioreactors various nitrogen removal processes pathways are possible, including autotrophic processes, e.g. anammox [17]. A sufficiently thick layer of biofilm or flocs is needed for complex nitrogen process transformations including denitrification. On the other hand a relatively thin biofilm (due to shearing stress) results in high activity of biomass [18]. Some authors [19,18] found that in sequencing batch biofilm reactors (SBBR) the biofilm is fully penetrated by substrates and electrons acceptors can be released.

Similarly as in case of other attached biomass systems, e.g. trickling filters in MBBR design procedure, surface area loading rate should be the design parameter [5,11]. This approach is based on some typical range of biofilm thickness (in this case surface area can be the indicator of the biomass concentration). From this point of view the size and shape of carriers seem to be less important. The substrate to biomass loading rate is base but not sole design criterion. Important but poorly recognised factors are: access to total biofilm surface area and access to

aerobic biofilm surface area. Some authors indicated that carriers of high total surface area thanks to micropores should have some amount of macropores, enabling fluid reach in oxygen contact with deeper inner spaces of carriers, e.g foamed cellulose carriers [20]. The macropores are important for nitrifying biomass, which needs contact with dissolved oxygen. Micropores are often filled completely with biofilm preventing the oxygen penetration. Oxygen access factor is crucial for biofilm thickness, porosity and surface roughness.

The ratio of the suspended to the attached biomass can vary accordingly to many factors and conditions. The amount of attached biomass can reach over 90%. Plattes et al. [21] indicated 93% of biomass in form of biofilm attached to the carrier elements and only 7% of biomass – as suspended in the bulk liquid. Detachment (or sloughing) of biomass is variable in time [5]; probably this phenomenon is similar to sloughing of excess biomass from biofilm growing in others attached biomass systems, e.g. trickling filters. Some authors [22] suggested that in such systems detachment process occurs periodically.

Due to mechanical contact with others carriers and shear stress, the biomass grows mainly on the internal area of carriers, what was reported by several authors [16, 23], excepting carriers having outgrowths on the outside walls surface.

The common forms in typical activated sludge system are aggregated flocs and planktonic free-swimming cells, and bacterial communities are dominated by: *Betaproteobacteria, Alphaproteobacteria, Gammaproteobacteia* and more less frequent: *Bacteroidetes and Firmicutes* [24]. Some authors [24] observed in biofilms in MBBR limited bacterial diversity and *Firmicutes* domination. The research of Biswas and Turner [24] indicated that MBBR communities differ from communities existing in conventional activated sludge reactors. The characteristic feature of MBBR bacteria community was a presence of two distinct communities: suspended biomass with fast-growing aerobic bacteria and biofilm biomass, which was dominated by anaerobic bacteria [24]. In biofilms of WWTP which were studied by these authors the prevailing forms were *Clostridia* (38% of clones) and sulfate-reducing bacteria (*Deltaproteobacteria* members). The another forms were less abundant: *Desulfobacterales* (11-19%), *Syntrophobacterales* (8-10%), *Desulfovibrionales* (0.5-1.5%). The other groups were also observed: *Bacteroidetes*, *Synergistes*, *Planctomycetes*, *Verrucomicrobia* and *Acidobacteria*.

The suspended biomass observed in two MBBR reactors by Biswas and Turner [24] was consisted mainly of aerobic microorganisms: *Alphaproteobacteria* (*Rhizobiales, Rhodobacterales*), Gammaproteobacteria (*Pseudomonadales, Aeromonadales*), *Betaproteobacteria* (*Burkholderiales, Rhodocyclales*). Majority of *Firmicutes* was represented by *Clostridia* and one MBBR reactor suspended biomass was reach in *Campylobacteraceae* (54% of clones).

The differences in microbial composition can appear not only between biofilm and activated sludge in MBBR reactor but also between MBBR bioreactors themselves. Biswas and Turner [24] observed the biomass, both black with sulfurous odour in one MBBR reactor and grayish-brown without obvious odour - in other MBBR reactor. Some authors indicated that in continuous-flow MBBR in which SND process was established, the microbial community structures of biofilm are related to C/N ratios [25]. In MBBRs the volume concentration ratio

of biofilm to the activated sludge flocs cab be even higher: 5-13 [26] than for separated attached biomass and suspended biomass systems. Some important differences between biofilm and flocs features in MBBRs were found by Xiao end Garnczarczyk [26]. They observed 3 - 5 times higher geometric porosity in biofilm than in activated sludge flocs. Biofilm boundary fractal dimension was higher than activated flocs one. These authors observed also some similarities: two different space populations both in biofilm and in flocs were indicated and both attached and suspended biomass shifted some of their structural properties to larger values (thickness, density) with the increased hydraulic loading.

2.5. Carriers material characteristics and impact on attachment conditions and biomass structure

For the MBBR pollutants removal efficiency and biomass concentration the crucial role plays the material of which carriers were made (table 1). The basic features are as follow: material type, specific surface area, shape and size of carriers and other features: porous surface, e.g. polyurethane or not porous material surface e.g. polyethylene [27,28].

Material	Density, g/cm^3	Specific surface area, m^2/m^3	Type of carrier	References
Cellulose (foamed)	-	-	continuous macro-porous, Aquacel, 1-5 mm	[20]
Polyvinylformal (PVF)	-	-	cubic, 3 mm	[20]
PVA-gel beads	-	-	-	[23]
Polyethylene (PE)	-	-	K1, EvU-Perl	[55]
Nonwoven fabric	-	900	-	[56]
Reticulated polyester urethane sponge (foam)	0.028 (volumetric density)	-	S45R, S60R, S90R, Joyce Foam Products	[57]
Polypropylene (PP)	1.001	230-1400	cylindrical rings 4 mm	[58]
Polyethylene (PE)	0.95	230-1400	cylinders 7 mm/10 mm	[59]
Polyethylene (PE)	-	-	Kaldnes, Natrix, Biofilm-Chip	[60]
Polyurethane coated with activated carbon	-	35,000	cubes (1.3 cm), Samsung Engineering Co	[61]

Table 1. Selected types of carriers and material of which carriers were made

2.6. Carbon and nitrogen compounds removal

The basic role in biochemical transformations have three types of processes:

(*i*) hydrolysis (slow decomposition of polymeric substances to easy biodegradable substances, (*ii*) substrates assimilation by microorganisms correspondingly with Monod equation and (*iii*) growth and decay of microorganisms, what can be written in form:

$$\frac{dX}{dt} = \mu_{max} \cdot f(S) \cdot X - K_d \cdot X \tag{5}$$

where: $f(S)$ – substrate concentration related function,
X – biomass concentration, g_{dm}/m^3,
K_d – biomass decay constant, 1/d.

Organic substrates in the wastewater are in the form of: suspended solids, colloids and soluble matter. In overall form it can be described as $C_{18}H_{19}O_9N$. Due to their different forms (easy degradable, slowly degradable and not biodegradable fractions) they can be oxidized, assimilated or not biologically decomposed.

The organic substrate fractions, relating to the form and decomposition pathways can be identified correspondingly to commonly used methodology [29-31]. Typical wastewater consists 10-27% of soluble easy biodegradable substances (S_S), 1-10% of not biodegradable soluble substances (S_I), 37-60% of slowly biodegradable suspended solids (X_S) and 5-15% of very slowly biodegradable suspended solids (X_I) [31-34].

Easy biodegradable organic substances are an energy sources for denitrifying and phosphorus accumulating bacteria (PAB) and its concentrations have direct impact on the nitrogen and phosphorus removal.

Nitrogen in wastewater appear usually in form of soluble non-organic forms (mainly ammonium nitrogen, seldom nitrites and nitrates), organic soluble (degradable and not degradable) and as the suspended solids (slowly degradable, not degradable and as a biomass). Nitrogen compounds transformations are carried by autotrophic and heterotrophic bacteria and elementary processes need to preserve adequate technological conditions for these microorganisms.

The polymeric substances hydrolysis is catalysed by extracellular proteolytic enzymes to transform into simple monomers, which can be assimilated by microorganisms.

This process and ammonia nitrogen assimilation is related to fraction of nitrogen in biomass (5-12%). The nitrogen assimilation rate depends on the C/N ratio.

Another process of nitrogen transformation is nitrification – oxidation of ammonium nitrogen to nitrites and nitrates by chemolithotrophs: *Nitrosomonas, Nitrosococcus* and *Nitrosospira* in first phase (hydroxylamine is the intermediate product) and *Nitrobacter, Nitrospira, Nitrococcus* in the second phase, what can be described in form [35]:

$$NH_4^+ + 1{,}5O_2 \rightarrow NO_2^- + H_2O + 2H^+ - 278\,kJ/mol$$

$$NO_2^- + 0{,}5O_2 \rightarrow NO_3^- - 73\,kJ/mol$$

Nitrifying bacteria, as autotrophs, take the energy from carbon dioxide and carbonates. The utilisation rate for *Nitrosomonas* is equal to 0.10 $g_{dmo}/g_{N\text{-}NH4}$, and for *Nitrobacter* – 0.06 $g_{dmo}/g_{N\text{-}NO2}$ [1]. The important process parameters are: oxygen supply (4.6 g $O_2/1_gN\text{-}NH_4$), temperature (5 – 30^{0}C), sludge/biomass age (more than 6 days is recommended), biomass organic compounds loading (over 0.2 g BZT_5/g_{dm} is recommended) and BOD/N ratio: when the value is more than five – organic compounds removal dominates, when is lower than three – the nitrification is a prevailing process. The decrease in alcanity is the result of nitrification (theoretically: 7.14 g $CaCO_3$/1 g N-NH_4) and it causes decrease in pH from 7.5 – 8.5 to 6.5.

Nitrates are transformed in the dissimilation reduction process (*Pseudomonas, Achromobacter, Bacillus* and others) to the nitrogen oxides and gaseous nitrogen correspondingly to the path:

$$2NO_3^- \xrightarrow{-4e} 2NO_2^- \xrightarrow{-2e+2H^+} 2NO \xrightarrow{-2e} N_2O \xrightarrow{-2e} N_2$$

In the terms of dissolved oxygen deficiency (anaerobic or anoxic conditions) those organisms use nitrates as H^+ protons acceptors.

Denitrifying bacteria, as the heterotrophs, need organic carbon for their existence. The source of organic carbon can be: organic compounds in wastewater (internal source), easy assimilated external source of organic carbon, e.g. methanol/ethanol, or intracellular compounds as an energetic source. This ratio of organic carbon should be in range of 5 – 10 g COD/g N-NO_3 [35].

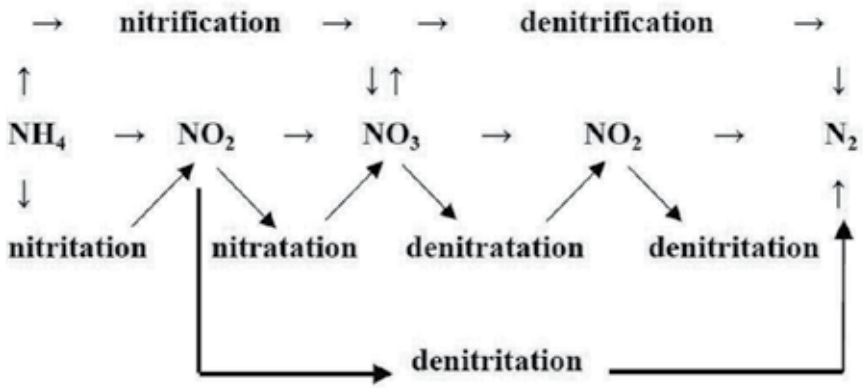

Figure 3. Elementary processes of nitrification and denitrification in nitrogen treatment

In the figure 3 the elementary processes of nitrification and denitrification in nitrogen removal are shown [11]. It is easy to recognise that denitrification is partly the reverse process to the nitrification. Due to the fact that some nitrifying bacteria can live without oxygen and some denitrifying bacteria can survive in oxygen conditions there is the possibility to carry out the simultaneous nitrification and denitrification (SND) in one reactor. As the SND reactor both continuous and sequencing batch reactors can be used. In

the SBR-SND reactor high removal efficiency for organic and nitrogen compounds can be achieved – 79% and 96% respectively [36]. The biomass growth rate can be in range of 0.3 – 0.75 $g_{SDM}/g_{sub\ rem}$ [37]. Similarly high nitrogen removal efficiency in SND process in continuous reactor (about 90%) can be achieved at certain pH and N-NH_4 concentration [38]. The nitrite concentration rise indicates that the nitrification process has stopped after the first phase. The process can run at low C/N ratio.

The short version of SND process needs preservation of certain technological conditions. Important conditions are aerobic and anoxic conditions, what in one reactor system can be achieved by intermittent aeration and non aeration. It causes the characteristic variability of parameters such: pH, redox potential or nitrogen compounds concentration [39]. Examples of changes of some variables in bioreactor with intermittent aeration are presented in figure 4 [40].

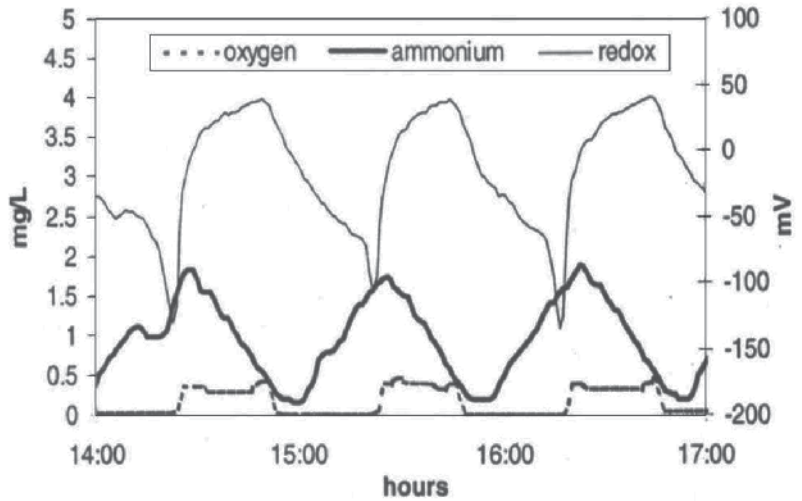

Figure 4. Change of sewage parameters for oxic and aerobic phase of biological reactor [40]

The blockage or limitation of second phase of nitrification can be achieved by limitation of oxygen availability up to approximately 0.7 mg O_2/dm^3 [41] or by free ammonia inhibition, which concentration is impacted by the temperature and pH of wastewater. Anthonisen et al. [42] identified the limiting values of partial and full inhibition of second phase of nitrification: 0.1 g N-NH_4/m^3 and 1.0 g N-NH_4/m^3 respectively. They proposed the description of free ammonia concentration in the reactor in form [42]:

$$S_{NH_3} = \frac{17}{14} \cdot \frac{S_{N-NH_4} \cdot 10^{pH}}{\exp(6344/T) + 10^{pH}} \tag{6}$$

where: $S_{N\text{-}NH4}$ – ammonium nitrogen concentration, mg/dm^3,
T – temperature, K.

The free ammonia concentration has an impact on the ammonium nitrogen removal velocity, what is described by substrate inhibition model presented by Haldane [43]:

$$r_{NH} = r_{NH\,max} \cdot \frac{S_{NH_3}}{K_{sNH3} + S_{NH_3} + S^2_{NH_3} / K_{iNH3}} \tag{7}$$

where: r_{NH} – ammonia nitrogen and ammonium removal velocity, mgN/mg$_{sm}$h,
r_{NHmax} – ammonia nitrogen and ammonium removal maximum velocity, mgN/mg$_{sm}$,
K_{sNH3} – saturation constant, mgN-NH_3/dm^3,
K_{iNH3} –inhibition constant, mgN-NH_3/dm^3.

For the mathematical description of organic and nitrogen compounds removal many existing models are used with certain modifications, e.g.: substrate inhibition in Brigs–Haldane model [44] or ASM1 model [45], intermittent aeration or oxygen limitation inhibition in ASM1 [46-48], or two stages process of nitrification and denitrification in ASM3 model [49].

3. Laboratory research

The aim of this study was to determine elementary processes related to the organic and nitrogen compounds removal in hybrid reactors with intermittent aeration, to assess removal efficiency under various organic and hydraulic loadings and organic and nitrogen compounds' utilization rates. The utilitarian aim was to determine technological conditions which could make the process shorter and more economically efficient. The attempts to modeling using various technical parameters (together or separately) were conducted.

3.1. Laboratory model and methods

Carriers used in the research were corrugated cylindrical rings made of PP diameter and length of 13 mm, 0.98 g/m^3 density and 0.86 porosity (figure 5).

The research studies were conducted in four stages of 10 months duration. Each stage was consisted of three or four series. In each stage three reactors worked simultaneously as continuous flow system in stage I and III and as a sequencing batch reactor in stage II. The volume of reactors was equal to 75 dm^3 and volume of settler for the continuous flow was equal to 20 dm^3.

The studies were focused on an intermittent aeration. The most attention was put on the last stage – with increased wastewater pH value using lime ($Ca(OH)_2$). The aim of higher pH maintaining was to inhibit the second phase of nitrification by ammonia. The wastewater originated from one family household. The retention time before the sewage discharging into the reactors was relatively high – about 6 days (septic tank and retention tank). The activated sludge originated from Poznań Central WWTP and was inoculated to each reactor at the same amount in each stage beginning. Mixing and aeration of the reactors was made using large-bubble diffusers. The air was supplied by compressor of 0.1-2.0 m^3/h capacity. The sludge recirculation was made using an air-lift cooperating with a membrane pump. Characteristic research parameters are shown in table 2.

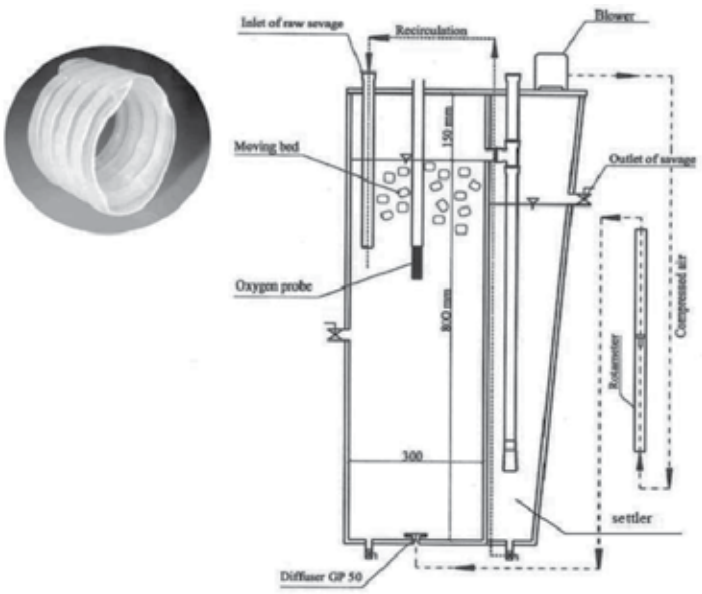

Figure 5. Scheme of laboratory model

	Continuous flow reactor (CFR)	Batch reactor (SBR)	Continuous flow reactor (CFR) with increased pH
Sewage volume per day, dm^3/d	70 - 290	45 - 270	140
Number of series	4	3	3
Variable factor for series	time of aeration	length of reactor cycle	number of carriers
Variable factor for reactors	hydraulic load pollution load	active volume of reactor	pH value
C/N	1.31 ± 0.09	1.67 ± 0.10	1.39 ± 0.07
Total solids, g/m^3	34.95 ± 6.65	62.30 ± 3.30	37.00 ± 5.20
Organic compounds as COD, $g\ O_2/m^3$	188.25 ± 1.62	172.80 ± 3.7	184.00 ± 8.00
Nitrogen compounds as N_{tot}, $g\ N/m^3$	46.56 ± 1.35	41.65 ± 1.09	52.92 ± 2.43

Table 2. Technological characteristics of model investigation and average concentrations of pollutants in sewage

The characteristic feature of the used sewage was a low C/N ratio caused by pretreatment in a septic tank. The pollutants in sewage and suspended biomass concentrations were measured according to the standard methods. The attached biomass concentration was identified via the Kjeldahl nitrogen measurement: 1 g N_{TKN} corresponds to 0.11 g_{dm} [50]; pH and oxygen were measured using calibrated electrodes.

The detailed description of research results of all experimental stages is included in the Makowska's monography [11]. In this chapter only the most important processes and parameters related to the carbon and nitrogen removal efficiency are presented. Results related to the parameters like: biomass loading, pollutants' removal efficiency and substrates utilization rates were analyzed statistically.

3.2. Carbon and nitrogen compounds removal efficiency in continuous and sequencing flow MBBR reactors

The oxygen accessibility play a very important role in bioreactor performance. Four aeration/nonaeration time intervals were tested: 75/45, 45/45, 30/30 and 15/15 minutes. The most effective was the last interval in which oxygen deficit lasted 10 min was observed in nonaeration phase and maximum for SND process oxygen concentration (0.8 mg O_2/dm^3) was achieved. In these conditions for continuous flow, the maximum removal efficiencies for carbon and nitrogen compounds were equal to 98% and 85% respectively [16,11]. The optimal hydraulic retention time was equal to 12 hours.

The outflow pollutants concentrations were related mainly to biomass loading: the higher loading – the lower the removal efficiency, especially for medium and high loaded reactors (the most evident for sequencing batch reactor). This relationship was more evident for nitrogen compounds removal (figure 6) excepting total nitrogen removal in SBR.

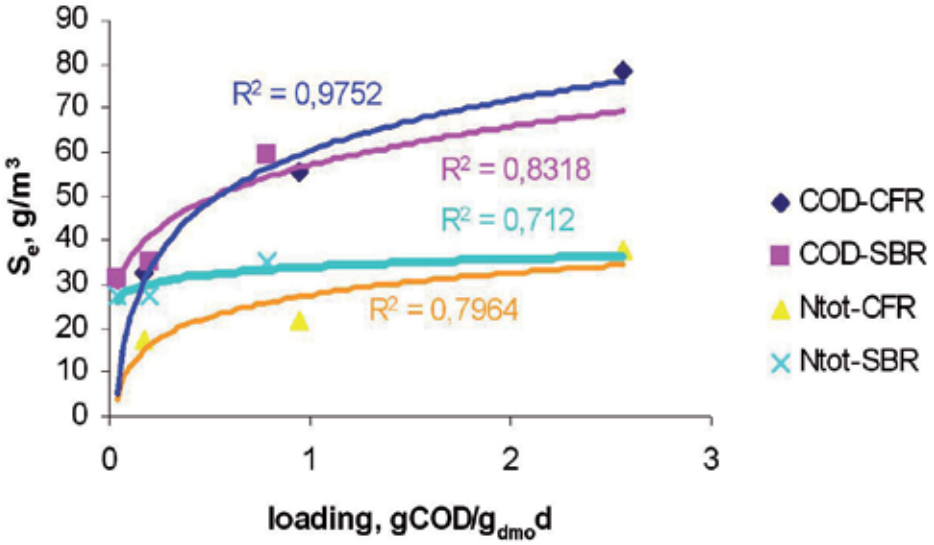

Figure 6. Relationship between pollutants' concentration in purified sewage and biomass loading

The increase in loading up to 2.5 g COD/g_{dm}d caused the rise of contaminants removal rate (figure 7), although it was partly related to biomass concentration decrease as a result of lading rise. The removal efficiency in SBR was related to the volumetric exchange ratio (0.2-0.5 range); the higher ratio – the lower removal efficiency.

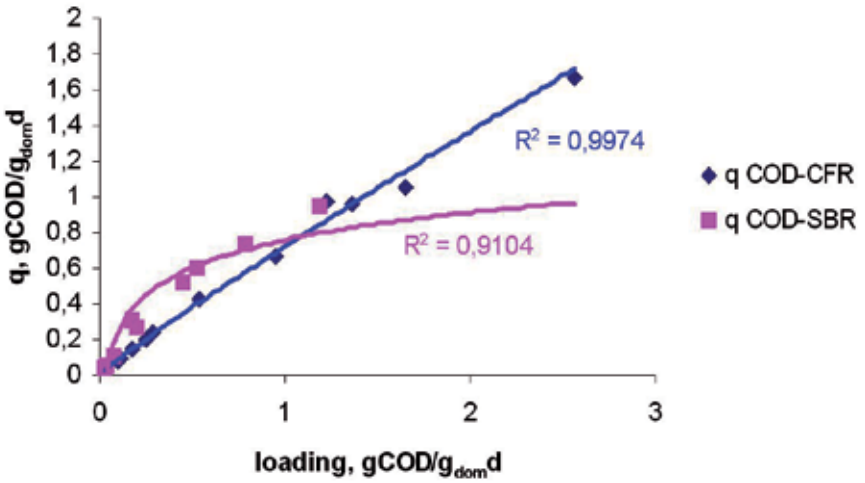

Figure 7. Relationship between pollution removal rate and biomass loading

Some authors stated the higher resistance for hydraulic overloading and more stable nitrification in hybrid reactors than in conventional activated sludge reactors [12]. These research showed that suspended/attached biomass ratio was related to the biomass organic compounds loading (figure 8). The higher biomass loading – the higher attached biomass concentration (the same - lower suspended biomass concentration). This phenomenon was also observed by other authors [26].

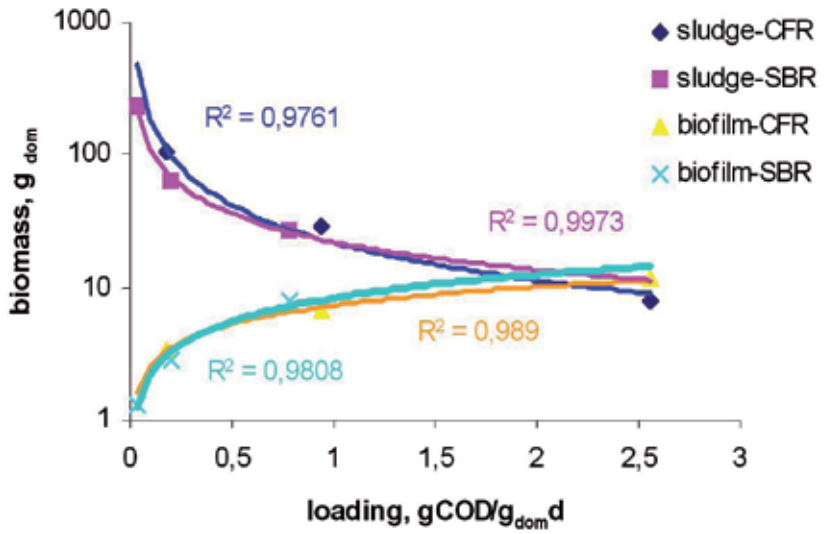

Figure 8. Relationship between biomass in reactors and organic load

So the conclusion can be drown that highly loaded reactors (especially SBR) do not need excess sludge removal, although biomass growth yield can reach values in range 0.31 - 0.50 $g_{dm}/g_{sub\ rem}$.

3.3. Carbon and nitrogen compounds removal efficiency in continuous flow reactor with elevated pH of sewage

Continuous flow reactor with elevated wastewater pH was maintained at 12 hours HRT and 15/15 minutes aeration/nonaeration intervals. Values of pH were in the range of 8.0-8.5. Three rates of volume reactor filling by carriers were investigated: 60%, 40% and 20%.

The elevated pH caused the ammonia release and inhibition of the second phase of nitrification and this way the total nitrogen removal process was shorten by elimination of two elementary processes: nitratation and denitratation (figure 8).

The free ammonia concentration value, calculated accordingly to equation 6 was equal to appr. 1 mg N/dm^3, what is known as a limiting value for inhibition of nitrification second phase [42]. The part of free ammonia could be released as the result of amino acids denaturization during alkalinity process, but due to relatively low concentration of organic nitrogen (mainly amino acids) in inlet wastewater (maximum 10% of total nitrogen) this factor can be neglected. In these conditions the SND process was achieved (shortened nitrogen removal process) what had been indicated by temporary nitrites accumulation. It is known and was stated by several authors [51] that as a result of ammonia inhibition of second phase of nitrification, mediate and final products are released simultaneously.

The higher removal efficiency was achieved at higher volume carriers filling of reactor (figure 9). The rise in pH value versus the rise of nitrogen compounds removal rate, what was observed by other authors [52].

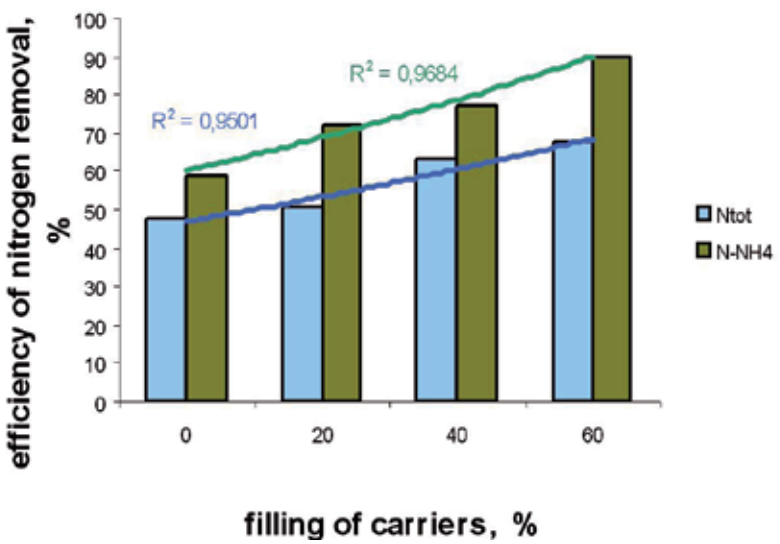

Figure 9. Relationship between effect of nitrogen removal efficiency and filling by carriers

The lime addition resulted in some changes in biomass, e.g. reducing organic fraction rate in biomass of 25% comparing to the process without lime addition. The lime in the liquid phase and on the carriers surface were some kind of "condensation centers" and caused the

higher concentration of both biomass form. The smaller amount of carriers enabled more undisturbed carriers movement (figure 10). The lime addition caused also the less susceptibility of carriers pores for clogging by biomass.

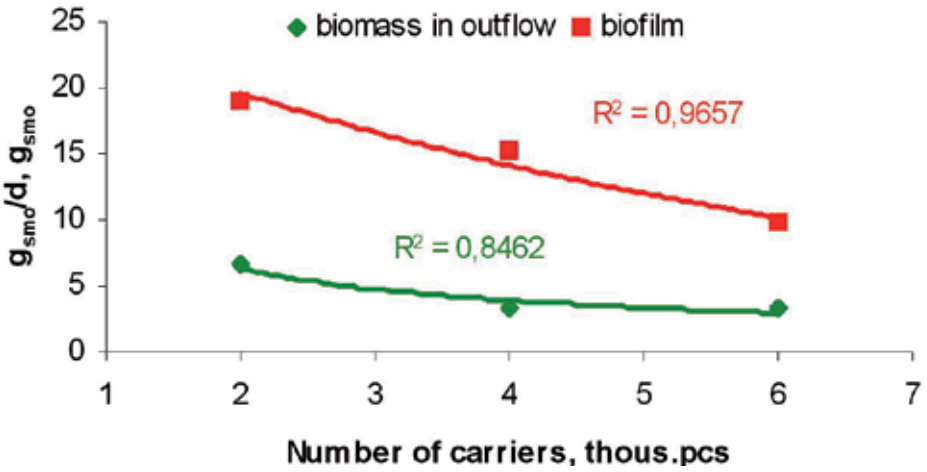

Figure 10. Biomass removed from reactor and amount of biofilm with quantity of carriers

3.4. Hybrid bioreactors biomass characteristic

In this research relatively wide (but typical for activated sludge reactors) range of activated sludge flocs size was observed: 150-500 μm in all reactors.

The biomass attached to the moving bed carriers surfaces was poorly developed. Attached biomass did not cover the outside carriers surface and existed only on the inert surface not as continuous film but as separate small mushroom shape colonies. Small colonies of stalked ciliates (figure 11) were observed on the inner surface of carriers. There were observed some differences between biofilm and activated sludge flocks groups of organisms, in both biomass forms. Stalked, creeping and free-swimming ciliates, filamentous microorganisms, rotifers and nematodes were observed.

Epistylis and *Vorticella* were dominating genera of ciliates in both suspended and attached biomass. Stalked ciliates were observed in relatively high number both in attached and suspended biomass. The domination of this form of *Ciliata* was probably related to good pollutant removal efficiency, what was reported by other authors [1, 54].

The observed number of rotifers in the attached biomass was much higher than in suspended biomass (t-Student statistics; t calculated: 2.83, critical value: 2.78, α: 0.05, replications no.: 5, df: 4), what was the most evident in period 3 in SBRs, and was probably related to the long time of growth of rotifers. Also the differences in concentrations of filamentous microorganisms were observed - in continuous flow reactors the number of filamentous microorganisms was often lower in attached biomass than in suspended biomass (figure 12). Filamentous microorganisms concentration was the highest in the

highest COD loaded reactor: R3 (t-Student statistics; t calculated: 8.98, critical value: 2.2, α: 0.05, replications no.: 13, df: 11).

Figure 11. The inner surface of a carrier covered by a small stalked ciliates colony [16]

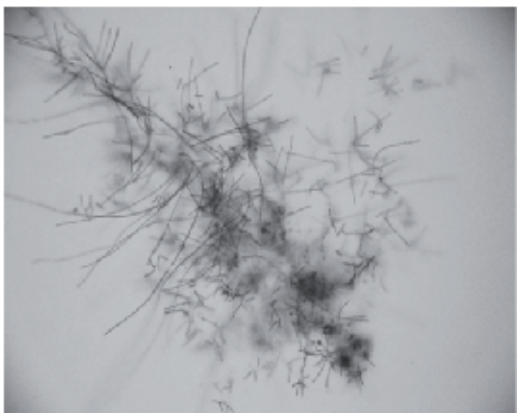

Figure 12. Filamentous organisms in reactor R2 during stage II [16]

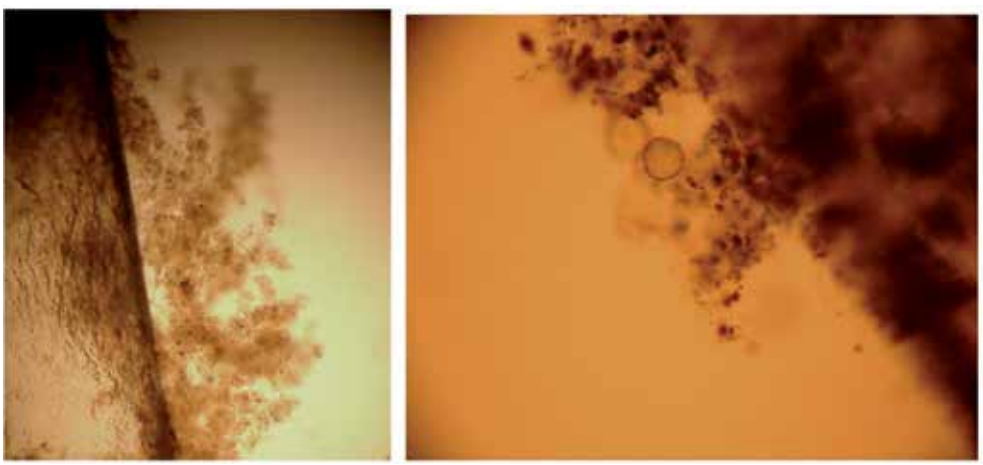

Figure 13. Biofilm on the MBBR carrier

3.5. Hybrid bioreactor mathematical modeling

The mathematical modeling is a very useful method of process simulation, because there is no need time and costs consuming experimental methods using. However, there are many problems in adequate mathematical description of complex biochemical processes and parameters' estimation.

For hybrid bioreactor modeling the ASMH1 model [11] can be applied. It is based on the ASM1 model, but significantly modified: nitrogen removal processes are more completely treated by implementation of two stages nitrification and denitrification (figure 14). The intermittent aeration and oxygen accessibility to the second phase of nitrification was considered. The free ammonia inhibition was also implemented.

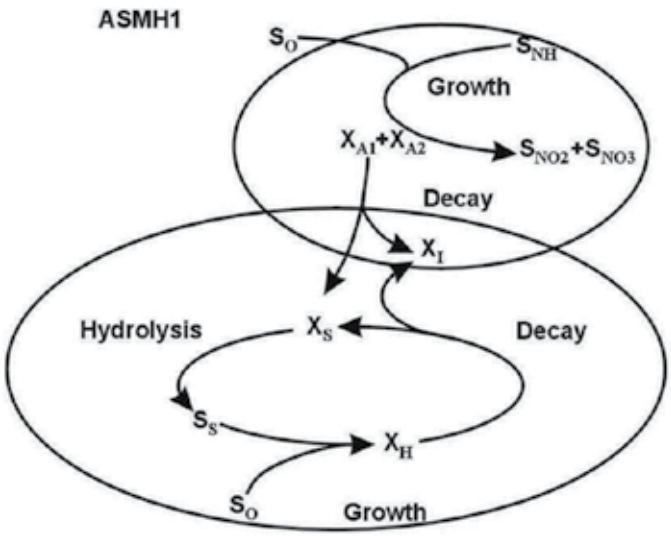

Figure 14. Scheme of process in the ASMH1 model

The model calibration using laboratory data allowed to identify the kinetic and stechiometric parameters values. The model was implemented in to the POLYMATH program and relatively good agreement with experimental results was achieved (st. dev. 10%).

4. Conclusions

The basic technological parameters related to removal efficiency of pollutants from septic tank in hybrid MBBR, at intermittent aeration, continuous/sequencing flow and elevated pH were presented in table 3. The parameters' values concerning the purified sewage fulfill the Polish law requirements for 2000 p.e. WWTPs.

The hybrid MBBR has occurred an effective system for carbon and nitrogen compounds removal from septic tank effluent. The carbon and nitrogen compounds can be removed

with at least 80% and 50% removal efficiency respectively. It can be achieved even at loading of 2 g COD/g_{dm}d and 12 hours HRT. Similar results were reported by [53] for partial nitrification-denitrification process in combination of aerobic and anoxic reactors with Kaldnes carriers. The system needed internal recirculation. Thanks to attached biomass the nitrification process in the hybrid MBBR was effective at low and high loaded reactors. The remaining of ammonium nitrogen in treated wastewater appeared at high loadings only. The intermittent aeration and dissolved oxygen limitation enabled simultaneous nitrification-denitrification process (SND) in one reactor. The inhibition of second phase of nitrification by free ammonia has intensified nitrogen removal and resulted in energy savings and internal source of carbon using as a sole source. The shortened process of carbon compounds removal was confirmed by medium products appearance. The long time aeration cycles and long time operating cycles can result in denitrification disturbances due to the organic substances oxidation and limitation of that energy source for denitrifying bacteria.

Parameter	Continuous flow reactor CFR	Batch reactor	CFR with increased pH
Pollution load -organic compounds, g COD/d -nitrogen compounds, g N_{tot}/d	 19.29 – 29.25 5.93 – 6.42	 15.56 – 31.57 2.77 – 7.87	 26.51 – 27.89 7.30 – 8.25
Hydraulic load, dm^3/dm^3d	1.53 – 1.90	1.65 – 3.05	1.85 – 2.04
Concentration in purified sewage -organic compounds, g COD/m^3 -nitrogen compounds, g N_{tot}/m^3	 26.91 – 56.22 21.57 – 31.07	 31.31 – 59.46 27.49 – 34.94	 23.00 – 37.00 17.10 – 25.28
Biomass: -activated sludge density, g/dm^3 -biofilm mass, g/m^2	 0.62 – 1.18 2.58 – 5.46	 0.48 – 4.03 0.62 – 3.70	 5.58 – 6.52 1.55 – 3.58
Biomass loading of -organic compounds, g COD/g_{dm}d -nitrogen compounds, g N_{tot}/g_{dm}d	 0.247 – 0.945 0.067 – 0.201	 0.081 – 1.080 0.011 – 0.155	 0.075 – 0.082 0.011 – 0.021
Efficiency of removal,% -organic compounds -nitrogen compounds	 75 – 84 37 – 54	 69 – 82 20 – 35	 80 – 87 51 - 68
Pollution removal rate -organic compounds, g COD/g_{dm}d -nitrogen compounds, g N_{tot}/g_{dm}d	 0.206 – 0.663 0.030 – 0.113	 0.110 – 0.620 0.004 – 0.032	 0.064 – 0.124 0.011 – 0.021
Yield coefficient, g_{dm}/g_{CODrem}	0.31 – 0.43	0.42 – 0.50	0.34 – 0.47

Table 3. Technological parameters and treatment efficiency in hybrid reactors

The pH elevation brought about higher treatment efficiency. The higher volumetric fraction of moving media (carriers) – the better performance. The continuous flow reactor was more effective in treatment and more stable than the sequencing batch reactor.

It was stated and statistically confirmed that: aeration regime, biomass loading and media volume fraction have an impact on the pollutants (especially organic compounds) removal efficiency.

Advantages of hybrid MBBR reactors operating in the modified conditions are as follows: lower energy consumption (up to 40%) related to the shorter aeration time, possibility of specific wastewater treatment (low N/C ratio), simultaneous processes maintaining in one reactor (internal recirculation elimination), overloading resistance (stable performance) and reduction in smaller reactors' volume. The mathematical model ASMH1 allows to simulate the reactor performance at the specific conditions.

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Chapter 10

Animal Manures: Recycling and Management Technologies

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Additional information is available at the end of the chapter

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1. Introduction

Many environmental problems of current concern are due to the high production and local accumulations of organic wastes that are too great for the basic degradation processes inherent in nature. With adequate application rates, animal manure constitutes a valuable resource as a soil fertilizer, as it provides a high content of macro- and micronutrients for crop growth and represents a low-cost, environmentally- friendly alternative to mineral fertilizers [1]. However, the intensification of animal husbandry has resulted in an increase in the production of manure - over 1500 million tonnes are produced yearly in the EU-27 [2] as reported by Holm-Nielsen et al. [3]- that need to be efficiently recycled due to the environmental problems associated with their indiscriminate and untimely application to agricultural fields. The potentially adverse effects of such indiscriminate applications include an excessive input of harmful trace metals, inorganic salts and pathogens; increased nutrient loss, mainly nitrogen and phosphorus, from soils through leaching, erosion and runoff-caused by a lack of consideration of the nutrient requirements of crops; and the gaseous emissions of odours, hydrogen sulphide, ammonia and other toxic gases [4]. In fact, the agricultural contribution to total greenhouse gas emissions is around 10%, with livestock playing a key role through methane emission from enteric fermentation and through manure production. More specifically, around 65% of anthropogenic N_2O and 64% of anthropogenic NH_3 emissions come from the worldwide animal production sector [5].

The introduction of appropriate management technologies could thus mitigate the health and environmental risks associated with the overproduction of organic wastes derived from the livestock industry by stabilizing them before their use or disposal. Stabilisation involves the decomposition of an organic material to the extent of eliminating the hazards and is normally reflected by decreases in microbial biomass and its activity and in concentrations

of labile compounds [6]. Composting and vermicomposting have become two of the best-known environmentally appropriate technologies for the recycling of manures under aerobic conditions [6-7], by transforming them into safer and more stabilised products (compost and vermicompost) with benefits for both agriculture and the environment. Unlike composting, vermicomposting depends on the joint action between earthworms and microorganisms and does not involve a thermophilic phase [8]. However, more than a century had to pass before vermicomposting was truly considered a field of scientific knowledge or even a real technology, despite Darwin [9] having already highlighted the important role of earthworms in the decomposition of dead plants and the release of nutrients from them.

Although microbial degradation under oxygen is usually faster and, as such aerobic processes are thermodynamically more favorable than anaerobic processes, in recent years, anaerobic digestion (AD) has become an upcoming technology for the treatment of animal manures [3, 10-13]. On the one hand, pretreatment of manure by anaerobic digestion can involve some advantages including malodor reduction, decreased biochemical oxygen demand, pathogen control, along with a reduction in the net global warming potential of the manure [4,14]. AD reduces the risk of water pollution associated with animal manure slurries (i.e., eutrophication) by removing 0.80–0.90 of soluble chemical oxygen demand and it improves human/farm cohabitation in rural regions by reducing odor emissions by 70–95% [4]. This process has other direct advantages beyond these, which are related to biogas production for renewable energy and the enrichment of mineral fractions of N and P during digestion [4,10], resulting in a more balanced nutrient mix and increased nutrient bioavalability for plants compared with undigested manure [15].

Therefore, the purpose of this chapter is to give an overview of the three major management technologies of manure recycling, including the aerobic processes of composting and vermicomposting and the anaerobic digestion for biogas production. The main changes that occur in the substrate from a chemical and microbial viewpoint during the specific phases of each degradation process are addressed, as such changes determine the degree of stability of the end product and in turn its safe use as an organic amendment. Different methods that have been proposed to evaluate compost stability are summarised. Also, the influence of the end products derived from each process on the soil microbiota and disease suppressiveness are discussed.

2. Aerobic degradation: Composting and vermicomposting processes

Under aerobic conditions, the degradation of organic matter is an exothermic process during which oxygen acts as a terminal electron acceptor and the organic materials are transformed into more stable products, carbon dioxide and water are released, and heat is evolved. Under field conditions, aerobic degradation takes place slowly at the soil surface, without reaching high temperatures; but this natural breakdown process can be accelerated by heaping the material into windrows to avoid heat losses and thus allowing for temperature increases (composting) or by using specific species of earthworms as agents for turning,

fragmentation, and aeration (vermicomposting). Although both aerobic processes, composting and vermicomposting, have been widely used for processing different types of animal manure either separately or in combination with each other (see Table 1), most of the studies are not comparable mainly due to differences in the applied experimental designs, parent material, earthworm species, as well as the length of the experiments and the parameters used for analysis, among others. Despite these limitations, all these findings have largely contributed to better understand the changes that the material undergoes during these biological stabilisation processes, which is of great importance for their optimisation, and ultimately to obtain a high quality final product. In line with this, certain chemical characteristics of the animal manures can limit the efficiency of these processes, such as an excess of moisture, low porosity, a high N concentration in relation to the organic C content or high pH values [6]. Therefore, different aeration strategies, substrate conditioning-feedstock formulation, bulking agents and process control options have been considered in manure composting and vermicomposting so as to reduce the time and costs of both processes and enhance the quality of the end-products [6-7].

2.1. The composting process

Composting is defined as a bio-oxidative process involving the mineralization and partial humification of the organic matter, leading to a stabilised final product, free of phytotoxicity and pathogens and with certain humic properties, which can be used to improve and maintain soil quality and fertility [25]. Composting of animal manures has been traditionally carried out by the farmers after manure collection for better handling, transport and management [6]. Frequently, the wastes were heaped up and very little attention was paid to the process conditions (aeration, temperature, ammonia loss, etc.) and using rudimentary methodology.

From a microbial viewpoint continuous composting processes may be described as a sequence of continuous cultures, each of them with their own physical (temperature), chemical (the available substrate), and biological (i.e., the microbial community composition) properties and feedback effects. These changes make it difficult to study the process, which is virtually impossible to simulate in the laboratory since temperature, moisture, aeration, etc., are directly related to the surface/volume ratio. However, in general, composting may be described as a four-phase process in which the energy-rich, abundant and easily degradable compounds like sugars and proteins are degraded by fungi and bacteria (referred to as primary decomposers) during an initial phase called the *mesophilic phase* (25-40 °C). Although there exists a competition between both microbial groups regarding the easily available substrates, fungi are very soon outcompeted because the maximum of specific growth rates of bacteria exceed those of fungi by one order of magnitude [26]. The importance of bacteria (with the exception of Actinobacteria) during the composting process has long been neglected, probably because of the better visibility of mycelial organisms. A review on the microbial groups involved in the first mesophilic phase is given by [27]. Provided that mechanical influences (like turning) are small, compost fauna including earthworms, mites and millipedes may also act as catalysts, thereby contributing

to the mechanical breakdown and offering an intestinal habitat for specialized microorganisms. The contribution of these animals may be negligible or, as in the special case of vermicomposting, considerable (see section 2.2). The number of mesophilic organisms in the original substrate is three orders of magnitude higher than the number of thermophilic organisms; however, the activity of primary decomposers induces a temperature rise and in turn, mesophilic microbiota is, along with the remaining easily degradable compounds, degraded by the succeeding thermophiles. The temperature rise continues to be fast and accelerates up to a temperature of about 62 °C during this second phase of composting, known as the *thermophilic phase*.

When a temperature exceeding 55 °C is reached in a compost pile, fungal growth is usually inhibited and the thermophilic bacteria and Actinobacteria are the main degraders during this peak-heating phase. Moreover, oxygen supply affects fungi to a greater extent than bacteria, and even in force-aerated systems, temporary anoxic conditions may occur. Hence, fungi play a negligible role during this phase, except for the composting of lignocellulosic residues. Bacteria of the genus *Bacillus* are often dominant when the temperature ranges from 50 to 65 °C. Moreover, members of the *Thermus*/*Deinococcu*s group have been found in biowaste composts [28] with an optimum growth between 65 and 75 °C. A number of autotrophic bacteria that obtain their energy by the oxidation of sulfur or hydrogen have been isolated from composts [28]. Their temperature optimum is at 70-75 °C and they closely resemble *Hydrogenobacter* strains, which were previously found in geothermal sites. Furthermore, obligate anaerobic bacteria are also common in composts, but up to now, there is still a gap of knowledge concerning this microbial group. It is believed that the longer generation times of archaea, in comparison with bacteria, made the archaea unsuitable for the rapidly changing conditions in the composting process. Nevertheless, in recent works, and using the right tools, a considerable number of cultivable (*Methanosarcina termophila*, *Methanothermobacter* sp., *Methanobacterium formicicum*, among others) and yet uncultivated archaea have been detected in composting processes [29-30].

The final temperature increase may exceed 80 °C and it is mainly due to the effect of abiotic exothermic reactions in which temperature-stable enzymes of Actinobacteria might be involved. Such high temperatures are crucial for compost hygienisation in order to destroy human and plant pathogens, and kill weed seeds and insect larvae [31]. The disadvantage of temperatures exceeding 70 °C is that most mesophiles are killed, and therefore the recovery of the decomposer community is retarded after the temperature peak. The inoculation with matter from the first mesophilic stage might, however, solve this problem.

When the activity of thermophilic organisms ceases due to the exhaustion of substrates, the temperature starts to decrease. This constitutes the beginning of the third stage of composting, called the *cooling phase* or *second mesophilic phase*. It is characterised by the recolonisation of the substrate with mesophilic organisms, either originating from surviving spores, through the spread from protected microniches, or from external inoculation. During this phase there is an increased number of organisms with the ability to degrade cellulose or starch, such as the bacteria *Cellulomonas*, *Clostridium* and *Nocardia*, and fungi of the genera *Aspergillus*, *Fusarium* and *Paecilomyces* [27]. Finally, during the *maturation phase*, the ratio of

fungi to bacteria increases due to the competitive advantage of fungi under conditions of decreasing water potential and poorer substrate availability. Compounds that are not further degradable, such as lignin-humus complexes, are formed and become predominant. Some authors have proposed a fifth composting phase, known as the *curing phase* (or *storage phase*), during which the physico-chemical parameters do not change, but changes in microbial communities still occur [32]. Therefore, the chemical and microbial changes that the substrate undergoes during the different phases of the composting process will largely determine the stability and degree of maturity of the end product and in turn, its safe use as an organic amendment. There exists a wide range of parameters that have been proposed to evaluate compost stability/maturity, as shown in the next section.

2.1.1. Evaluation of compost stability and maturity

The stability and maturity of compost is essential for its successful application, particularly for composts used in high value horticultural crops [33]. Both terms are usually used interchangeably to describe the degree of decomposition and transformation of the organic matter in compost [34], despite the fact that they describe different properties of the composting substrate. Stability is strongly related to the degree to which composts have been decomposed to more stable organic materials [35]. Unstable compost, in contrast, contains a high proportion of biodegradable matter that may sustain a high microbial activity [36]. Typically, compost stability is evaluated by different respirometric measurements and/or by studying the transformations in the chemical characteristics of compost organic matter [6]. On the other hand, compost maturity generally refers to the degree of decomposition of phytotoxic organic substances produced during the active composting stage and to the absence of pathogens and viable weed seeds [37]. This property is often characterised by germination indexes [38] and/or by nitrification [6] and has been related to the degree of compost humification. Wu et al. [37] reported that the low CO_2 evolution is not always an indicator of a non-phytotoxic compost, which suggests that a stable compost may not always be at a level of maturity suitable for its use as a growing medium for certain species of plant.

Several authors highlight that there is no one single method that can be applied successfully for determining compost stability mainly due to the wide range of raw materials used to produce compost, as reviewed by [6]. Therefore, the integration of different parameters seems to be the most reliable option for evaluating the stability/maturity stage of composted materials. For instance, physical parameters including temperature, odor and color constitute a very simple and rapid method for stability evaluation, giving a general idea of the decomposition stage reached; however, little information is achieved as regards to the degree of maturation. In addition, chemical parameters including pH, electrical conductivity, cation exchange capacity (CEC), the ratios of C to N and NH_4^+ to NO_3^- and humification parameters have also been widely used as indicators of stability [39]. Nevertheless, several drawbacks have been found regarding these parameters, thereby preventing their use as accurate indicators. According to Wu et al. [37], pH and electrical conductivity may be used to monitor compost stabilisation, as long as the source waste

composition is relatively consistent and other stability tests are conducted. Moreover, Namkoong et al. [40] established that the C to N ratio could not be considered as a reliable index of compost stability, as it changed irregularly with time. In fact, when wastes rich in nitrogen are used as the source material for composting, like sewage sludges or manures, the C to N ratio can be within the values of a stable compost even though it may still be unstable. Zmora-Nahum et al. [34] reported a C to N ratio lower than the cutoff value of 15 very early during the composting of cattle manure, while important stabilisation processes were still taking place. The increase in CEC with composting time is related to the formation of carboxyl and phenolic functional groups during the humification processes; however, the wide variation in CEC values among the initial substrates prevent to establish a threshold level and to use it as a stability indicator [41].

Biological parameters such as respiration rates (CO_2 evolution rate and/or O_2 uptake rate) and enzyme activities have been proposed to measure compost stability [6-39]. The principle of the respirometric tests is that unstable compost has a strong demand for O_2 and high CO_2 production rates as a consequence of the intensive microbial development due to the presence of easily biodegradable compounds in the raw material. Then, as composting proceeds, the decrease in the amount of degradable organic matter is accompanied by a decline in both O_2 and CO_2 respirometry. The Solvita test, which measures CO_2 evolution and ammonia emissions simultaneously have been found to be a simple and easily used procedure for quantifying soil microbial activity in comparison with both titration and infrared gas analysis [42]; this test has also been used for determining the stability degree in diverse composts [43]. Enzymatic activities have also been found suitable as indicators of the state and evolution of the organic matter during composting, as they are implicated in the biological and biochemical processes through which the initial organic substrates are transformed (Tiquia, 2005). Important enzymes during composting are related to the C-cycle (cellulases, β-glucosidase, β-galactosidase), the N-cycle (protease, urease, amidase) and/or the P-cycle (phosphatase) [44]. These latter authors established that the formation of a stable enzymatic complex, either in moist or air-dried compost samples, could represent a useful index of stabilisation. Additionally, enzymatic activities, especially dehydrogenases, are considered easy, quick and cheap stability measurements [36]; however, the wide range of organic substrates involved in the composting process makes it difficult to establish general threshold values for these parameters. The hydrolysis of fluorescein diacetate (FDA), which is a colourless fluorescein conjugated that is hydrolyzed by both free (exoenzymes) and membrane bound enzymes [45], has been suggested as a valid parameter for measuring the degree of biological stability of the composting material, as it showed a good correlation with other important stability indexes [46]. The analysis of phospholipid fatty acid (PLFA) composition has also been proposed for determining compost stability [47-48]. These authors found a positive correlation between the proportion of PLFA biomarkers for Gram-positive bacteria and the germination index during the maturation of composting of poultry manure and cattle manure, respectively. The strength of this lipid-based approach, as compared to other microbial community assays, is that PLFAs are rapidly synthesized during microbial growth and quickly degraded upon microbial death and they are not found in storage molecules, thereby providing an accurate 'fingerprint' of the current living

community [49]. The potential ability of the microbial community to utilise select carbon sources by determining the community-level physiological profiles (CLPPs) with the Biolog® Ecoplate has also been considered for compost stability testing [50]. The principle is that compost extracts are inoculated onto microtiter plates that contain 31 different C substrates [51-52]. Ultimately, molecular techniques are becoming increasingly useful in composting research. For example, as in reference [32] the authors used three different cultivation-independent techniques based on 16S rRNA gene sequences, i.e. PCR-denaturing gradient gel electrophoresis (DGGE), clone libraries, and an oligonucleotide microarray (COMPOCHIP), in order to evaluate the dynamics of microbial communities during the compost-curing phase. Specific compost-targeted microarrays are suitable to investigate bacterial [53-54] and fungal community patterns [55], including plant growth promoting organisms and plant and human pathogens.

2.1.2. Influence of compost amendments on the soil microbiota

Composted materials have gained a wide acceptance as organic amendments in sustainable agriculture, as they have been shown to provide numerous benefits whereby they increase soil organic matter levels, improve soil physical properties (increased porosity and aggregate stability and reduced bulk density) and modify soil microbial communities [56]. Substantial evidence indicates that the use of compost amendments typically promotes an increase in soil microbial biomass and activity, as reviewed in [56-57]. This enhancing effect may be attributed to the input of microbial biomass as part of the amendments [58]; however, the quantity of organic matter applied with the compost is very small in comparison with the total organic matter present in the soil and, in turn it is believed that the major cause is the activation of the indigenous soil microbiota by the supply of C-rich organic compounds contained in the composting materials [58]. Such effects on microbial communities were reported to be dependent on the feedstocks used in the process [59]. However, other authors did not find significant differences between soil plots that had been amended with four different compost types (green manure compost, organic waste compost, manure compost and sewage sludge compost) over 15 years [60]. Similarly, Ros et al. [61] observed that different types of composts had a similar effect on the fungal community and microbial biomass in soil in a long-term field experiment. This fact suggests that the soil itself influences the community diversity more strongly than the compost treatments. Such discrepancies between the previous findings may be due to differences in soil properties, land-use and compost type (i.e., different starting material and process parameters), frequency and dose of application, length of the experiment and parameters chosen for analysis, among others.

Furthermore, C addition to soil seems to select for specific microbial groups that feed primarily on organic compounds. Therefore, it can be expected that the addition of organic amendments not only increases the size of the microbial community but also changes its composition, as has already been observed in previous experiments [61-63]. As shown by [62] higher amounts of composts resulted in a more pronounced and faster effect in the structure of microbial communities, as revealed by PLFA analysis, indicating that the compost application rate is a major factor regarding the impact of compost amendments on

soil microbiota. Carrera et al. [63] found that soil PLFA profiles were influenced by both the treatment with poultry manure compost and the sampling date. Ros et al. [61] observed that the date of sampling contributed more to modifications in fungal community structure, assessed by PCR-DGGE analysis, than treatment effects. However, in contrast to fungi, the bacterial community structure, both on the universal and the Streptomycetes group-specific level, were influenced by compost amendments, especially the combined compost and mineral fertilisers treatments. This seems reasonable, as bacteria have a much shorter turnover time than fungi and can react faster to the environmental changes in soil. Bacterial growth is often limited by the lack of readily available C substrates, even in soils with a high C/N ratio, and are the first group of microorganisms to assimilate most of the readily available organic substrates after they are added to the soil [64]. CLPP profiles have also been used to evaluate the impact of compost amendments on the potential functional diversity of soil microbiota, as they are considered suitable indicators for detecting soil management changes [65]. As shown by [66] different types of compost (household solid waste compost and manure compost) affected differently the substrate utilization patterns of the soil microbial community relative to unamended control soils. Contrarily, no significant changes in CLPP profiles were found by [59]. Other authors also reported that the sampling date had more weight on CLPP results than compost treatments [63,67]. All these studies together highlight the importance of a multi-parameter approach for determining the influence of compost amendments on the soil microbiota, which is of utmost importance to understand the disease suppressive activity of compost and the mechanisms involved in such suppression [68].

Since the 1980s a large number of experiments have been addressed describing a wide array of pathosystems and composts from a broad variety of raw materials. Interestingly, Noble and Coventry [69] evaluated the suppression of soilborne plant pathogens by compost in both laboratory and field scale experiments. In general, they found that the effects in the field were smaller and more variable than those observed at lab-scale. Termorshuizen et al. [70] compared the effectiveness of 18 different composts on seven pathosystems and interestingly they found significant disease suppression in 54% of the cases, whereas only 3% of the cases showed significant disease enhancement. They highlighted that the different composts did not affect the pathogens in the same way and that no single compost was found to be effective against all the pathogens. Furthermore, in a study carried out with 100 composts produced from various substrates under various process conditions, it was found that those composts that had undergone some anaerobic phase showed the best results in terms of suppressing plant disease [71].

However, up to now, there is still a general lack of understanding concerning the suppressivity of compost [68], as it depends on a complex range of abiotic and biotic factors. Such factors are reviewed by [72]. Briefly, the main mechanisms by which compost amendments exert their suppressivity effect against soil-borne plant pathogens include hyperparasitism; antibiosis; competition for nutrients (carbon and/or iron); and induced systemic resistance in the host plant [73]. The first three affect the pathogen directly and reduce its survival, whilst the latter one acts indirectly via the plant and affect the disease cycle.

Manure type	Bulking agent	Composting process	Vermicomposting process	Duration of experiment	Investigated parameters	Remarks	References
Pig manure	-	-	Vertical continuous-feeding system (*E. fetida*)	36 weeks	Microbial biomass C, basal respiration, metabolic quotient (qCO_2), CLPPs, enzymatic activities	Increase in functional microbial diversity with earthworm presence associated with a decrease in qCO_2, indicative of high metabolic efficiency Increase in cellulase activity with earthworm presence accompanied by greater C losses throughout the process	[16-17]
Pig manure	-	-	Vertical continuous-feeding system (*E. fetida*)	36 weeks	PLFAs, basal respiration	Decrease in bacterial and fungal biomass, assessed by PLFA biomarkers, associated with an increase in microbial activity in the presence of earthworms	[18]
Sheep manure	Olive waste	Turned windrow	Vermicomposting bed (*E. fetida*)	36 weeks for both composting and vermicomposting	Enzymatic activities, PCR-DGGE, real time-PCR	Higher bacterial abundance and microbial diversity was found in vermicompost relative to the initial substrate than in compost	[19]

Manure type	Bulking agent	Composting process	Vermicomposting process	Duration of experiment	Investigated parameters	Remarks	References
Cow manure	Straw	Forced-ventilation	Vermicomposting bed (*E. andrei*)	Composting: 15 d (thermophilic phase) Vermicomposting: 40 d (active phase)	Microbial biomass C, basal respiration, enzymatic activities, ergosterol	Lower levels of microbial biomass and dehydrogenase activity indicative of a higher degree of stabilisation were found in the combined treatment (composting + vermicomposting)	[20]
Cow manure	Agricultural plant waste	In-vessel system	Semicontinuously vermicomposting system (*E. fetida*)	Composting: 3-4 weeks (thermophilic phase) Vermicomposting: not specified	Substrate-induced respiration, PCR-DGGE, CompoChip	Vermicompost tea composition was influenced by production and storage conditions Carbon substrate addition during the tea production process was identified to be of major importance for obtaining a vermicompost tea with a rich and diverse microbial community	[21]
Cow manure+poultry manure	Biochar	Turned windrow	-	12 weeks	PLFAs	Changes in microbial community composition depended on the origin of manure composted with biochar	[22]
Cow manure	Sawdust	Turned windrow	-	30 d	PCR-DGGE, clone libraries	Ammonia oxidizing archaea were found to be dominant during the composting process probably because they could adapt to increasing temperature and/or nutrient loss	[23]
Cow manure+Horse manure+Pig Manure	-	-	In-container system (*E. andrei*, *E. fetida* and *P. excavatus*)	30 d (active phase)	Basal respiration, microbial growth rates, PLFAs	Species-specific effects of earthworms on microbial community structure and bacterial growth rate	[24]

Table 1. Research studies focused on the stabilization of animal manures through the aerobic processes of composting and vermicomposting.

Two mechanisms of biological control, based on antibiosis, hyperparasitism, competition and induced protection, have been reported for compost amendments. On the one hand, diseases caused by plant pathogens such as *Phytophtora* spp. and *Phytium* spp. have been eradicated through a mechanism known as "general suppression", in which the suppressive activity is attributed to a diverse microbial community in the compost rather than to a population of a single defined species augmented to infested soil. Whilst, for *Rhizoctonia solani*, few microorganisms present in compost are able to eradicate this pathogen and, in turn this type of suppression is referred as "specific suppression". Overall, all of the above reinforces that the activity of microbial communities in composts is a major factor affecting the suppression of soilborne plant pathogens. Indeed, the disease suppressive effect is usually lost following compost sterilization or pasteurization [68]. Better understanding of the microbial behaviour and structure of the antagonistic populations in the compost will provide tools to reduce its variability. In line with this, Danon et al. [32] detected, using PCR-based molecular methods, distinctive community shifts at different stages of prolonged compost curing being Proteobacteria the most abundant phylum in all the stages, whereas Bacteroidetes and Gammproteobacteria were ubiquitous. Actinobacteria were dominant during the mid-curing stage, and no bacterial pathogens were detected even after a year of curing.

The addition of antagonistic microorganisms to compost is also a promising technique to improve its suppressivity. Already in 1983, Nelson et al. [74] increased the suppressive potential of compost by adding selected *Trichoderma* strains. They found that not only the addition of the antagonist is important, but also the strategy of inoculation of the antagonist in order to efficiently colonize the substrate, as the autochthonous microbial community can inhibit it. Ultimately, predicting disease suppression on the basis of pure compost is expected to be highly advantageous for compost producers. This would enable them to optimise the composting process based on the specific disease jeopardising the target crop. For this purpose, a further step could be the development of quality control criteria based mainly on bioassays designed for a specific pathogen or disease.

2.2. The vermicomposting process

Vermicomposting is defined as a bio-oxidative process in which detritivore earthworms interact intensively with microorganisms and other fauna within the decomposer community, accelerating the stabilization of organic matter and greatly modifying its physical and biochemical properties [75]. Epigeic earthworms with their natural ability to colonize organic wastes; high rates of consumption, digestion and assimilation of organic matter; tolerance to a wide range of environmental factors; short life cycles, high reproductive rates, and endurance and resistance to handling show good potential for vermicomposting [76]. The earthworm species *Eisenia andrei*, *Eisenia fetida*, *Perionyx excavatus* and *Eudrilus eugeniae* display all these characteristics and they have been extensively used in vermicomposting facilities.

Vermicomposting systems sustain a complex food web that results in the recycling of organic matter and release of nutrients [77]. Biotic interactions between decomposers (i.e., bacteria and fungi) and earthworms include competition, mutualism, predation and

facilitation, and the rapid changes that occur in both functional diversity and in substrate quality are the main properties of these systems [77]. The biochemical decomposition of organic matter is primarily accomplished by the microbes, but earthworms are crucial drivers of the process as they may affect microbial decomposer activity by grazing directly on microorganisms [78-79], and by increasing the surface area available for microbial attack after the comminution of organic matter [8]. Furthermore, earthworms are known to excrete large amounts of casts, which are difficult to separate from the ingested substrate [8]. The contact between worm-worked and unworked material may thus affect the decomposition rates [80], due to the presence of microbial communities in earthworm casts different from those contained in the material prior to ingestion [81]. In addition, the nutrient content of the egested materials differ from that in the ingested material [82], which may enable better exploitation of resources, because of the presence of a pool of readily assimilable compounds in the earthworm casts. Therefore, the decaying organic matter in vermicomposting systems is a spatially and temporally heterogeneous matrix of organic resources with contrasting qualities that result from the different rates of degradation that occur during decomposition [83].

The impact of earthworms on the decomposition of organic waste during the vermicomposting process is initially due to *gut- associated processes* (GAPs), i.e., via the effects of ingestion, digestion and assimilation of the organic matter and microorganisms in the gut, and then casting [79] (Figure 1). Specific microbial groups respond differently to the gut environment [84] and selective effects on the presence and abundance of microorganisms during the passage of organic material through the gut of these earthworm species have been observed. For instance, some bacteria are activated during the passage through the gut, whereas others remain unaffected and others are digested in the intestinal tract and thus decrease in number [78]. These findings are in accordance with a recent work that provides strong evidence for a bottleneck effect caused by worm digestion (*E. andrei*) on microbial populations of the original material consumed [79]. This points to the earthworm gut as a major shaper of microbial communities, acting as a selective filter for microorganisms contained in the substrate, thereby favouring the existence of a microbial community specialised in metabolising compounds produced or released by the earthworms, in the egested materials. Such selective effects on microbial communities as a result of gut transit may alter the decomposition pathways during vermicomposting, probably by modifying the composition of the microbial communities involved in decomposition, as microbes from the gut are then released in faecal material where they continue to decompose egested organic matter. Indeed, as mentioned before, earthworm casts contain different microbial populations to those in the parent material, and as such it is expected that the inoculum of those communities in fresh organic matter promotes modifications similar to those found when earthworms are present, altering microbial community levels of activity and modifying the functional diversity of microbial populations in vermicomposting systems [80]. Previous studies have already shown that a higher microbial diversity exists in vermicompost relative to the initial substrate [19,85]. Upon completion of GAPs, the resultant earthworm casts undergo *cast- associated processes* (CAPs), which are more closely related to ageing processes, the presence of unworked

material and to physical modification of the egested material (weeks to months; Figure 1). During these processes the effects of earthworms are mainly indirect and derived from the GAPs [17].

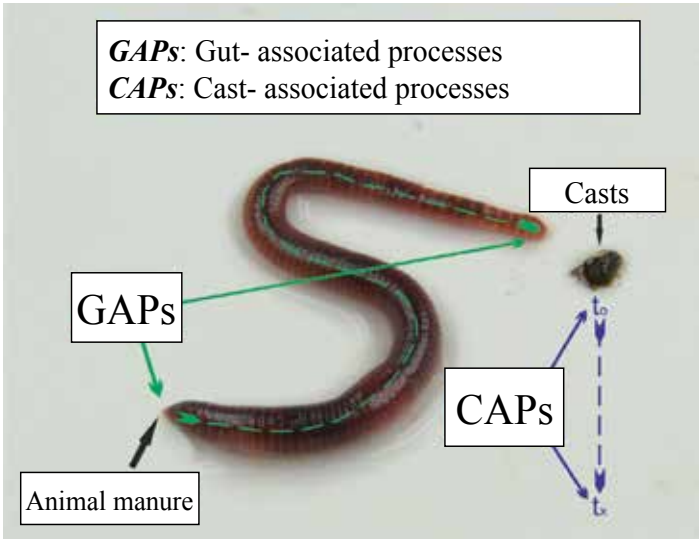

Figure 1. Earthworms affect the decomposition of the animal manure during vermicomposting through ingestion, digestion and assimilation in the gut and then casting (*gut- associated processes*); and *cast-associated processes,* which are more closely related with ageing processes.

Overall, vermicomposting includes two different phases regarding earthworm activity: (i) an *active phase* during which earthworms process the organic substrate, thereby modifying its physical state and microbial composition [86], and (ii) a *maturation phase* marked by the displacement of the earthworms towards fresher layers of undigested substrate, during which the microorganisms take over the decomposition of the earthworm-processed substrate [17-18]. The length of the maturation phase is not fixed, and depends on the efficiency with which the active phase of the process takes place, which in turn is determined by the species and density of earthworms, and the rate at which the residue is applied [8]. During this aging, vermicompost is expected to reach an optimum in terms of its nutrient content and pathogenic load, thereby promoting plant growth and suppressing plant diseases [8]. However, unlike composting, vermicomposting is a mesophilic process (<35 °C), and as such substrates do not undergo thermal stabilisation that eliminates pathogens. Nevertheless, it has been shown that vermicomposting may reduce the levels of different pathogens such as *Escherichia coli*, *Salmonella enteritidis*, total and faecal coliforms, helminth ova and human viruses in different types of waste [75]. In a recent work [78], a reduction by 98% in the number of faecal coliforms of pig slurry was detected after two weeks of processing in the presence of *E. fetida*, which indicates that the own earthworm digestive abilities play a key role in the reduction of the pathogenic load of the parent material. In a previous study, these authors found that the decrease in pathogenic bacteria (i.e. total coliforms) as a result of gut transit differed among four vermicomposting earthworm species (*Eisenia fetida*, *Eisenia andrei*, *Lumbricus rubellus* and *Eudrilus eugeniae*)

[87]. This was consistent with the fact that specific microbial groups respond differently to the gut environment, depending on the earthworm species. The pathogen considered is another important factor controlling the reduction in the pathogenic load during the process. Parthasarathi et al. [88] observed that earthworms did not reduce the numbers of *Klebsiella pneumoniae* and *Morganella morganii*, whereas other pathogens such as *Enterobacter aerogenes* and *Enterobacter cloacae* were completely eliminated. In a recent study [89] a decrease in the abundance of faecal enterococci, faecal coliforms and *Escherichia coli* was recorded across the layers of an industrial-scale vermireactor fed with cow manure; whereas no changes were reported for total coliforms, *Enterobacteria* or *Clostridium*. These findings are of great importance for the optimisation of the vermicomposting process because despite the pioneering studies of Riggle [90] and Eastman et al. [91], little is known about this process in industrial-scale systems, that is, vermicomposting systems designed to deal with large amounts of wastes. This selective effect on pathogens indicates that earthworms not only modify the abundance of such pathogenic bacteria but also alter their specific composition. According to [89], the unaffected pathogens could benefit as a result of the overall decrease in bacterial and fungal biomass across the layers of the reactor, thereby diminishing possible competition for resources.

Collectively, the aforementioned studies highlight the importance of monitoring the changes in microbial communities during vermicomposting, because if the earthworms were to stimulate or depress microbiota or modify the structure and activity of microbial communities, they would have different effects on the decomposition rate of organic matter, thereby influencing the vermicompost properties, which is critical to guarantee a safe use of this end-product as an organic amendment and thus benefit both agriculture and the environment.

2.2.1. Effects of earthworms on microbial communities during vermicomposting: a case study.

Animal manures are microbe-rich environments in which bacteria constitute the largest fraction (around 70% of the total microbial biomass as assessed by PLFA analysis), with fungi mainly present as spores [24]. Thus, earthworm activity is expected to have a greater effect on bacteria than on fungi in these organic substrates in the short-term [79]. In line with this, a significant increase in the fungal biomass of pig manure, measured as ergosterol content, was detected in a short-term experiment (72 h) with the earthworm species *E. fetida*, and the effect depended on the density of earthworms [82]. A higher fungal biomass was found at intermediate and high densities of earthworms (50 and 100 earthworms per mesocosm, respectively), which suggests that there may be a threshold density of earthworms at which fungal growth is triggered. This priming effect on fungal populations was also observed in previous short-term experiments in the presence of the epigeic earthworms *Eudrilus eugeniae* and *Lumbricus rubellus* fed with pig and horse manure, respectively [16,86]. These contrasting short-term effects on bacterial and fungal populations are thus expected to have important implications on decomposition pathways during vermicomposting because important differences exist between both microbial decomposers

related to resources requirements and exploitation [92]. This is based on the fact that fungi can immobilise great quantities of nutrients in their hyphal networks, whereas bacteria are more competitive in the use of readily decomposable compounds and have a more exploitative nutrient use strategy by rapidly using newly produced labile substrates [92].

The above-mentioned studies dealing with the effects of epigeic earthworms on microorganisms have focused on the changes before and after the active phase rather than those that occur throughout the whole vermicomposting process. Hence, in a current research study, and using a continuous-feeding vermicomposting system, we evaluated the different phases of interaction between earthworms and microorganisms and additionally, we monitored the stabilisation of the fresh manure during a period of 250 days. At the end of the experiment we obtained a profile of layers of increasing age, resembling a time profile, with a gradient of fresh-to-processed manure from the top to the bottom. This type of system allowed us to evaluate whether and when the samples reached an optimum value to be classified as vermicompost, as regards to the stabilisation of organic matter and the levels of microbial biomass and activity. Briefly, we used polyethylene reactors (n=5) with a volume of 1 m^3, which were initially comprised of a 10 cm layer of mature vermicompost (a stabilised non- toxic substrate that serves as a bed for earthworms), on which earthworms (*Eisenia fetida*) were placed and a layer containing 5 kg of fresh rabbit manure, which was placed over a plastic mesh (5 mm pore size) to avoid sampling the earthworm bedding. New layers with the same amount of fresh manure were added to the vermireactor every fifty days according to the feeding activity of the earthworm population. This procedure allowed the addition of each layer to be dated within the reactors. The reactors were divided into 4 quadrants and two samples were taken at random from each quadrant with a cylindrical corer (8 cm diameter). Each corer sample was divided into five layers of increasing age and the samples from the same layer and each reactor were gently mixed to analyse the changes in microbial communities. The structure of the microbial communities was assessed by PLFA analysis; some specific PLFAs were used as biomarkers to determine the presence and abundance of specific microbial groups [93]. The sum of PLFAs characteristic of Gram-positive (iso/anteiso branched-chain PLFAs), and Gram-negative bacteria (monounsaturated and cyclopropyl PLFAs) were chosen to represent bacterial PLFAs, and the PLFA 18:2ω6c was used as a fungal biomarker. Total microbial activity was also assessed by measuring the rate of evolution of CO_2, as modified for [17] for samples with a high organic matter content. Dissolved organic carbon was determined colorimetrically in microplates after moist digestion ($K_2Cr_2O_7$ and H_2SO_4) of aliquots of 0.5M K_2SO_4 extracts.

The earthworm species *E. fetida* had a strong effect in the decomposition of organic matter during vermicomposting, greatly modifying the structure of the microbial decomposer communities, as revealed by the phospholipid fatty acid analysis. The principal component analysis of the 27 identified PLFAs (10:0, 12:0, 13:0, 14:0, i14:0, 15:0, i15:0, a15:0, 16:0, i16:0, 17:0, a17:0, 18:0, 14:1ω5c, 15:1ω5c, 16:1ω7c, 17:1ω7c, 18:1ω7c, 18:1ω9c, 18:1ω9t, 18:2ω6c, 18:2ω6t, 18:3ω6c, 18:3ω3c, cy17:0, cy19:0, 20:0) clearly differentiated between the samples in function of the age of layers, explaining 51% of the variance in the data (Figure 2). Thus, the

upper layers (50 and 100 days old) along with the fresh manure were clearly distinguished from the intermediate (150 days old) and lower layers (200 and 250 days old) (Figure 2).

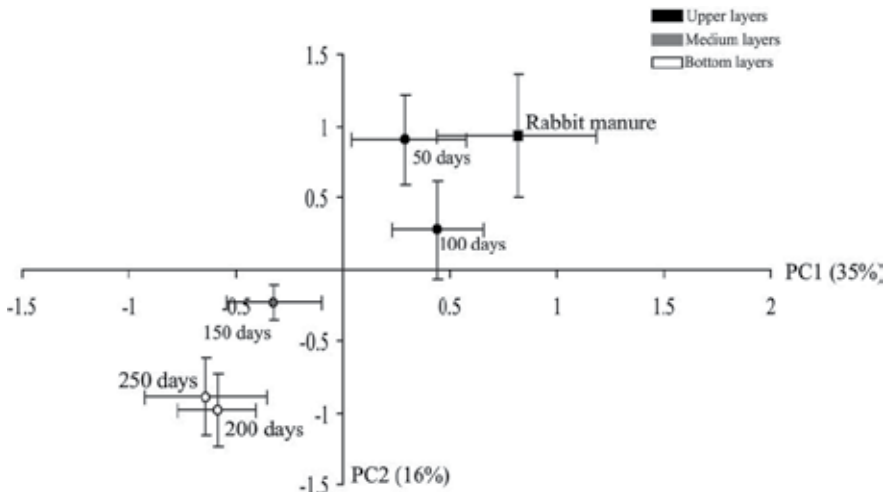

Figure 2. Changes in the microbial community structure throughout the process of vermicomposting assessed by the principal component analysis of the twenty-seven PLFAs identified in the layers of reactors fed with rabbit manure. The different layers represent different stages of the process. Values are means ± SE.

Such changes in the structure of microbial communities were accompanied by a decrease in the abundance of both Gram-positive and –negative bacterial populations with the depth of layers (Figure 3A,B), i.e. from upper to medium and lower layers; and the abundance of these groups were in the fresh rabbit manure 346 ± 49.0 and 336 ± 63 μg g^{-1} dw for Gram-positive and Gram-negative bacteria, respectively (Figure 3A,B). A similar trend was observed for fungal populations (Figure 3C), reaching an average value of 1.3 ± 0.1 at the end of the process (Figure 3C). These results are in accordance with previous studies based on PLFA profiles, with marked changes in the structure of microbial communities due to decreases in both bacterial and fungal populations throughout the process of vermicomposting [18, 89]. Recently, Fernández-Gómez et al. [94] observed that the structure of fungal communities, assessed by DGGE profiles differed at the stage of maximum earthworm biomass the most, suggesting the existence of a strong gut passage effect on the microbial communities through a continuous-feeding vermicomposting system in the presence of *E. fetida*.

Decreases in microbial activity were also detected with depth of layer (Figure 4A) and, after a maturation period for 250 d, basal respiration values dropped below 100 mg CO_2 kg^{-1} OM h^{-1} (Figure 4A), as previously shown by [18]. Accordingly, a reduction in the dissolved organic carbon content was detected from upper to lower layers (Figure 4B), reaching a value close to 7000 μg g^{-1} dw after 250 d of vermicomposting. In contrast, other authors [17] reported levels of DOC much more lower in a long-term experiment (252 days) with the epigeic earthworm *E. fetida*, with values below 1500 μg g^{-1} dw in the presence of

earthworms. Such differences could be due to the composition of the parent material (pig slurry *versus* rabbit manure) and/or to the experimental setup conditions. Unlike compost -a limit value of 4000 mg kg^{-1} is suggested for a stable compost according to [34]- there is still no threshold level of DOC for which vermicompost is to be considered stable.

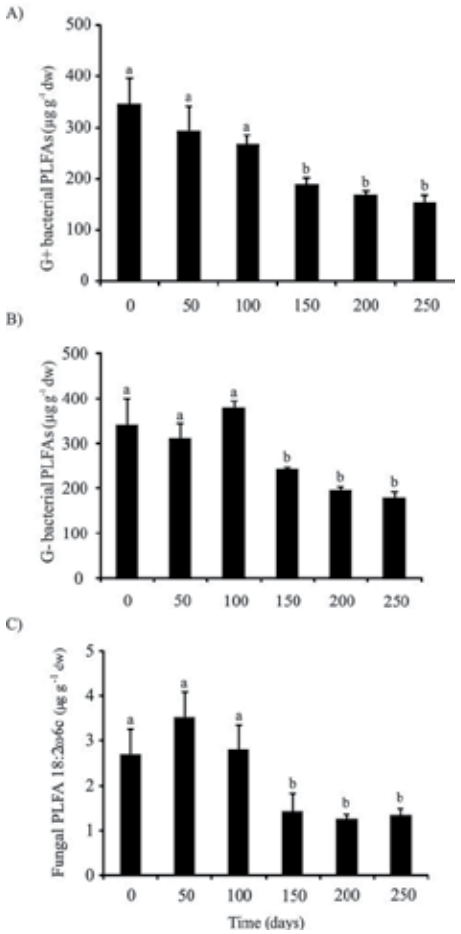

Figure 3. Changes in (a) Gram-positive bacterial, (b) Gram-negative bacterial and (c) fungal PLFAs in the layers of reactors fed with rabbit manure throughout the process of vermicomposting. The different layers represent different stages of the process. Different letters indicate significant differences between the layers based on post hoc test (Tukey HSD). Values are means ± SE.

Overall, in the present study a higher degree of stabilisation was reached in the rabbit manure after a period of between 200 and 250 days, as indicated by the lower values of microbial biomass and activity that are indicative of stabilized materials. These results underscore the potential of epigeic earthworms in the stabilisation of this type of organic substrates, which is of great importance for the application of animal manures as organic amendments into agricultural soils because, as already mentioned, it is widely recognised that the overproduction of this type of substrate has led to inappropriate disposal practices,

which may result in severe risks to the environment [6]. Furthermore, these findings constitute a powerful tool for the development of strategies leading to a more efficient process for the disposal and/or management of animal manures, thereby highlighting the continuous-feeding vermicomposting system as an environmentally sound management option for recycling such organic wastes, as previously reported by [94] for treating tomato-fruit waste from greenhouses. Ultimately, it should be borne in mind that the functioning of this type of reactors can lead to the gradual accumulation of layers and compaction of the substrate, thus minimizing earthworm- induced aeration, which can promote pathogen survival [89].

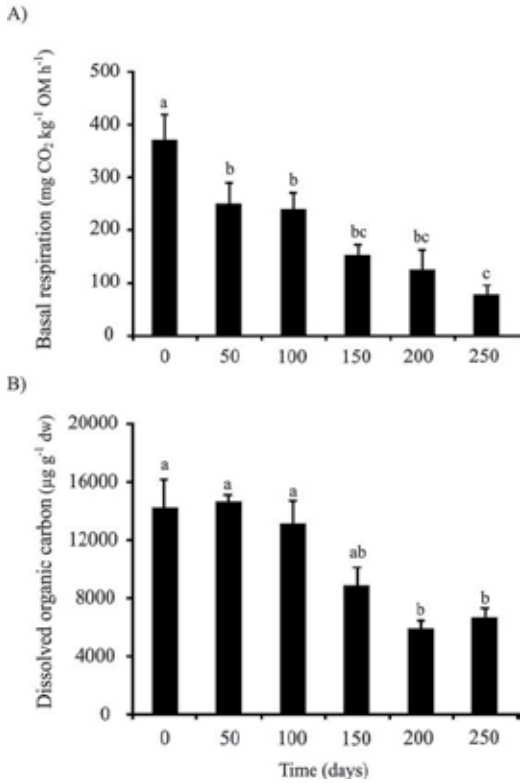

Figure 4. Changes in (a) microbial activity assessed by basal respiration, and (b) dissolved organic carbon content in the layers of reactors fed with rabbit manure throughout the process of vermicomposting. The different layers represent different stages of the process. Different letters indicate significant differences between the layers based on post hoc test (Tukey HSD). Values are means ± SE.

2.2.2. Influence of vermicompost amendments on the soil microbiota

As occurred with compost amendments, vermicompost has also been found to provide manifold benefits when used as a total or partial substitute for mineral fertiliser in peat-based artificial greenhouse potting media and as a soil amendment in field studies [95]. Among the advantages of vermicompost as a soil amendment is its potential to maintain soil

organic matter, foster nutrient availability, suppress plant diseases and increase soil microbial abundance and activity. However, although several studies have tried to disentangle the complex interactions between vermicompost application and soil microbial properties, most of them are frequently not comparable to each other due to differences in the experimental design, the land-use and vermicompost type (i.e., different starting material and earthworm species), the dose of application as well as the duration of experiments, among others. Despite these limitations, some recent findings have been made, thereby contributing to better understand whether and to what extent vermicompost amendments affect soil microbial biomass, activity and community structure. For instance, Arancon et al. [96] observed that a single application of vermicompost to a strawberry crop resulted in a significantly higher increase in soil microbial biomass than the application of an inorganic fertiliser, regardless of the dose used. Increases in the microbial activity and in the activity of the soil enzymes involved in the release of the main plant macronutrients with vermicompost amendments, have also been signalled in several studies [96-98]. Such increase could be due to the fact that soil microorganisms degrade organic matter through the production of a variety of extracellular enzymes and, in turn an input of organic matter is expected to be accompanied by a higher enzymatic activity. Moreover, the added material may contain intra- and extracellular enzymes and may also stimulate microbial activity in the soil [99]. Additionally, vermicompost has been found to promote the establishment of a specific microbial community in the rhizosphere different from that of plants supplemented with mineral fertilisers or other types of organic fertilisers such as manure [100]. Inorganic fertilisation only supplies N, P and K, whereas organic fertilisers also supply different amounts of C and macro- and micronutrients, which can select for microbial communities with different nutritional requirements [95]. Moreover, microbial communities in vermicompost are metabolically more diverse than those in manure [17], and may be incorporated, at least in the short-term, to soils [101]. Interestingly, Aira et al. [100] observed that the effect of the addition of vermicompost occurred despite the low dose used (25% of total fertilisation), and despite the short duration of the experiment (four months). Jack et al. [102] also examined how different organic transplant media amendments, including vermicompost, thermogenic compost and industry standard amendments affected the rhizosphere bacterial communities of organically produced tomato plants. They found differences in the bacterial community structure between the different amendments and these differences persisted for at least one month after seedlings were transplanted to the field. Since both compost and vermicompost were made from the same parent material, such differences could be due to the way in which the organic matter was processed prior to the amendment [102]. Previous comparisons between vermicompost and compost with respect to microbial communities [103-105] are difficult to interpret because different feedstocks were used for each process. Compost feedstocks are known to alter the material's effects on the structure of the microbial communities [86], so it is essential to use composts made from the same feedstock in order to draw valid comparisons between the two biological processes. Furthermore, it may be expected that different hybrids or plant genotypes will respond differently to vermicompost, considering that plant genotype determines important differences in nutrient uptake capacity, nutrient use efficiency and

resource allocation within the plant. Different genotypes may therefore enhance root growth or modify root exudation patterns in order to increase nutrient uptake [100], and all of these strategies will determine the establishment of different interactions with the microbial communities at the rhizosphere level. Indeed, after the application of vermicompost to sweet corn crops, these authors found important differences in the rhizosphere microbial community of two genotypes from cultivars of maize, with the sugary endosperm mutation (*su1*) and with the shrunken endosperm mutation (*sh2*), which differ in their C storage patterns.

Furthermore, recent studies have demonstrated the presence of various bacteria, which are useful for different biotechnological purposes, in diverse vermicomposts [106-107], reinforcing that the biological component (i.e., the microbial community composition) of a vermicompost largely determines its usefulness in agriculture and other applications, such as soil restoration and bioremediation. For instance, Fernández-Gómez et al. [106] detected the presence of *Sphingobacterium*, *Streptomyces*, Alpha-Proteobacteria, Delta-Proteobacteria and Firmicutes in diverse vermicomposts, irrespective of the parent material used for the process, by applying DGGE and COMPOCHIP, thereby demonstrating the usefulness of both techniques to assess the potential of vermicomposts as bioactive organic materials. Indeed, disease suppressiveness is obviously linked to the microbiota added with the vermicompost, along with the biological and physicochemical characteristics of the native soil microbial community. However, despite the large body of scientific evidence showing the positive effects of vermicompost regarding the suppression of soil-borne plant fungal diseases (reviewed in [75,108]), it is still necessary to obtain a deeper understanding of the mechanisms involved and the main factors influencing such suppressing effects. According to the mechanisms proposed for compost [68-69], disease suppression by vermicompost may be attributed to either direct effects or to the induction of systemic resistance in the plant. Direct suppression of the pathogen by the vermicompost-associated microflora and/or microfauna may be general or specific, depending on the existence of a single suppressive agent or the joint action of several agents, and the proposed mechanisms are competition, antibiosis and parasitism. Some of the indirect effects of vermicompost have been related to changes in the microbiological properties of the soil or the potting media. Processing by earthworms during vermicomposting has a strong effect on the microbial community structure and activity of the initial waste [8]. Vermicompost therefore has a rather different microbial community structure than the parent waste, with lower biomass but enhanced metabolic diversity [18]. Application of such a microbiologically active organic substrate may thus have important effects on the microbial and biochemical properties of soil or greenhouse potting media thereby influencing plant growth. Moreover, vermicompost may affect directly the plant growth via the supply of nutrients, as it constitutes a slow-release fertiliser that supplies the plant with a gradual and constant source of nutrients, and/or through the supply of plant growth regulating substances [95].

3. Anaerobic digestion

The process of anaerobic digestion (AD) has been extensively studied in natural and engineered ecosystems for more than a century. In natural habits, the anaerobic degradation

of organic matter takes place in sediments, waterlogged soils and animal intestinal tracts, in which the oxygen access is restricted; whilst in engineered environments it refers to the biotechnological process by which organic matter (i.e., organic waste, wastewater and/or a renewable resource) is degraded in the absence of oxygen for the commercial production of biogas that can be used as an eco-friendly energy source [109], thereby representing an important asset in times of decreasing fossil fuel supplies. According to [4], the anaerobic digestion from swine, bovine and poultry slurries resulted in the production of biogas at average rates of 0.30, 0.25 and 0.48 L/g volatile solids, respectively. Another valuable co-product derived from this process is the anaerobic digested slurry, which can be applied as an organic amendment into soil either in agricultural and non-agricultural lands [110](section 3.1). It is for this reason along with the production of biogas and the reduction in greenhouse gas emissions [10] that anaerobic digestion is becoming increasingly popular as a methodological alternative for manure recycling, which in turn has increased the number of farm-scale anaerobic bioreactors up to 4200 in central and northern Europe [111].

Bacteria represent over 80% of the total diversity in anaerobic digesters [112], and they are mainly composed by the phyla Firmicutes, Proteobacteria and Bacteroidetes; whereas most of the archaeal representatives belong to the phylum Euryarchaeota, which includes all the known methanogens. Anaerobic eukaryotes - particularly, fungi and protozoa- have received less attention probably because they are slower growers than bacteria and, as such, their abundance is lower in anaerobic reactors. As occurs with composting, anaerobic digestion may be described as a four-phase microbiologically driven process. Briefly, the first and rate limiting step of the anaerobic food chain is the *depolymerization* and *hydrolysis* of complex biopolymers, such as polysaccharides, lipids, proteins and nucleic acids into their corresponding structural units (sugars, fatty acids, amino acids, purines and pyrimidines) through the joint action of a complex community of fibrolytic bacteria and fungi, which produce extracellular hydrolitic enzymes (i.e., cellulases, xylanases, proteases, lipases) responsible for the disassembling of such polymers. Since polysaccharides, mainly cellulose, are the most abundant structural and storage compounds of biomass, their hydrolysis is considered as the most determinant enzymatic process regarding the efficiency of anaerobic reactors. The rate and efficiency of cellulose hydrolysis depends greatly on the particular microbial species composition involved [113], and under anaerobic conditions it proceeds slowly due to the heterogeneity of forms in which cellulose is present in nature and to the complexity of the hemicelluloses and lignin matrices in which it is embedded [113]. Similarly, protein hydrolysis to peptides and amino acids takes place slowly, whilst lipid hydrolysis into glycerol and long-chain fatty acids occurs rapidly compared to their subsequent fermentation or β-oxidation. As mentioned above, bacterial populations are more abundant and diverse and, hence, they are responsible for the majority of hydrolytic reactions, being *Clostridium*, *Acetivibrio*, *Bacteroides*, *Selenomonas* or *Ruminococcus* common examples of hydrolytic bacteria found in anaerobic reactors [112,114].

The monomeric compounds released after the hydrolysis of biopolymers can be taken up by microbial cells, in which they are either fermented or anaerobically oxidised into alcohols, short-chain fatty acids, CO_2 and molecular hydrogen (H_2). This step is known as *fermentation*

(*acidogenesis*) and usually occurs through the production of an energy-rich intermediate that is used to synthesise ATP, rendering a fermentation product that is excreted out of the cell. Since these products are typically acidic substances, the fermentative reactions are accompanied by a decrease in the extracellular pH. This fact along with the increase in short-chain fatty acids represents the most common reasons for reactor failure. Thus, for maintaining the pH balance of the system, it is of great importance to bear in mind the equilibrium between fermentative-acidogenic bacteria and acid scavenging microbes. Typically, the bacteria from the same group that hydrolyze biopolymers take up and ferment the resulting monomers. For instance, *Clostridium* sp. and enteric bacteria are common sugar fermenters in anaerobic reactors. *Streptococcus* sp. and *Lactobacillus* sp. also ferment sugars, producing lactate or lactate and ethanol, plus CO_2 and molecular hydrogen [114]. Fermentation of amino acids and purines and pyrimidines in anaerobic environments is mainly carried out by species of *Clostridium* [115].

Then, during *acetogenesis*, the fermentation products are mainly oxidised to acetate, formate, CO_2 and H_2 by acetogenic bacteria, most of them belonging to the low G+C branch of *Firmicutes* [116]. Certain acetogenic reactions are thermodynamically unfavourable under standard conditions, which make necessary a syntrophic relationship between the acetogen and a H_2-consuming methanogens in order to degrade the substrate and, in turn to obtain a net energy gain [117]. Finally, the last and most sensitive step during the anaerobic digestion of organic matter is the *methanogenesis*, i.e. the formation of methane from acetate, H_2/CO_2 and methyl compounds by the action of methanogenic organisms belonging to the phylum Euryarchaeota [118]. The orders Methanobacteriales, Methanococcales, Methanomicrobiales and Methanosarcinales include known methanogens commonly found in aerobic reactors. Members of the first three orders use CO_2 and H_2 as an electron acceptor and donor, respectively. Some species from these orders can also use formate and/or secondary alcohols (i.e., isopropanol or ethanol), but they cannot use acetate or C1 compounds such as methanol and methylamines (with the exception of the genus *Methanosphaera* from the order Methanobacteriales). However, Methanosarcinales are more diverse metabolically, and they can use acetate, hydrogen, formate, secondary alcohols and methyl compounds as energy sources. It is believed that the predominance of hydrogenotrophic or acetotrophic methanogens depends on the levels of their substrates and their tolerance to diverse inhibitors, including ammonia, hydrogen sulphide, or volatile fatty acids [119]. The aforementioned steps involved in the anaerobic digestion are explained in more detail by [109].

3.1. Influence of anaerobic digested slurry on the soil microbiota

The changes that occur in the parent waste during the process of anaerobic digestion largely depend on the dynamics of the abovementioned microbial groups, ultimately influencing the quality of the final products, i.e. biogas and anaerobic digested slurry. As mentioned before, anaerobic slurries are rich in partially stable organic carbon and can be used as organic amendments for crop production. However so far, many environmental issues relevant when these co-products are applied to agricultural land still have to be studied,

especially those related to the impact of the anaerobic digested slurry on the soil microbiota. In a recent work, Walsh et al. [120] observed that its application affected the fungal and bacterial growth in a very similar way to the application of mineral fertilizers in a 16- week greenhouse experiment. They found a pronounced shift towards a bacterial dominated microbial decomposer community, and such effects were consistent in different soils and different crop types. They conclude that mineral fertiliser could be thus exchanged for anaerobic digested slurry with limited impact on the actively growing soil microbial community, which is of great importance in the regulation of soil processes and consequently in soil fertility and crop yield. Recently, Massé et al. [4] gave an overview of the agronomic value of anaerobic digestion treated manures. In line with this, previous findings showed that the AD of animal slurries improved their fertilizer value [3], thereby leading to an increased forage yield and N uptake relative to raw liquid swine manure and mineral fertilizers [121]. Bougnom et al. [122] found a 20% increase in grass yield compared to conventional manure. Liedl et al. [123] also found that digested poultry litter resulted in similar or superior grass and vegetable yields versus N fertilizers. Therefore, all of the above provides evidence that the anaerobic digested slurry acts similarly to mineral fertiliser and should be considered as such in its application to land. Additionally, the enrichment of mineral fractions of N and P during digestion ultimately results in a higher concentration of plant-available nutrients compared with undigested manure and a subsequently elevated plant growth promotion ability, suggested to be similar to mineral fertilisers [4,123]. These latter authors also found that AD reduced swine manure total and volatile solid concentrations by up to 80% resulting in improved manure homogeneity and lowered viscosity allowing more uniform land application [4]. Nevertheless, the higher levels of mineral N found in the slurry, mainly ammonia, may also lead to an increase in the level of phytotoxicity of the slurry, thereby affecting seed germination and plant growth after land- spreading of this co-product into soils [124]. The presence of other phytotoxic substances, such as volatile fatty acids (i.e., acetic, propionic and butyric acids), as well as the high content of soluble salts may contribute to the slurry phytotoxicity [125]. Furthermore, Goberna et al. [126] found that amending soils with slurry resulted in greater nitrate losses during the first 30 d of a 100 d incubation period in 20 cm-depth lysimeters. In fact, around 23 and 45% of the total N contained in the soil (natural + added) was lost from soils amended with cattle manure and anaerobic slurry, respectively. Other authors also observed that N leaching was, along with NH_3 volatilisation, one of the most important sources of N leakage to the environment in a field-scale experiment, after having quantified the amount of mineral N at 1.7 m depth from grass cultivated plots amended with anaerobic digested slurry and mineral fertiliser [127]. Thus, the use of this co-product as an organic amendment should accurately match crop N demand because if not taken by the plants, nitrates could be drained to surface waters, leached to ground waters or denitrified into gaseous forms and emitted to the atmosphere.

The presence of pathogenic bacteria in agricultural amendments also represents a potential threat and their screening is thus of great importance mainly in those produced from animal manures, as it has been shown that such pathogenic organisms constitute a common fraction of the microbial community in manure [128]. In fact, it has been shown that some

pathogenic bacteria can survive the process of anaerobic digestion and persist in the slurry, as previously reported by [129]. In line with this, those microorganisms with a spore-forming capacity such as *Clostridium* and *Bacillus* species, which are commonly found in the intestinal flora of most warm-blooded animals and can harbor some highly pathogenic members for animals and humans [12,129], cannot be reduced during the process [130]. Accordingly, Olsen and Larsen [131] observed that the spores of *Clostridium perfringens* were not inactivated in either mesophilic or thermophilic biogas digesters. Similar results were observed by other authors [132-133] in a reactor operating under mesophilic and thermophilic conditions, respectively. It is acknowledged that bacterial spores can survive in extreme conditions and germinate after long periods, when the conditions become more favourable [131]. The non-hygienic conditions of the storage/transporting tanks can also favour pathogen regrowth [134]. The composition of the substrate fed into the reactor, as well as the reactor conditions such as pH, digestion temperature, slurry hydraulic retention time, ammonium concentration, volatile fatty acids content and nutrient supply are expected to have a significant influence on the sanitation of the end-product [130]. This indicates that there exists a potential risk of spreading potentially pathogenic microbes after the application of anaerobic slurries into soil. Indeed, Crane and Moore [135] stated that amending soils with raw and treated manures, even with a low pathogenic load, still posed a threat for the environment because a period of regrowth of some pathogens including *Escherichia coli*, enterococci, faecal streptococci and *Salmonella enterica* have been shown after manure deposition to soil [136]. Goberna et al. [126] also found that the levels of *Listeria* in soils amended with either cattle manure or anaerobic slurry were significantly higher than those in the control treatment after having been incubated for a month. They observed, however, that the cultivable forms of *Listeria* in the studied soils could correspond to *L. innocua* instead of *L. monocytogenes*, as shown by the polymerase chain reaction assays. However, as recently summarised by [137], anaerobic digestion generally reduces the pathogen risk when compared to untreated substrates.

In a current study, we evaluated, at a microcosm level, the short and long-term effects of the anaerobic digested slurry on soil chemical and microbiological properties compared to its ingestate (i.e., raw manure) and the two widely-recognized products, compost and vermicompost. All of the organic substrates were mixed with soil by turning at a rate of 40 mg N kg^{-1} soil (dry weight). A control treatment that consisted of soil without the addition of any organic amendment was also included. A total of 45 experimental units (5 amendment levels x 3 incubation times x 3 replicates) were set-up in the present study. After an equilibration period of 4 days at 4 °C, 15 columns were dismantled and the sample was collected to analyze (incubation time 0 days). The remaining thirty columns were then maintained in a room at 22 °C, which is the average temperature of the hottest and wettest month in this area and the most suitable for the survival of pathogens. These columns were destructively sampled after 15 and 60 d incubation corresponding to short and medium-term effects. The survival of selected pathogens was then determined according to standard protocols [138-140] (ISO 16649-2, 2001 for *Escherichia coli*; ISO 4832, 1991 for faecal coliforms; and ISO 7937, 2004 for *Clostridium perfringens*) in all the organic materials and amended soils.

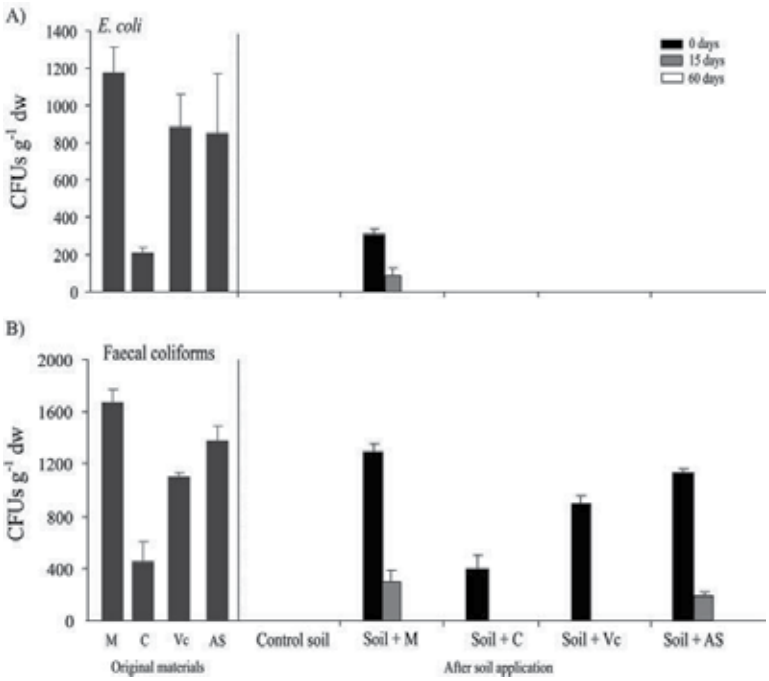

Figure 5. Abundance of *Escherichia coli* and faecal coliforms in the original materials (manure (M), compost (C), vermicompost (Vc) and anaerobic digested slurry (AS)) and in the unamended and amended soils at the three incubation times (0, 15 and 60 days). Values are means ± SE.

Briefly, culturable forms of both faecal coliforms and *E. coli* were isolated from all the initial materials, although their levels were greatly lower in compost relative to the other substrates (Figure 5). This is not surprising taking into account that the composting process, unlike vermicomposting, involved a four-day thermophilic phase, during which the process reached a temperature of 70 °C. Those pathogens were also detected in the anaerobic digested slurry after 40 days of anaerobic digestion (Figure 5). This fact suggests that feeding the reactor with four to five m^3 cattle manure d^{-1} could have provided enough nutrients to maintain a large population of the studied pathogens. Indeed, nutrient availability is one of the major factors influencing pathogen survival in biogas digesters, as previously reported by [130]. Once applied to soils, *E. coli* CFUs were detected in manure-amended soils at the start of the experiment and after incubation for 15 d (Figure 5A); whilst faecal coliforms CFUs were recorded in both manure and slurry-amended soils in the short-term, even though at lower values in comparison with the start of incubation (Figure 5B).

However, the spore-forming *C. perfringens* persisted in all the amended soils (Figure 6), which supports the fact that this bacterium has more resistance to environmental stresses and the capacity to outcompete the native soil microbiota. After 60 d CFU of *C. perfringens* were much closer to those in the control in the slurry-amended soils (Figure 6), which suggests that this time period could be considered as a safe delay between land-spreading the product into soil and crop harvesting with respect to its pathogenic load.

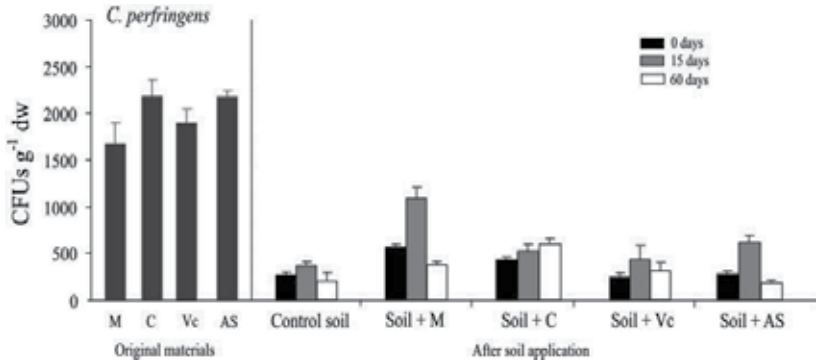

Figure 6. Abundance of *Clostridium perfringens* in the original materials (manure (M), compost (C), vermicompost (Vc) and anaerobic digested slurry (AS)) and in the unamended and amended soils at the three incubation times (0, 15 and 60 days). Values are means ± SE.

4. Conclusions

The intensity and concentrated activity of the livestock industry generate huge amounts of biodegradable wastes, which must be managed with appropriate disposal practices to avoid a negative impact on the environment. Composting is one of the best-known processes for the biological stabilization of solid organic wastes under aerobic conditions. Vermicomposting, i.e. the processing of organic wastes by earthworms under aerobic and mesophilic conditions, has also proven to be a low-cost and rapid technique. Although aerobic processes are thermodynamically more favorable, the manure treatment by anaerobic digestion has become increasingly important due to its energetic potential. A stabilised end product that can be used as an organic amendment is obtained under both aerobic and anaerobic conditions. A multi-parameter approach applying diverse methods constitutes the best option for evaluating the stability/maturity degree of the organic matter, which is of utmost importance for its safe use for the agriculture and the environment. During biodegradation all organic matter goes through the microbial decomposer pool and thus, further knowledge about the changes occurring during the process from a microbial viewpoint will contribute to further develop efficient strategies for the management of animal manures.

5. Outlook

Waste management continues to be a topic of increasing importance. Deeper knowledge of the different biological processes involved in the recycling and recovery of waste components is thus of utmost importance in order to contribute towards more sustainable production and consumption systems. For example, biowaste may be used as a resource to produce high quality lactic acid and protein, as well as biogas in a cascade procedure. Briefly, biowaste is separated into two phases, i.e. a solid phase that is used to feed *Hermetia illucens* larvae that may be harvested as an excellent source of protein for feeding chicken or fish, and the liquid phase that is microbially fermented to the platform chemical lactic acid.

The remaining residuals may eventually be used for biogas production, a cascade process that utilizes the organic waste at its highest level. Furthermore, although an interest in vermicompost research and technology has been increasing over recent years, and the body of knowledge available is quite large, there are still some important topics to be investigated. During vermicomposting, earthworm activity helps microbial communities to use the available energy more efficiently and plays a key role in shaping the structure of the microbial communities during the process. Hence, it is of future interest to evaluate whether the changes in the composition of microbiota in response to earthworm presence are accompanied by a change in the microbial community diversity and/or function. Ultimately, this knowledge will help us to understand the functional importance of earthworms on the stabilization of organic matter from a microbial viewpoint, thereby contributing to minimize the potential risks related to the use of animal manures an organic amendments.

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Chapter 11

Methods and Applications of Deoxygenation for the Conversion of Biomass to Petrochemical Products

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Additional information is available at the end of the chapter

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1. Introduction

Depleting reserves, uncertain economics, and environmental concerns associated with crude oil have prompted an extensive search for alternatives for producing transportation fuels. Biomass has been given close scrutiny due to the emphasis on climate change and the ability of biomass based energy systems to mitigate greenhouse gas (GHG) emissions. Further, its ability to produce fuels and chemicals that are identical to those produced using petroleum resources, makes biomass an important alternative raw material [1, 2]. Biomass can be considered clean, as it contains negligible amounts of sulfur and nitrogen. Consequently, the emissions of SO_2, NO_x are extremely low compared to conventional fossil fuels. The overall CO_2 emission is considered to be neutral, as CO_2 is recycled by the plants through photosynthesis [3]. Moreover, substitution of fossil fuels with biomass-based counterparts could lead to net CO_2 emission reductions [4].

1.1. Biomass production

Biomass encompasses all plant and plant-derived materials as well as animal matter and animal manure. Due to its abundance biomass has to be considered as a vital source of energy to satisfy the global energy demand. Table 1 shows the breakdown of primary energy sources and their contribution to total world energy demand during 1973 and 2009. It is evident that the contribution from biomass is only 10% over this period and in the global scale bioenergy has not yet made any significant impact. However, according to a report published by the United States Department of Agriculture, biomass derived energy has become the highest contributor among all the renewable sources during 2003. According to US statistics 190 million dry tons of biomass is consumed per year, which is equivalent to

3% of current energy consumption [5]. Even though it is hard to conceive that biomass can totally eliminate the use of fossil fuel consumption, it can complement the renewable energy sector together with other sources like wind, solar and geothermal energy.

Primary energy sources	1973	2009
The total world energy demand (Mtoe)	6111	12150
Oil	46.00%	32.80%
Natural gas	16.00%	20.90%
Nuclear	0.90%	5.80%
Hydro	1.80%	2.30%
Biofuels and waste	10.60%	10.20%
Coal / peat	24.60%	27.20%
other	0.10%	0.80%

Table 1. The world's primary energy sources with its contribution. [6]

1.2. Biomass to energy conversions

Biomass has been intimately connected with the everyday life of people since prehistoric times. With the advent of fire by rubbing splinters, humans began to exploit the chemical energy of biomass to produce heat. Ever since, various methods have been developed to convert chemical energy present in biomass to useful heat energy. These conversion methods can be summarized as shown in figure (1).

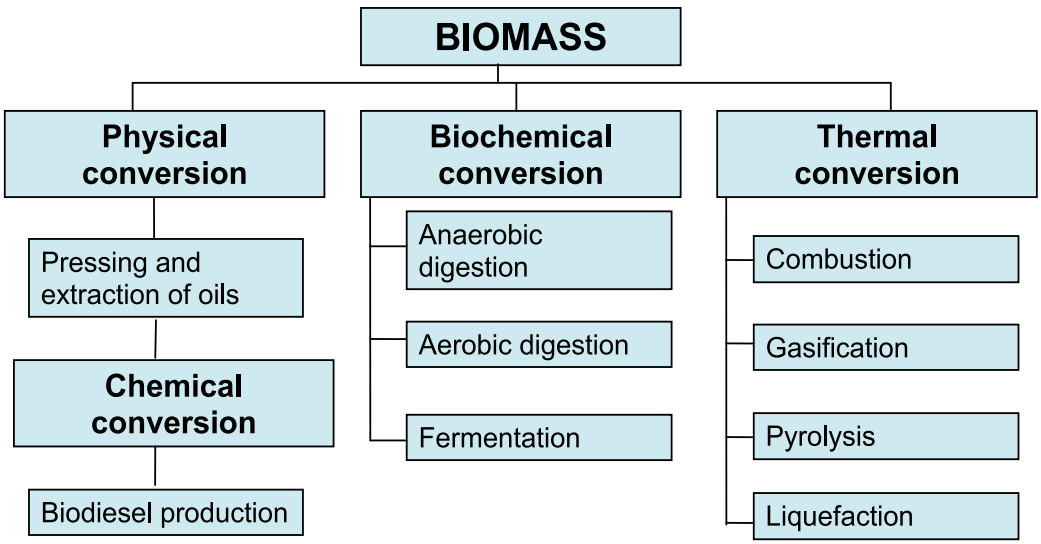


Figure 1. Different categories of biomass conversion

Physical conversion of biomass typically involves pressing the plant (or animal) matter to produce triglyceride oils. Triglycerides cannot be used directly as transport fuels and needs to be processed further. Triglycerides can be converted into a renewable fuel widely known

as biodiesel using the transesterification process. This process converts triglyceride in the presence of alcohol to fatty acid alkyl esters .

Biochemical conversion primarily involves using microorganisms or enzymes to breakdown complex chemicals present in biomass into simpler sugars or alcohols. Biomass conversion to alcohols such as ethanol has attracted wide interest in the recent past. The corn to ethanol technology is mature and is commercial in the US. Nevertheless, this technology has created some debate in the context that corn is still a primary food to many around the world. To circumvent this issue, significant strides have been made in the use of lingo-cellulosic biomass as a source for ethanol. In this method enzymes are used to breakdown cellulose to its monomers and subsequently subjected to fermentation under anaerobic conditions using microorganisms.

Thermochemical conversion is another key process that uses heat to induce chemical transformations in the biomass constituents to produce energetically useful intermediate and/or end products. Conversion techniques available under this umbrella can be categorized into four main processes as represented in figure (1). Energy generation by combustion of biomass can be considered as the most archaic [7]. However, increasing demand for transport fuels has led to the development of other processes that involve converting biomass into liquid and gaseous products [8] such as gasification, pyrolysis and liquefaction. Gasification is the conversion of biomass to a mixture of gases called synthesis gas (or syngas) that primarily consists of hydrogen, carbon monoxide, carbon dioxide and methane. During gasification, biomass is heated under an oxygen-lean environment [9]. Synthesis gas can be directly used in an internal combustion engine or can be converted to liquid fuels using a method known as Fisher-Tropsch (FT) synthesis [7, 10]. Fisher-Tropsch process is considered to be quite energy intensive and therefore, is not yet believed to be economical to compete with petroleum fuels. Nevertheless, active research is still in progress to improve the process [11, 12]. Fermentation of synthesis gas to alcohols (primarily ethanol) using microorganisms is also an active area of research [13].

Pyrolysis and liquefaction are two closely related routes targeted towards producing liquids – called bio-oil or bio-crude [14, 15]. Although not universally accepted, the term bio-oil generally refers to the highly oxygenated liquid product that directly exits a pyroloysis reactor. The term bio-crude represents a more deoxygenated liquid product.

Pyrolysis, unlike gasification, takes place in an oxygen-free atmosphere. The most common technique for producing a large volume of condensable fraction is the fast pyrolysis process. Several reactor configurations used for fast pyrolysis include: ablative, entrained flow, rotating core, vacuum pyrolysis, circulating fluidized bed, and deep bubbling fluidized bed reactors [16]. During fast pyrolysis, biomass is heated at a very high heating rate (eg. 10^3–10^4 K/s)[17]. The temperature at which the thermal scission of biomass material such as cellulose and lignin take place is around 450–550 °C. The bio-oil portion is originally in the vapor-phase and obtained by quenching the volatile output. The yields of the condensable-fraction are reported to be as high as 70–80%. According to some kinetics studies conducted for different biomass, the frequency factor for pyrolysis varies between 10^9-10^{11} orders of

magnitude. This is an indication of how fast the reaction would occur during a short residence time [18].

Since its chemical complexity, the biomass pyrolysis reaction has been studied by using model compounds like cellulose. A set of possible reactions paths under different heating conditions is presented in figure (2)[19, 20].

The composition of bio-oil is substantially different from crude petroleum due to the presence of high concentrations of oxygenates. Biomass to bio-oil pyrolysis stoichiometry can be represented using an empirical formula as shown in eq.(1).

$$\underset{}{CH_{1.46}O_{0.67}} \rightarrow \underset{\text{Bio oil}}{0.71CH_{1.98}O_{0.76}} + \underset{\text{Char}}{0.21CH_{0.1}O_{0.15}} + \underset{\text{Gas}}{0.08CH_{0.44}O_{1.23}} \quad (1)$$

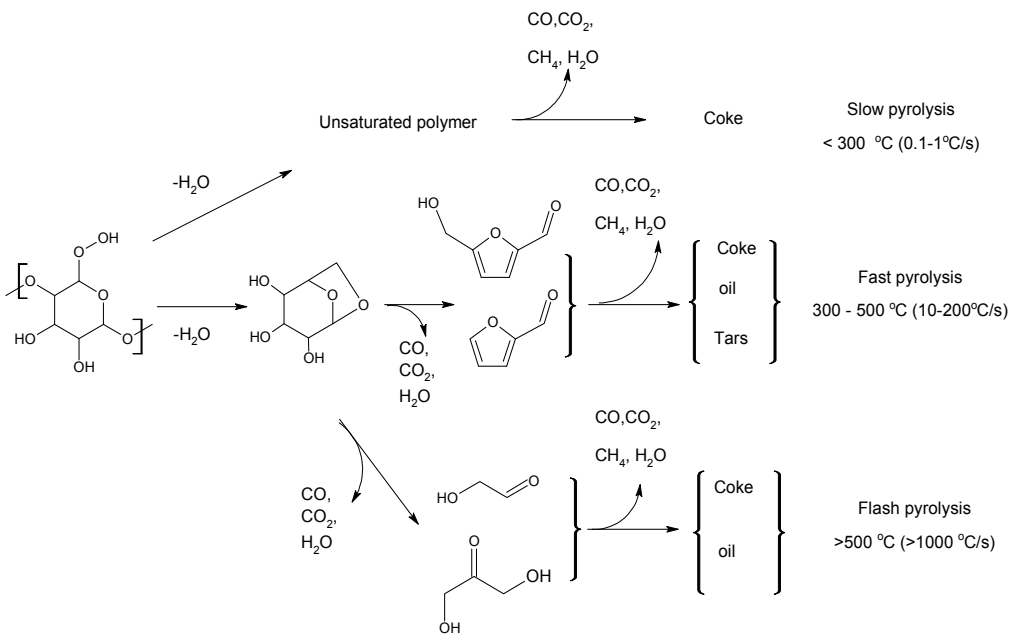


Figure 2. Cellulose pyrolysis with the products at different heating conditions. (Information for scheme was adapted from Huber et al. [20])

Bio-oil properties are highly variable and depend heavily on the type of biomass. High fibrous biomass that contains high amounts of lignin is considered to be the most effective for the production of bio-oil for fuel applications. Further, the oil-yields and composition of bio-oils highly depend on the process (pyrolysis/ liquefaction) used. Fast pyrolysis yields higher amounts of bio-oil compared to slow pyrolysis whereas liquefaction produces low amounts of oxygenated compounds as compared to fast pyrolysis. Oxygenated compounds such as aldehydes, alcohols, ketones, and carboxylic acids can be seen in bio-oils in varying degrees. A typical product distribution of bio-oil is depicted in figure (4). It can be seen that bio-oil contains large amounts of ketones, carboxylic acids and aldehydes [22].

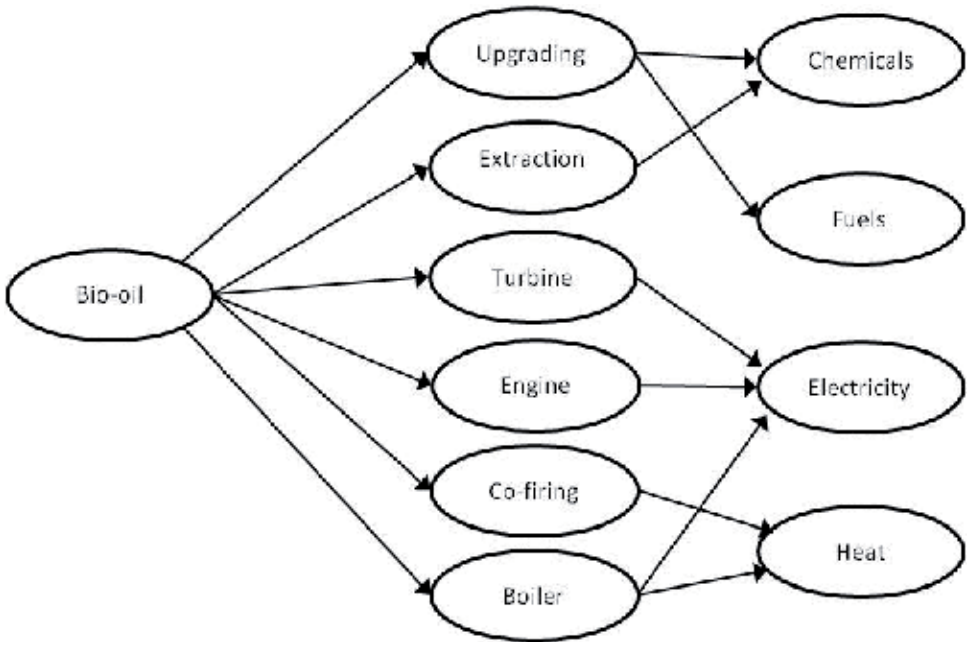


Figure 3. Different industrial applications of bio-oil derived from biomass (In formation adapted from Bridgwater et al. [21].)

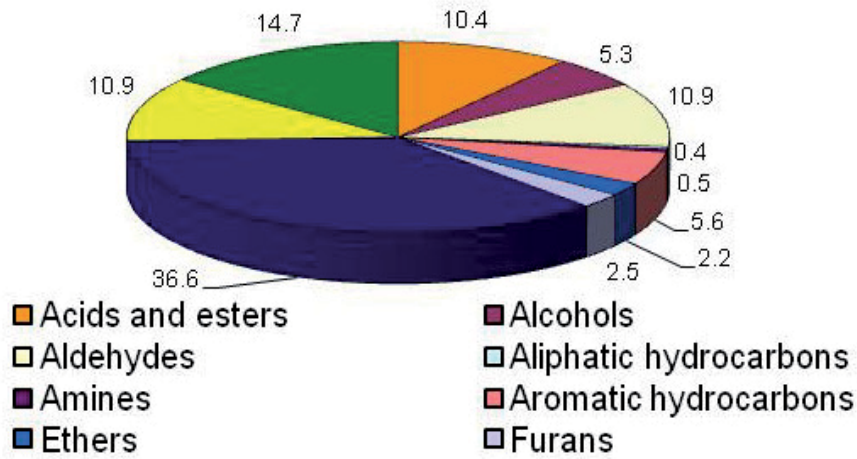


Figure 4. Relative distribution of chemical compounds in bio-oil (In formation adapted from Adjaye et al. [23].)

Bio-oil has already been tested in furnaces and gas turbines [21], as well as in space heaters and in boilers[24, 25], as represented in figure (3). Although bio-oil has potential as a crude-oil alternative, problems have been reported when bio-oil was used in such applications. These include, blocking filters by high levels of char particulates, high viscosity causing pumping issues, and corrosion from the low pH [26].

Liquefaction is another approach to producing bio-oils. The concept of oil production using biomass in hot water surfaced in the early 1920's. However, a more robust and effective method was not available until Pittsburg Energy Research Center (PERC) in the 70's demonstrated the use of carbon monoxide, steam and sodium carbonate catalyst at Albany Biomass Liquefaction facility in Oregon USA. Detailed information on this method can be found elsewhere [27].

The quality of bio-oil from the liquefaction process is reported to be superior to that obtained from pyrolysis. Liquefaction of biomass using super -critical methods can be considered as one of the more recent techniques under investigation.

Conversion of biomass to a product that is compatible with existing petroleum refinery infrastructure is prudent in several fronts. First, this will allow biomass to be converted into fuels and chemicals that are identical to what we use today (such as gasoline, diesel and jet fuels). So, such fuels could be used in present-day automobiles with no engine modifications. On the other hand, usage of existing fuel production and distribution of infrastructure helps long-term sustainability of biomass-to-fuels technology [28].

The challenge of converting bio-oil into a hydrocarbon fuel has been effective for the removal of functional groups that contain oxygenates (–OH, -COH, -COOH, etc.). The oxygen content of biomass-derived bio-oil is estimated to be 35-40% with a heating value between 16 and 19 MJ/kg [8]. In the recent years, there has been an unprecedented growth of research and developmental efforts related to conversion technologies and the information is scattered. Accordingly, the overall objective of this review is to assimilate this information and compare the status of key deoxygenation technologies.

2. Deoxygenation

The presence of oxygen in cellulose, hemicelluloses and lignin is the primarily reason for biomass derived bio-oil to be highly functionalized. Cellulose and hemicellulose have common structural building blocks which are glucose and xylose respectively. Cellulose which is the most abundant component in terrestrial biomass is a crystalline polymer, and therefore, is quite difficult to chemically transform. Lignin which is abundant in biomass is mainly an amorphous poly aromatic polymer. Because of this complex heterogeneity of biomass, the liquid derived from the pyrolysis or liquefaction process contains a variety of different chemical species which can be roughly estimated to be around 400.

2.1. Importance of deoxygenation

The properties of bio-oil are significantly affected by its chemical composition. Unlike crude petrolium, bio-oil constituents have numerous functional groups as shown in figure (4). The high functionality decreases the stability of the oils and results in polymerization. A direct consequence is the increase of viscosity of bio-oils with time which in turn raises concerns about the feasibility of using bio-oil products as substitutes for petrochemical fuels. In addition to instability, low pH values and the presence of a high water content and ash

content are considered problematic [29, 30]. Additionally, the presence of oxygenates and water in the bio-oil reduces the heating value. Consequently, upgrading is necessary to make it useful as a fuel.

As shown in the figure (5) upgrading improves the H/C molar ratio together with increasing the heating value. In contrast, crude petroleum is lean in oxygenated compounds and therefore, most of the chemistries developed to date are based on adding functional groups to increase its activity. As a result, there is only limited knowledge on techniques available to remove functionality from highly oxygenated compounds. However the upgrading of bio-oil by removing of oxygen (deoxygenation) is considered as one of the most intricate challenges we face today in getting bio-oil to a form that is applicable as a fuel intermediate.

As discussed above, chemical species present in biomass derived liquid bio-oil can be categorized into alcohols, aldehyde / ketones, carboxylic acids, phenols and furans. Therefore the upgrading process of bio-oil involves removal of various oxygenated species and can be collectively called as deoxygenation. The deoxygenation process predominantly involves three reaction classes, i.e., dehydration, decarbonilation and decarboxylation.

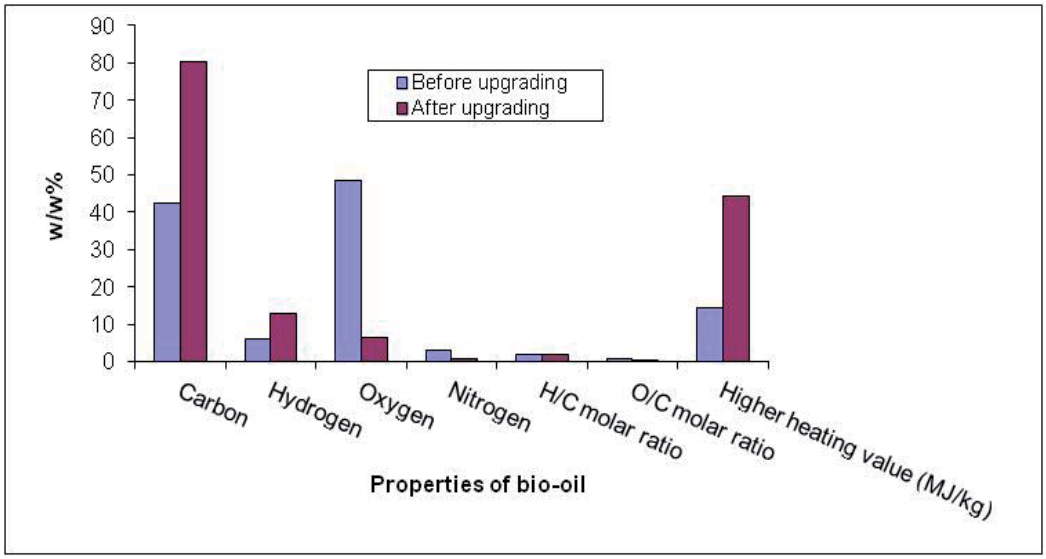


Figure 5. Change of characteristics before and after upgrading bio-oil derived from soybean stalk (Information adapted from Li et al. [8].)

2.2. Reactions involved in deoxygenation

As previously mentioned, deoxygenation involves the removal of functionality of biomass constituents associated with -OH, -COOH, and -C=O. The bond dissociation energies (BDE) for these functional groups are quite high and can be written in descending order as follows:

C-O (1076.5 kJ/mol) > C=O (749 kJ/mol) > C-C (610.0 kJ/mol) > O-H (429.99 kJ/mol) > C-H (338.4 kJ/mol).

Higher BDE for a particular functional group implies that the activation energy required for dissociating this bond (deoxygenation) would also be high. This would dictate rigorous reaction conditions for particularly C-O and C=O bond scissions. The presence of large amount of C=O groups in the pyrolysis products can be related to the higher activation energies required for dissociation of these bonds. However, using appropriate catalysts, these high activation energies could be overcome.

2.2.1. Dehydration

Bio-oil has significant amounts of –OH groups in its components that require dehydration, i.e., removal of oxygen in the form of water, to make hydrocarbons. Dehydration occurs even during HDO process but what is considered here is the spontaneous removal of oxygen as water in the absence of extraneous H_2.

Studies on dehydration as a method to produce motor fuel initiated several decades ago. With the advent of new catalysts, this area grew into new heights. A wide range of studies have been carried out with oxygenates ranging from simple methanol, to polyols like glycerol. The presence of light-molecular-weight alcohols such as methanol and ethanol in bio-oil is less common while phenolic compounds and polyols can be considered to be more abundant. However, studies on light alcohols give good insights into the chemistry and will be discussed in more detail below.

Dehydration of methanol to produce gasoline products such as benzene, toluene and xylene has been reported by different research groups [31-35]. In this regard, different heterogeneous catalysts have been studied. In particular, the molecular sieve ZSM-5 has received great attention for dehydration reactions. It has been widely accepted that Brønsted acid sites of the ZSM-5 catalyst play a crucial role for the dehydration reaction. Acid sites donates protons to the hydroxyl group of the oxygenate as shown in figure (7) to form water instigating dehydration.

Addition of metal oxides onto the catalyst framework was reported to increase the acidity of the support material. As a result, this would enhance dehydration reactions in turn facilitating the formation of higher molecular weight hydrocarbons. For example, methanol conversion to gasoline range hydrocarbons over metal oxides (such as ZnO and CuO) supported on HZSM-5 at temperature 400 ^{O}C and 1 atm pressure was reported [36]. The results indicate that pure HZSM-5 produced the lowest yields compared to metal-oxide-promoted catalyst such as CuO/ HZSM-5, CuO /ZnO/HZSM-5, ZnO/HZSM-5. The presence of CuO significantly increased the yields of aromatics. It was further concluded that addition of ZnO over CuO significantly reduced the catalyst deactivation potential [36]. Studies conducted to find the effect of different CuO loadings on methanol conversion indicate that the highest conversion of 97% was obtained when the CuO loading was at 7%. It was observed that further increase of oxide loading decreased methanol conversion. This behavior was attributed to loss of acid sites on the support. A subsequent catalyst deactivation study indicated that the catalyst deactivation increased with the increase in CuO loading. It was concluded that the deactivation of the catalyst occurred mainly due to

the deposition of large molecular weight hydrocarbons known as coke blocking the catalyst pores[37].

Dehydration of ethanol has also been studied extensively with other catalysts. A study of the effect of different additives such as ZnO, Ga_2O_3, Mo_2C and Re, on ZSM-5 on deoxygenation of alcohols has provided new insights on renewable aromatic hydrocarbons production [38]. The product selectivities for different catalysts are depicted in figure (8). It is clear that Ga_2O_3 performed better in terms of selectivity toward benzene, toluene and xylenes found in gasoline.

Figure 6. The two form of acid sites that exist in zeolites.

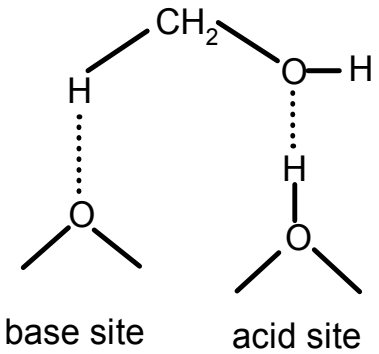
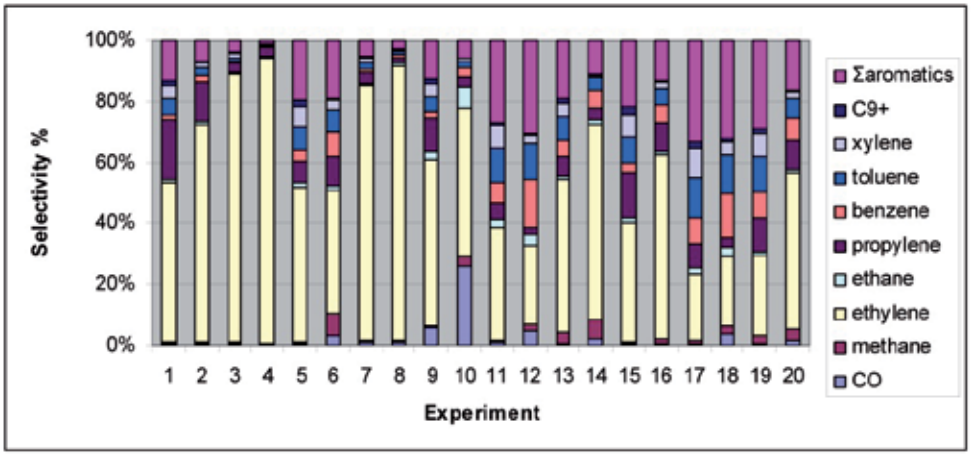


Figure 7. The proposed adsorption of methanol on ZSM-5.(Information was adapted from Chang et al. [31])

Ethanol dehydration has been further studied in order to make ethylene as the precursor to make ethyl-tetra-butyl-ether (ETBE) [39, 40]. Ethers have gained much attention as a substitute for petroleum diesel. A study on ethanol dehydration to ethylene over dealuminated modernite (DM) and metals such as Zn, Mn, Co, Rh, Ni, Fe and Ag loaded DM has shown that Zn/DM and Zn/Ag/DM gives the highest selectivity to ethylene formation [40]. This indicates that incorporation of single metal or metal combinations onto dealuminated modernite makes the catalyst more selective toward ethylene production. Further , the results suggest that such combinations of metal and support would lower coke formation as high molecular weight compounds were not produced significantly.



(a)

Experiment No:	Catalyst used	Reaction Temperature -K
1	ZSM-5(80)	773
2	ZSM-5(80)	873
3	ZSM-5(280)	773
4	ZSM-5(280)	873
5	2% Mo_2C/ZSM-5(80)	773
6	2% Mo_2C/ZSM-5(80)	873
7	2% Mo_2C/ZSM-5(280)	773
8	2% Mo_2C/ZSM-5(280)	873
9	2% Re/ZSM-5(80)	773
10	2% Re/ZSM-5(80)	873
11	2% Ga_2O_3/ZSM-5(80)	773
12	2% Ga_2O_3/ZSM-5(80)	873
13	2% ZnO/ZSM-5(80)	773
14	2% ZnO/ZSM-5(80)	873
15	ZSM-5(80) + 2% Mo_2C/ZSM-5(80)	773
16	ZSM-5(80) + 2% Mo_2C/ZSM-5(80)	873
17	ZSM-5(80) + 2% Ga_2O_3/ZSM-5(80)	773
18	ZSM-5(80) + 2% Ga_2O_3/ZSM-5(80)	873
19	ZSM-5(80) + 2% ZnO/ZSM-5(80)	773
20	ZSM-5(80) + 2% ZnO/ZSM-5(80)	873

(b)

Figure 8. (a) Selectivity towards different products from ethanol dehydration. (b) The catalysts and respective reaction conditions for each reaction.(Prepared with data from Barthos et al.[38])

There has been some studies on phenol dehydration [41, 42]. In once such study where HZSM-5 was used it has been observed that the reactivity of phenol and 2-methoxyphenol has been very low and that the catalyst had a greater tendency to form coke [42]. The rate of deactivation of the catalyst by coke formation reduced with increased water formation. In a separate study, lignin derivative guaiacol was attempted to be transformed to phenol at a temperature of 350 ^{o}C and 1atm pressure. In this study, first row transition metals (V to Zn) supported on alumina was tested [43]. Results indicate that vanadium oxide on alumina

gave the highest yield of phenol. They concluded that vanadium, as an early transition metal, has the oxophilic property and had helped the efficient removal of oxygen from guaiacol in the form of water. Nevertheless, water formed during dehydration has a tendency to adsorb onto acid sites dramatic decreasing the catalyst activity.

2.2.2. Decarboxylation

Bio-oil contains considerable amounts of acids such as acetic, formic, and butyric acid, resulting in low pH values (c.a. pH 2-3). The presence of these acids creates various practical challenges for bio-oil applications. Highly corrosive nature makes bio-oil not suitable for applications with metals and rubber. Further, the presence of acids would increase O/C ratio and make bio-oil more reactive. Accordingly, it is critical to develop chemistries that can deal with carboxylic acids when upgrading bio-oil. Decarboxylation refers to the removal of oxygen in the form of CO_2 from a carboxylated compound and can be given in a general equation as follows (eq.(2)):

$$RCO_2H \rightarrow RH + CO_2 \tag{2}$$

Insights on effective catalysts for removing oxygen in carboxylic acids can be obtained from studies on decarboxylation of model systems that include stearic, palmetic, benzoic, and heptanoic acids. Equations (3) and (4) depict the thermodynamic favorability of the decarboxylation reactions. The negative values for ΔG^o implies that the decarboxylation reactions of acetic and benzoic acids have a significant activation energy barrier to overcome[44].

$$CH_3CO_2H \rightarrow CH_4 + CO_2 \qquad \Delta G^o = -68.5 \text{ kJ / mol} \tag{3}$$

$$C_6H_5CO_2H \rightarrow C_6H_6 + CO_2 \qquad \Delta G^o = -54.3 \text{ kJ / mol} \tag{4}$$

Biodiesel research is an important area to look for information related to decarboxylation. Fatty acids and fatty acid methyl esters from biodiesel industry have been subjected further deoxygenation with the intension of obtaining higher quality liquid fuels [45, 46]. In one such study, Pd has been identified to be an active metal for decarboxylation of fatty acids present in plant oil. In this study, the ability to convert heptanoic acid to octane was investigated using Pd/SiO_2 and Ni/Al_2O_3 catalysts [47-50]. It was reported that 98% acid conversion was obtained with Pd/SiO_2 at 330°C but only 64% was reported by Ni/Al_2O_3 [44].

Pd supported on active carbon has also been tested as the catalyst for decarboxylation of stearic acid at 300°C. The results indicated that the reaction was selective toward *n*-heptadecane [47]. Further, they claimed that conducting the reaction in the presence of hydrogen increased the rate of decarboxylation. A comparative study performed with thermal and catalytic deoxygenation of stearic acid further proved that catalytic deoxygenation is highly selective toward hydrocarbons. In this study, 5%Pd supported on mesoporous silica, SBA-15, MCM-41and zeolyte-Y has been used as the catalyst. It was reported that SBA-15 had a selectivity of 67% for n-pentadecane [48]. The study further

revealed that the deoxygenation activity reduces in the order as SBA -15 > MCM-41 > zeolite-Y.

In an analogous study, pure palmitic acid, stearic acid, and a mixture of 59% of palmitic and 40% of stearic acid was deoxygenated over 4 % Pd/C catalyst at 300 oC and 5% H_2 in argon at 17 bar of pressure. The conversion of the catalyst was reported to be over 94% after 180 min of the reaction time with a selectivity of 99% [51]. The kinetic behavior of decarboxylation of ethyl stearate over Pd / C has been investigated with the aim to verify the reaction mechanism. As shown in figure (9), decarboxylation of ethyl stearate proceeded through fatty acid decarboxylation to the desired *n*-heptadecane. The produced paraffin simultaneously dehydrogenated to unsaturated olefins and aromatics. A kinetic model has been developed based on the proposed reaction network in figure (9) using Langmuir–Hinshelwood mechanism with the assumptions that the surface reaction is rate limiting and the adsorption reaction is rapid compared to surface reaction [52]. The rate equation for the proposed reaction scheme can be represented in a simplified form as shown in eq.(5). According to the rate information, step 6 in figure (9) can be considered as the rate limiting step with the rate constant of $1.45x10^{-12}$/min. Both decarboxylation steps which were represented in step 4 and 5 is shown to be the fastest steps in the scheme.

$$r_i = \frac{k_i \bullet C_i}{\left(1 + \sum_i K_i \bullet C_i\right)} \tag{5}$$

(r_i: reaction rate, k_i: lumped reaction rate, C_i: concentration, K_i: equilibrium constant)

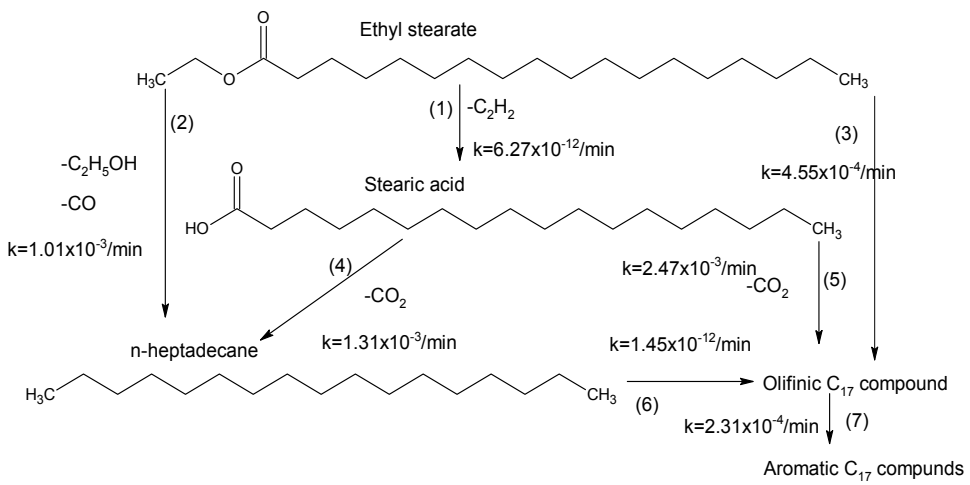


Figure 9. Decarboxylation of elthyl stearate (Information was adapted from Snare et al.[52])

HZSM-5 can be considered as a versatile catalyst that has the ability to do both dehydration and decarboxylation. For example, decarboxylation of methyl esters to hydrocarbon fuels has been studied using methyl octonoate (MO) on HZSM-5 [53]. The catalyst showed strong

signs of MO adsorption on to the catalyst surface. This reaction produced significant amounts of C1-C7 hydrocarbon compounds and aromatics. Formation of octonic acid as a primary product indicates that acidic hydrolysis reaction has taken place. However, it was noted that these primary products further undergo conversion into aromatic compounds. The proposed reaction scheme for the MO conversion is presented in figure (10).

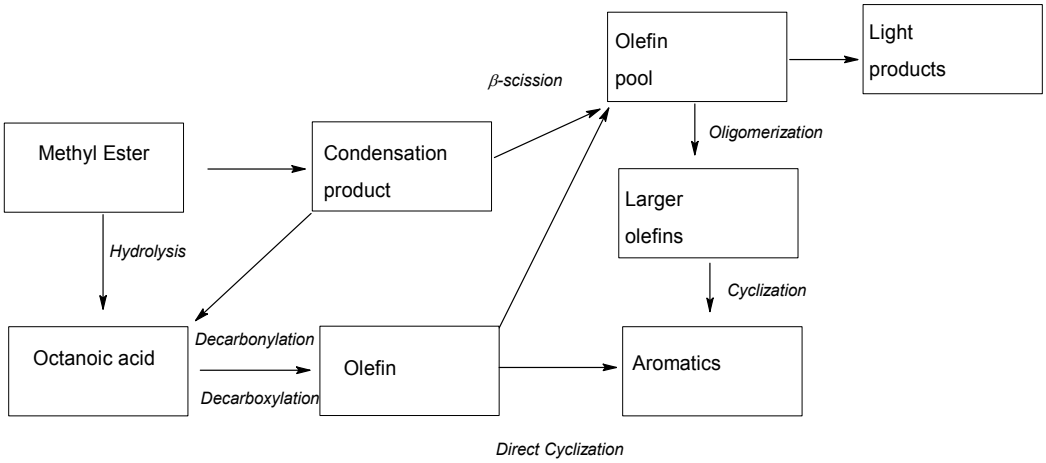


Figure 10. A possible reaction pathway for the deoxygenation of methyl octonoate (Information was adapted from Danuthai et al. [53]).

Rather than complete removal, partial removal of oxygen to aldehydes or ketones would also be useful during upgrading since the latter product(s) can go through HDO pathway relatively easily. Various studies have been conducted in this regard and many have used benzoic acid as the model compound [54-58]. In such a study, different weak base catalysts such as MnO_2, CeO_2, MgO, ZnO, Fe_2O_3, K_2O supported on SiO_2, Al_2O_3, TiO_2 have been tested for upgrading the acid-rich phase of bio-oil through ketonic condensation. The study further evaluated the effect of the presence of water on ketonic condensation of three model components, phenol, *p*-methoxyphenol, and furfural (typically seen in bio-oil). They reported that CeO_2 on Al_2O_3 and TiO_2 had better catalytic activity and tolerance to water. Although the presence of water and phenol did not have a significant impact on the ketonic condensation of acetic acid, the presence of furfural exhibited a strong inhibitory effect on the reaction [54].

Recent studies reported that the best catalysts for the conversion of carboxylic acids to aldehyde /alcohol(s) were Al_2O_3, SiO_2, TiO_2 or MgO supported transition/noble metals such as Pt, Pd, Cu, or Ru. For example, in deoxygenation of methyl stearate and methyl octanoate over alumina-supported Pt [59], 1% Pt/γ-Al_2O_3 reported to be highly active and selective toward deoxygenation. They reported that 1% Pt/TiO_2 displayed a higher C_8 hydrocarbon selectivity than 1% Pt/Al_2O_3. This was attributed to the presence of larger oxygen vacancies on the TiO_2 support [59]. Results of a similar screening study for the decarboxylation of stearic acid are depicted in the figure (11). It is apparent that Pd, Pt on activated carbon and 5% Ru on MgO resulted in the highest conversion of stearic acids to hydrocarbons.

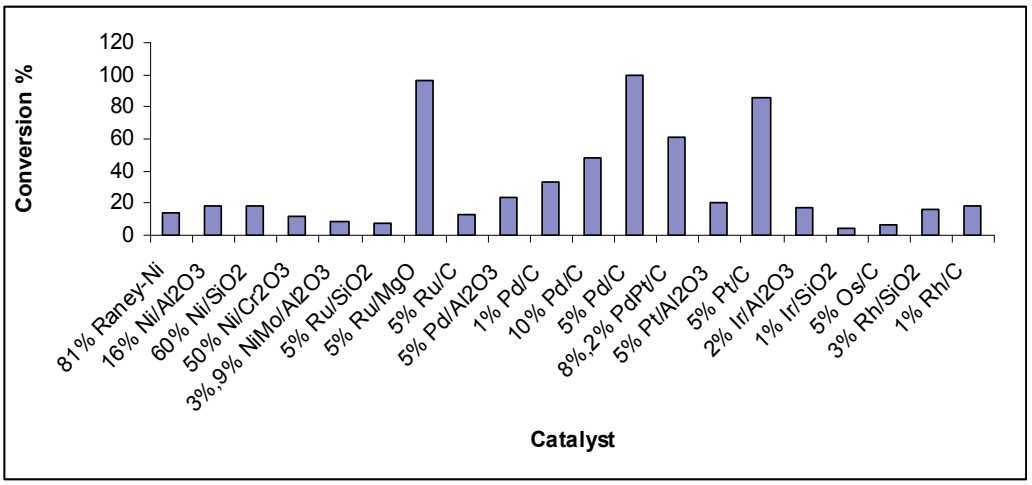


Figure 11. Conversion of stearic acid on different catalysts (Information was adapted from Snare et al. [45].)

2.2.3. Decarbonylation

In general, bio-oil contains significant amounts of aldehydes and ketones c.a. 10.9% and 36.6% respectively. The presence of carbonyl groups in the structure reduces the heating value and stability of bio-oil. Therefore, selective removal of carbonyl group as carbon monoxide as given in eq. (6) is another route to make bio-oil a more favorable fuel intermediate. However, the level of understanding of decarbonylation as a route for the upgrading bio-oil is still quite limited.

$$RCOH \rightarrow RH + CO \quad (6)$$

According to literature, decarbonylation and decarboxylation are integral reactions in the deoxygenation of carboxylic acids and esters. At times, instead of removing CO_2, removal of CO and H_2O can take place in the deoxygenation step and is considered as decarbonylation. Moreover, product(s) derived by decarbonylation / decarboxylation are not significantly different from those obtained from hydrogenolysis [47].

Decarbonylation usually takes place over supported noble metal catalysts such as Pd/C at elevated temperatures [47]. A study on decarbonylation reaction has been carried out to understand the effect of the presence of Cs on zeolyte-X for the deoxygenation of methyl octanoate (MO) as well as the effect of methanol co-feeding with MO [60]. The results indicated that the decarbonylation of MO occurs at a higher rate and for extended periods over CsNaX when co-fed with methanol. The surface analysis revealed that MO strongly adsorbed on basic sites of CsNaX and Cs improved the basicity of the catalyst. It was concluded that not only the basicity of the catalyst but also the polar nature of the zeolite catalyst assisted the decarbonylation process [60].

Deoxygenation of aldehyde, ketone and carboxylic acid containing bio-oil constituents has been studied using model compounds such as acetaldehyde, acetone, butanone, and acetic

acid [61]. In this study, HZSM-5 was used as the catalyst. Acetone was considered to undergo a reaction via a mechanism as depicted in figure (12). The results indicated that acetone is less reactive than alcohols and that a higher space velocity was needed to achieve higher conversion into aromatics. A significant increase in coke formation had been observed for both aldehyde and carboxylic acids compared to alcohol.

The ongoing interest in understanding decarbonylation mechanism(s) under the umbrella of organometalic chemistry has resulted in some useful insights. For example, a theoretical and an isotope labeled experimental study of decarbonylation of benzaldehyde and phenyl acetaldehyde on rhodium surface in the presence of bidentate phosphine ligand indicate that decarbonylation mechanism consists of oxidative addition, migratory extrusion, and reductive elimination with migratory extrusion as the rate-determining step [62].

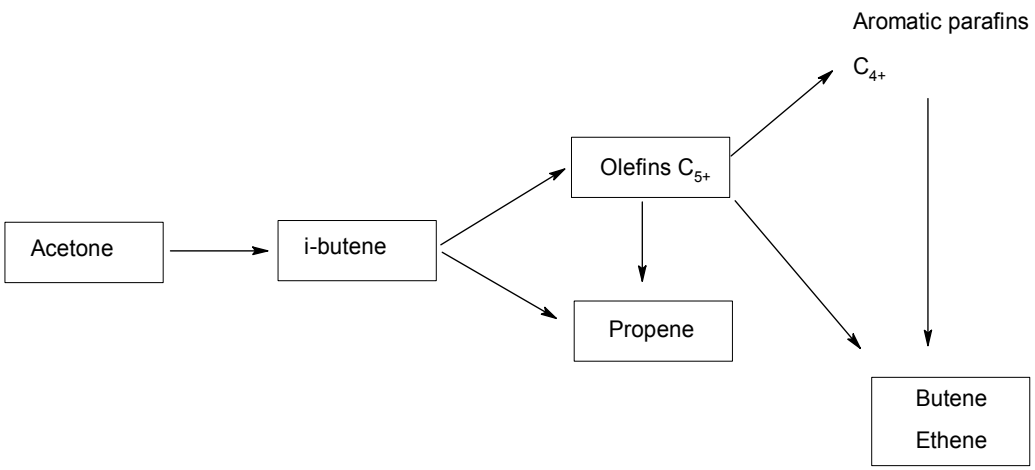


Figure 12. The reaction scheme for acetone decarbonylation on HZSM-5 (In formation was extracted from Gayubo et al. [61].)

Using DFT calculations, it has been deduced that decarbonylation of acetaldehyde is assisted by Co^{+} as the representative transition metal ion. The study concludes that decarbonylation of acetaldehyde follows four steps, i.e., complexation, C–C activation, aldehyde H-shift and nonreactive dissociation [63].

Furan, C_4H_4O is one of the common oxygenated compounds in the biomass derived bio-oils that has been used to study decarbonylation. Adsorption and desorption steps of furan on pure metal surfaces during the deoxygenation reaction can be found in many publications [64-66]. Some studies on furan decarbonylation has been conducted on different single crystal metal surfaces such as Cu (110), Ag (110) and Pd(111). It was observed that furan absorbs on Cu, Ag and, under mild temperatures, on Pd. Under mild conditions, it was observed that furan desorbs on the metal surface without disrupting the molecule, but, at elevated temperatures, undergoes a deoxygenation reaction [67].

3. Upgrading techniques for fuel production

In any biomass to biofuels conversion process, above mentioned three reaction pathways can be considered to be quite significant. Depending on the process conditions such as temperature, pressure, the resident time and the type of catalyst, the degree to which these reactions would take place may vary. Basically the major processes that are exploited during deoxygenation pathways are secondary cracking, fast catalytic cracking and hydrodeoxygenation.

3.1. Secondary pyrolysis

This is the simplest process with no catalyst involved for the deoxygenation of biomass oxygenates and occurs with fairly low efficiencies. The concept behind this process is simply to route the pyrolysis vapor through a second reactor which is maintained at a high temperature. The thermal cracking reaction initiated in the secondary reactor would remove oxygen as H_2O, CO_2 and CO. Due to the thermodynamic nature of this reaction, increased resident times would favor formation of thermodynamically more stable species such as CO_2 and CO. The significant drawback of this method is the higher tendency of losing carbon in the forms of CO_2 and CO.

3.2. Catalytic upgrading

Catalytic upgrading process is conducted in a number of different ways. Most commonly practiced method would be injection of bio-oil into a tubular reactor packed with a catalyst capable of deoxygenating the substrates. Due to the inefficacies associated with condensing and reheating processes, a reactor capable of accepting a direct feed of pyrolysis vapor into its catalytic chamber has become more popular. Numerous forms of Zeolites are known to be effective for deoxygenation[68] of which ZSM-5 is widely touted to be the most effective catalyst for deoxygenation (via decarboxylation, decarbonylation or dehydration pathways). ZSM-5 is a molecular sieve with 5.5 Å pore channels. This pore structure is responsible for the high selectivity of ZSM-5 toward aromatic hydrocarbons. Deoxygenation reaction on ZSM-5 is believed to be catalyzed at the Bronsted acid sites and its structure is depicted in fig.6.

3.3. Hydrodeoxygenation (HDO)

Due to the hydrogen deficient nature of biomass (C:H<1), catalytic upgrading of bio-oil often leads to deoxygenation via decarboxylation and/or decarbonylation routes leading to losing of precious carbon assimilated during photosynthesis. Analogously, dehydration, in a hydrogen-lean environment leads to formation of large unsaturated compounds commonly known as coke. In order to circumvent these issues, extra hydrogen is supplied to the reactor - and this process is called hydrodeoxygenation.

So far the most reliable and extensively studied method for deep deoxygenation is hydrodeoxygenation which involves gaseous hydrogen and heterogeneous catalyst such as

sulfided NiMo, CoMo supported on alumina[69] [70].The idea of using hydrogen to upgrade bio-oil originates from the use of hydrogen in the petrochemical industry. Hydroprocessing is a crucial step in petroleum refining process that basically involves five types of reaction classes: hydrodenitrogenation (HDN), hydrodesulfurization (HDS), hydrodeoxygenation (HDO), hydrodemthylation (HDM) and hydrogenation (HYD)[70-72].

The process where oxygen in the feed is removed via dehydration using gaseous hydrogen is called hydrodeoxygenation (HDO). In a typical hydro processing process, the order of these reaction classes are HDS>HDO>HDN. This is because in a conventional petroleum feed, the sulfur and nitrogen content is significantly higher compared to oxygen. Therefore, HDO chemistry has received only little attention in petrochemical refining [27]. Hydrotreatment of crude petroleum is challenging for the catalyst due to the presence of sulfur and nitrogen in the feed in significant amounts. The products of hydrotreatment such as water, ammonia, hydrogen sulfide has been reported to poison hydrotreating catalysts [73]. Nevertheless, since bio-oil contains less sulfur and nitrogen, HDO would be a better fit for bio-oil upgrading. The presence of significant amounts of oxygen and C=C compounds in bio-oil increases chances of simultaneous occurrence of HDO as well as the hydrogenation reactions. More negative Gibbs free energies of deoxygenation reactions compared to hydrogenation reactions implies that deoxygenation is more favorable. However, saturation of aromatic rings is not desirable as it consumes large amounts of hydrogen and reduces the octane number of the fuel reducing the fuel quality.

Much of the studies on HDO are based on catalysts such as Co-Mo, Ni-Mo, Ni-W, Ni, Co, and Pd. A catalyst, to be effective for HDO, should ideally perform two tasks, i.e., activating the dihydrogen molecule as well as activating the oxygen group of the compound. The oxygen group activation usually occurs on the transition metal oxides such as Mo, W, Co, Mn, Zr, Ce, Y, Sr and La while the activation of hydrogen is known to happen on noble metals such as Pt, Pd, and Rh [74].

Studies on HDO chemistry has mostly been done using model compound such as phenol, cresole, guaiacol, napthol etc. [75, 76] which are abundantly present in bio-oil. Some of these model compounds and their proposed reaction pathways are shown in Table (2).

Most of the earlier work in HDO used sulfided forms of Mo as the active element and Co or Ni as promoters on $\gamma.Al_2O_3$ [27, 70, 79]. However, sulfide catalyst would not work for bio-oil since the feed does not contain significant amounts of sulfur. In the HDO process, a sulfide catalyst would soon be deactivated if an external sulfur source is not provided [80]. Nevertheless, the oxygen content of the feed is said to have a negative effect on the sulphide structure resulting in losses such as the catalyst deactivation and changes in product distribution. In-depth studies on Co (or Ni)-promoted MoS_2 catalysts have revealed that the edges on MoS_2 is also important in terms of catalyst activity since they are dominated by promoter atoms in the so-called Co–Mo–S structures [81]. The studies have revealed that poly-condensation products formed have shortened the life by deactivation. Alumina in this regard is quite susceptible to deactivation by coke formation. Therefore, investigations on new catalysts that do not require sulfidation and supports such as activated carbon that are tolerant to deactivation are needed[82].

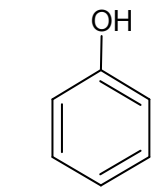
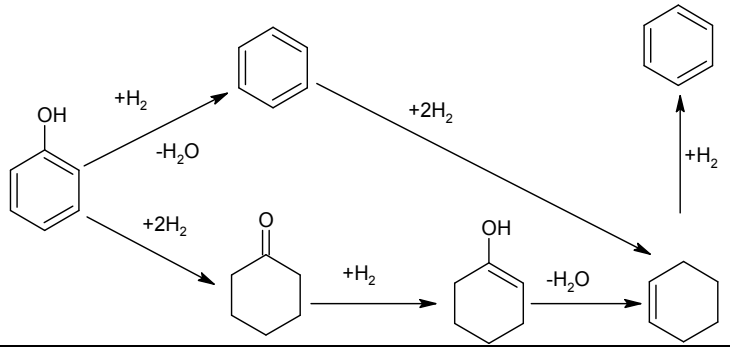
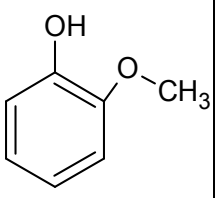
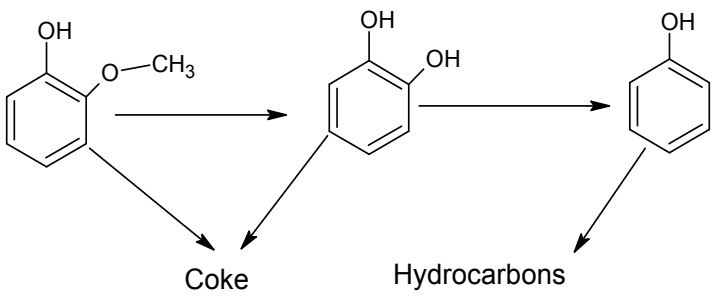
Oxygenate compound	Proposed hydro-deoxygenation reaction	Ref.
 Phenol		[77]
 Guaicol		[78]

Table 2. Common reaction pathways proposed for HDO reactions. (Reaction schemes were extracted from Senol et al [77]and Laurent et al.[78])

The suitability of Ni as a catalyst to activate the dihydrogen molecule in HDO has been reported by several groups[79, 83]. In comparison to noble metals, the use of Ni is extremely economical especially for large scale applications. For example, the gas-phase hydrodeoxygenation of a series of aromatic alcohols that include aldehydes and acids has been reported with Ni/SiO_2. This study analyzed kinetic effects of the gas phase hydrogenolysis of –CH_2OH, –CHO and –COOH groups attached on to aromatic ring structures in the presence of Ni/SiO_2 [79]. They concluded that the oxygenated aromatics get weakly adsorbed on the catalyst and the surface mobility facilitates reaction with adsorbed hydrogen atoms. Further, the adsorption reactions of H_2 and aromatic species were considered to be a competitive adsorption.

In a separate study, HDO of model compound anisole using Ni-Cu on Al_2O_3, CeO_2 and ZrO_2 supports found that Ni–Cu supported on CeO_2 and Al_2O_3 was the most active catalyst in comparison to pure Ni catalyst[74]. The significance of this study was that the catalysts

tested were not in the sulfided form and therefore would be highly suitable for bio-oil-type application(s).

The need for hydrogen in HDO process has always been a point of concern due to high expenses associated with hydrogenations. A study in this regard attempted using hydrogen generated in situ for performing HDO. A study conducted using Pt on TiO_2, CeO_2, and ZrO_2 supports showed that the oxygenates undergo dehydrogenation and subsequently HDO using the produced H_2. The catalysts tested were Pt/CeO_2, $Pt/CeZrO_2$, Pt/TiO_2, Pt/ZrO_2, Pt/SiO_2-Al_2O_3, and Pt/Al_2O_3. Of all catalysts tested, Pt on Al_2O_3 showed the highest activity with a reduction of oxygen content from 41.4 wt% to 2.8 wt% after upgrading [84].

It has also been shown that Pd on supports such as carbon, Al_2O_3, ZSM-5, MCM 41 [76, 85, 86] are active for HDO. A study conducted with benzophenone with 5% Pd on active carbon and on ZSM-5 supports proved to be very active toward hydrogenation as opposed to supports such as Al_2O_3, and MCM 41. However, the HDO of benzophenone was significantly higher with Pd on supports like active carbon and acid zeolytes. Furthermore, it was concluded that the acidity of the zeolite support affects the HDO reaction [76].

Co processing of bio-oil with straight run gas oil (SRGO) can be considered to have more practical significance. The concept behind this method is the simultaneous use of HDS process with the HDO of biocrude [69, 87-89]. The model compound guaiacol (5000 ppm) representing oxygenates in bio-oil has been used together with SRGO (containing13,500 ppm of S) in a trickle bed reactor. At low temperature and low space velocities, a decrease in the HDS reaction has been observed with $CoMo/Al_2O_3$. The possible explanation is the competition of intermediate phenol with the sulfur containing molecules for adsorption on hydrogenation/ hydrogenolysis sites. At higher temperatures (above 380^0C), the HDO of guaiacol was observed along with HDS taken place without further inhibition [88] .

Although the role of sulfur on HDS is well understood, the effect of sulfur on HDO is not yet well explained. Certain studies indicate that the presence of H_2S has an inhibitory effect on the HDO while other studies support maintaining the sulfidasation level of the catalyst [90-92]. For example, the effect of using a sulfiding agent, H_2S has been studied with model compounds like phenol and anisole during hydrotreatment. The results conclude that the presence of H_2S decreases the HDO activity of the sulfided $CoMo/\gamma–Al_2O_3$ catalyst and the product yield depends on the concentration of H_2S [80]. A similar study conducted using H_2S for HDO of phenol on Co-Mo and Ni-Mo arrived at the same conclusion. However, quite interestingly, the presence of H_2S during the HDO of aliphatic oxygenates has shown a promoting effect. The reason for this observation is that the sulfiding agent , H_2S, enhances the acid catalyzed reactions of aliphatic oxygenates. However, direct hydrogenolysis reaction of phenol is suppressed due to competitive adsorption of both phenol and H_2S [77, 93].

4. Concluding remarks

During pyrolysis or liquefaction, biomass undergoes a series of complex conversion processes producing bio-oil. Bio-oils are highly diverse in its composition and has a

spectrum of oxygenates. Due to the presence of a collective of different oxygenated compounds, an upgrading process to remove oxygen would likely involve a series of reactions. This review introduces several possibilities for upgrading bio-oil including dehydration, decarboxylation and decarbonylation. A summary of the best catalyst contenders for each reaction class is given in the Table 3.

Reaction class	Reaction details	Catalyst	Best performance
Dehydration	Methanolconversion to gasoline range products	HZSM-5, ZnO/HZSM-5, CuO/HZSM-5	CuO/HZSM-5 (7% loading of CuO is said to be best)
	Ehtanol conversion to hydrocarbon	HZSM-5 ZnO/ ZSM-5 Ga_2O_3/ZSM-5 Mo_2C/ ZSM-5 Re/ ZSM-5	Ga_2O_3/ZSM-5
	Ehtanol to ethylene	dealuminated modernite (DM) Zn/DM Mn/DM Co/DM Rh/DM Ni/DM Fe/ DM Ag/DM	Zn on dealuminated modernite
Decarboxylation	Conversion of heptanoic acid to octane	Pd/SiO_2 Ni/Al_2O_3	Pd/SiO_2
	Deoxygenation of stearic acid	5%Pd supported on messoporous silica - SBA15, -MCM41 zeolyte-Y	5%Pd -SBA15
	Ketonic condensation of acetic acid	MnO_2, CeO_2, MgO, ZnO, Fe_2O_3, K_2O supported on SiO_2, Al_2O_3, TiO_2	CeO_2 supported on Al_2O_3, or on TiO_2
Decarbonylation	Deoxygenation of methyl octanoate	Cs on NaX zeolyte	Cs on NaX zeolyte
	Deoxygenation of acetaldehyde, acetone, butanone, and acetic acid	HZSM-5	HZSM-5

Table 3. Summary of best performing catalyst for each reaction class.

Hydrodeoxygenation has been the most frequently studied and one of the most reliable methods that could be used for deoxygenation of oxygenates. However, the drawbacks of hydrodeoxygenation include: the need for continuous replenishment of sulfur (for sulfided catalysts), and the non-selective deoxygenation of all bio-oil chemical moieties resulting in a spectrum of short-chained and long-chained hydrocarbons that are less useful as liquid fuels. The need of hydrogen makes resulting biofuels less competitive with existing petroleum fuels. More research is needed on development of effective non-sulfided hydrodeoxygenation catalysts. Preliminary investigations with Ni-Cu on CeO_2 or ZrO_2 may provide new directions on this front. Further research should be directed on identifying materials and processes will allay the need for using direct hydrogen.

According to the analysis, metal promoted or unpromoted HZSM-5 proves to be one of the most versatile catalysts that is capable of catalyzing all three deoxygenation reaction classes, i.e., dehydration, decarboxylation and decarbonylation. Nevertheless, the nanopore structure of HZSM-5 (~5.4-5.5 Å), while assisting size selectivity for gasoline range hydrocarbons, also promotes pore blockages resulting in rapid catalyst deactivation. Developing catalysts of the same class, with larger and consistent pore structure and/or higher activity/functionality should be closely looked at.

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Aquatic Biomass

Chapter 12

Biofloc Technology (BFT): A Review for Aquaculture Application and Animal Food Industry

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Additional information is available at the end of the chapter

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1. Introduction

The aquaculture industry is growing fast at a rate of ~9% per year since the 1970s [1]. However, this industry has come under scrutiny for contribution to environmental degradation and pollution. As a result, requirement for more ecologically sound management and culture practices remains fully necessary. Moreover, the expansion of aquaculture is also restricted due to land costs and by its strong dependence on fishmeal and fish oil [2,3]. Such ingredients are one of the prime constituents of feed for commercial aquaculture [4]. Feed costs represent at least 50% of the total aquaculture production costs, which is predominantly due to the cost of protein component in commercial diets [5].

Interest in closed aquaculture systems is increasing, mostly due to biosecurity, environmental and marketing advantages over conventional extensive and semi-intensive systems [6]. When water is reused, some risks such as pathogen introduction, escapement of exotic species and discharging of waste water (pollution) are reduced and even eliminated. Furthermore, because of high productivity and reduced water use, marine species can be raised at inland locations [6]. A classic example is the currently expansion of marine shrimp farms at inland location in USA, which allows local farmers market fresh never frozen shrimp in metropolitan locations with good profitability.

The environmental friendly aquaculture system called "Biofloc Technology (BFT)" is considered as an efficient alternative system since nutrients could be continuously recycled and reused. The sustainable approach of such system is based on growth of microorganism in the culture medium, benefited by the minimum or zero water exchange. These microorganisms (biofloc) has two major roles: (i) maintenance of water quality, by the uptake of nitrogen compounds generating "*in situ*" microbial protein; and (ii) nutrition, increasing culture feasibility by reducing feed conversion ratio and a decrease of feed costs.

As a closed system, BFT has primordial advantage of minimizing the release of water into rivers, lakes and estuaries containing escaped animals, nutrients, organic matter and pathogens. Also, surrounding areas are benefitted by the "vertically growth" in terms of productivity, preventing coastal or inland area destruction, induced eutrophication and natural resources losses. Drained water from ponds and tanks often contains relatively high concentrations of nitrogen and phosphorous, limiting nutrients that induce algae growth, which may cause severe eutrophication and further anaerobic conditions in natural water bodies. In BFT, minimum water discharge and reuse of water prevent environment degradation and convert such system in a real "environmentally friendly system" with a "green" approach. Minimum water exchange maintain the heat and fluctuation of temperature is prevented [7], allowing growth of tropical species in cold areas.

Currently, BFT has received alternate appellation such as ZEAH or Zero Exchange Autotrophic Heterotrophic System [8-10], active-sludge or suspended bacterial-based system [11], single-cell protein production system [12], suspended-growth systems [13] or microbial floc systems [14,15]. However, researches are trying to keep the term "BFT or Biofloc Technology" in order to establish a key reference, mainly after the book release "*Biofloc Technology – A Practical Guide Book*" in 2009 [16]. Moreover, BFT has been focus of intensive research in nutrition field as a protein source in compounded feeds. Such source is produced in a form of "biofloc meal", mainly in bioreactors [17]. In addition, the fast spread and the large number of BFT farms worldwide induced significant research effort of processes involved in BFT production systems [14].

The objective of this chapter is to review the application of Biofloc Technology (BFT) in aquaculture; and describes the utilization of biofloc biomass (also described in this chapter as "biofloc meal") as an ingredient for compounded feeds. An addition goal is to help students, researchers and industry to clarify the basic aspects of such technology, aiming to encourage further research.

2. History of BFT

According to [18], BFT was first developed in early 1970s at Ifremer-COP (French Research Institute for Exploitation of the Sea, Oceanic Center of Pacific) with different penaeid species including *Penaeus monodon*, *Fenneropenaeus merguiensis*, *Litopenaeus vannamei* and *L. stylirostris* [19,20]. Such culture system was compared with an "external rumen", but now applied for shrimp [21]. At the same period, Ralston Purina developed a system based on nitrifying bacteria while keeping shrimp in total darkness. In connection with Aquacop, such system was applied to *L. stylirostris* and *L. vannamei* both in Crystal River (USA) and Tahiti, leding considerations on benefits of biofloc for shrimp culture [22]. In 1980, a French scientific program 'Ecotron' was initiated by Ifremer to better understand such system. Several studies enabled a comprehensive approach of BFT and explained interrelationships between different compartments such as water and bacteria, as well as shrimp nutritional physiology. Also in 1980s and beginning of 1990s, Israel and USA (Waddell Mariculture Center) started R&D in BFT with tilapia and white shrimp *L. vannamei*, respectively, in

which water limitation, environmental concerns and land costs were the main causative agents that promoted such research (Fig. 1).

Figure 1. Biofloc technology at Ifremer, Tahiti (A), Sopomer farm, Tahiti (B), Waddell Mariculture Center (C) and Israel (D) (Photos A and B: Gerard Cuzon; C: courtesy of Wilson Wasielesky; and D: courtesy of Yoram Avnimelech)

Regarding to commercial application of BFT, in 1988 Sopomer farm in Tahiti (French Polynesia) using 1000m^2 concrete tanks and limited water exchange achieved a world record in production (20–25 ton/ha/year with two crops) [22, 23]. On the other hand, Belize Aquaculture farm or "BAL" (located at Belize, Central America), probably the most famous case of BFT commercial application in the world, produced around 11-26 ton/ha/cycle using 1.6 ha lined grow-out ponds. Much of know-how of running worldwide commercial scale BFT shrimp ponds is derived from BAL experience. In small-scale BFT greenhouse-based farms, Marvesta farm (located at Maryland, USA), probably is the well-known successful indoor BFT shrimp farm in USA, can produce around 45 ton of fresh never frozen shrimp per year using ~570 m^3 indoor race-ways [24]. Nowadays, BFT have being successfully expanded in large-scale shrimp farming in Asia, Latin and Central America, as well as in small-scale greenhouses in USA, South Korea, Brazil, Italy, China and others (Fig 2). In addition, many research centers and universities are intensifying R&D in BFT, mostly applied to key fields such as grow-out management, nutrition, BFT applied to reproduction, microbial ecology, biotechnology and economics.

Figure 2. Biofloc technology commercial-scale at BAL (A) and Malaysia (B), and pilot-scale in Mexico (C and D) (Photos A, B and D: Maurício Emerenciano; and C: courtesy of Manuel Valenzuela)

3. The role of microorganisms

The particulate organic matter and other organisms in the microbial food web have been proposed as potential food sources for aquatic animals [25]. In BFT, microorganisms present a key role in nutrition of cultured animals. The macroaggregates (biofloc) is a rich protein-lipid natural source available *"in situ"* 24 hours per day [14]. In the water column occurs a complex interaction between organic matter, physical substrate and large range of microorganisms such as phytoplankton, free and attached bacteria, aggregates of particulate organic matter and grazers, such as rotifers, ciliates and flagellates protozoa and copepods [26] (Fig 3). This natural productivity play an important role recycling nutrients and maintaining the water quality [27,28].

The consumption of biofloc by shrimp or fish has demonstrated innumerous benefits such as improvement of growth rate [10], decrease of FCR and associated costs in feed [9]. Growth enhancement has been attributed to both bacterial and algae nutritional components, which up to 30% of conventional feeding ration can be lowered due to biofloc consumption in shrimp [29]. In reference [9] was reported that more than 29% of daily food consumed for *L. vannamei* could be biofloc. In tilapia, in [30] was estimated that feed utilization is higher in BFT at a rate of 20% less than conventional water-exchange systems.

Also, consumption of macroaggregates can increase nitrogen retention from added feed by 7-13% [31, 32]. In this context, BFT has driven opportunities to use alternative diets. Low protein feeds and feeds with alternative protein sources different than marine-based products (i.e. fishmeal, squid meal, etc) have been successfully applied in BFT [28, 33-35], leading "green" market opportunities.

Figure 3. Grazers often observed in BFT such as flagellates protozoa (A), ciliates protozoa (B), nematodes (C) and copepods (D) (10x magnification) (Source: Maurício Emerenciano)

Regarding to maintenance of water quality, control of bacterial community over autotrophic microorganisms is achieved using a high carbon to nitrogen ratio (C:N) [30], which nitrogenous by-products can be easily taken up by heterotrophic bacteria [36]. High carbon to nitrogen ratio is required to guarantee optimum heterotrophic bacteria growth [14, 37], using this energy for maintenance (respiration, feeding, movement, digestion, etc), but also for growth and to produce new cells. High carbon concentration in water could supersede the carbon assimilatory capacity of algae, contributing to bacteria growth. Aerobic microorganisms are efficient in converting feed to new cell material (40-60% of conversion efficiency), rather than higher organisms that spend about 10-15% to rise in weight [16]. Bacteria and other microorganisms act as very efficient "biochemical systems" to degrade and metabolize organic residues [36]. In other words, they recycle very efficiently nutrients in a form of organic and inorganic matter (un-consumed and non-digested feed, metabolic residues and carbon sources applied as fertilizers) into new microbial cells.

The carbon sources applied in BFT are often by-products derived from human and/or animal food industry, preferentially local available. Cheap sources of carbohydrates such as molasses, glycerol and plant meals (i.e. wheat, corn, rice, tapioca, etc) will be applied before fry/post-larvae stocking and during grow-out phase, aiming to maintain a high C:N ratio (~15-20:1) and to control N compounds peaks. Also, a mix of plant meals can be pelletized ("green-pellet") and applied into ponds [38]; or low protein diets containing high C:N ratio can also be carried out [16,33]. The carbon source serves as a substrate for operating BFT systems and production of microbial protein cells [36]. There are many considerations for its selection such as costs, local availability, biodegradability and efficiency of bacteria assimilation. In Table 1 is summarized some studies with different species and carbon source applied in BFT system.

Carbon source	Culture specie	Reference
Acetate	*Macrobrachium rosenbergii*	[39]
Cassava meal	*Penaeus monodon*	[40]
Cellulose	Tilapia	[12]
Corn flour	Hybrid bass and hybrid tilapia	[41, 42]
Dextrose	*Litopenaeus vannamei*	[43]
Glycerol and Glycerol+*Bacillus*	*M. rosenbergii*	[39]
Glucose	*M. rosenbergii*	[39]
Molasses	*L. vannamei and P. monodon*	[9, 29, 44]
Sorghum meal	Tilapia	[12]
Tapioca	*L. vannamei* and *M. rosenbergii*	[31, 45]
Wheat flour	Tilapia (*O. niloticus*)	[33]
Wheat bran + molasses	*Farfantepenaeus brasiensis, F. paulensis and F. duorarum*	[37, 46, 47]
Starch	Tilapia *O. niloticus x O. aureus* and tilapia (Mozambique)	[7, 14]

Table 1. Different carbon sources applied on BFT system (Source: adapted from [36])

Not all species are candidates to BFT. Some characteristics seems to be necessary to achieve a better growth performance such as resistance to high density, tolerance to intermediate levels of dissolved oxygen (~3-6 mg/L), settling solids in water (~10 with a maximum of 15 mL/L of "biofloc volume", measured in Imhoff cones) [38] and N-compounds, presence of filtering apparatus (i.e. tilapia), omnivorous habits and/or digestive system adaptable to better assimilate the microbial particles.

4. Applications in aquaculture

4.1. Nursery and grow-out

Nursery phase is defined as an intermediate step between hatchery-reared early postlarvae and grow-out phase [48]. Such phase presents several benefits such as optimization of farm land, increase in survival and enhanced growth performance in grow-out ponds [49-51]. BFT has been applied successfully in nursery phase in different shrimp species such as *L. vannamei* [44, 48], *P. monodon* [51], *F. paulensis* [15, 46], *F. brasiliensis* [37, 52] and *F. setiferus* [34]. The primary advantage observed is related to a better nutrition by continuous consumption of biofloc, which might positively influence grow-out performance *a posteriori* [53], but was not always the case [54]. In addition, optimization of farm facilities provided by the high stocking densities in BFT nursery phase seems to be an important advantage to achieve profitability in small farms, mainly in cold regions or when farmers are operating indoor facilities.

In [46] was observed that presence of bioflocs resulted in increases of 50% in weight and almost 80% in final biomass in *F. paulensis* early postlarval stage when compared to conventional clear-water system. This trend was observed even when postlarvae were not fed with a commercial feed (biofloc without commercial feed). In *L. vannamei* nursery in BFT conditions, references [48] and [55] reported survival rates ranging from 55.9% to 100% and 97% and 100%, respectively. In [51] was demonstrated that the addition of substrates in BFT systems increased growth and further enhanced production, while also contributing to more favorable water quality conditions. According to the same study, growth and survival was not affected by stocking density (2500 *vs* 5000 PL/m^2), therefore greater production outputs were achieved at the higher density. Furthermore, in [37] was found that *F. brasiliensis* postlarvae grow similarly with or without pelletized feed in biofloc conditions during 30-d of nursery phase, which was 40% more than conventional clear-water continuous exchange system.

In grow-out, BFT has been also shown nutritional and zootechnical benefits. In [9] was estimated that more than 29% of the daily food intake of *L. vannamei* consisted of microbial flocs, decreasing FCR and reducing costs in feed. The reference [10] showed that juveniles of *L. vannamei* fed with 35% CP pelletized feed grew significantly better in biofloc conditions as compared to clear-water conditions. In [28] was showed that controlling the concentration of particles in super-intensive shrimp culture systems can significantly improve shrimp production and water quality. Also, the same authors demonstrated that environmentally friendly plant-based diet can produce results comparable to a fish-based feed in BFT conditions. In [56] was evaluated the stocking density in a 120d of *L. vannamei* BFT culture, reporting consistent survival of 92, 81 and 75% with 150, 300 and 450 shrimp/m^2, respectively. Moreover, the study [57] performed in a heterotrophic-based condition detected no significant difference in FCR when feeding *L. vannamei* 30% and 45% CP diets and 39% and 43% CP diets, respectively. With these results in mind, floc biomass might provide a complete source of cellular nutrition as well as various bioactive compounds even at high density. It is not known exactly how microbial flocs enhance growth. Growth might be enhanced by continuous consumption of “native protein”, protein source without

previous treatment [18], which could possess a "growth factor" similar to the one investigated in squid [58]. Is well known that protein, peptides and aminoacids participate fully in synthesis of new membranes, somatic growth and immune function and biofloc can potentially provide such ingredients.

For fish and other species, BFT also has been demonstrated encouraged results. Intensive BFT *Oreochromis niloticus* tilapia culture could produce an equivalent of 155 ton/ha/crop [11]. Besides high yields, decrease of FCR and decreased of protein content in diets have also been observed. In [30] was estimated that feed utilization by tilapia is higher in BFT with a ration 20% less than conventional water exchange system. Studying the effect of BFT in juveniles tilapia, the reference [33] showed no difference in fish growth/production between 35% and 24% CP fed tanks under BFT, but both were higher than clear-water control without biofloc with 35% CP. Moreover, in [7] was investigated the effectiveness of BFT for maintaining good water quality in over-wintering ponds for tilapia. The authors concluded that BFT emerge as an alternative to overcome over-wintering problems, particularly mass mortality of fish due to low temperatures. In the study [14] was observed that biofloc consumed by fish (tilapia) may represent a very significant feed source, constituting about 50% of the regular feed ration of fish (assuming daily feeding of 2% body weight).

In *M. rosenbergii* larviculture was evaluated the effect of different carbon sources in a BFT culture conditions [39]. The authors found that using glucose or a combination of glycerol plus *Bacillus* as a carbon source in bioreactors led to higher biofloc protein content, higher n-6 fatty acids, which resulted in improved survival rates. In a study with a Brazilian endemic tropical fish species tambaqui (*Colossoma macropomum*) was observed that BFT did not improve fish growth/production as compared to clear-water conditions [59], although some water quality problems in such study remained unsolved (i.e. turbidity and nitrite). The authors showed no differences in 44% CP fed tanks under BFT and clear-water conditions, as well as 28% CP BFT. Certainly further research is needed to clarify the effect of BFT in *Colossoma macropomum*. On the other hand, *Piaractus brachypomus* or pirapitinga seems to be a candidate species to BFT [60].

4.2. Breeding

The BFT has been successfully applied for grow-out, but little is known about biofloc benefits on breeding. For example, in the shrimp industry with the global spread of viruses, the use of closed-life cycle broodstock appeared as a priority to guarantee biosecurity, avoiding vertical transmissions. Moreover, such industry places a considerable interest on penaeid breeding program, often performed in closed facilities, controlling the production plan through successive generations. These programs were frequently associated with large animals, disease resistance as well as the enhancement of reproductive performance. However, nutritional problems remain unresolved [61] and alternatives should be evaluated.

As an alternative for continuous *in situ* nutrition during the whole life-cycle, breeders raised in BFT limited or zero water exchange system are nutritional benefited by the natural productivity (biofloc) available 24 hours per day. Biofloc in a form of rich-lipid-protein

source could be utilized for first stages of broodstock's gonads formation and ovary development. Furthermore, production of broodstock in BFT could be located in small areas close to hatchery facilities, preventing spread of diseases caused by shrimp transportation.

In conventional systems breeders used to be produced in large ponds at low density. However, risks associate with accumulation of organic matter, cyanobacteria blooms and fluctuations of some water quality parameters (such as temperature, DO, pH and N-compounds) remains high and could affect the shrimp health in outdoor facilities. Once the system is stable (sufficient particulate microbiota biomass measured in Imhoff cones), BFT provides stabilized parameters of water quality when performed in indoor facilities such as greenhouses, guaranteeing shrimp health.

According to studies performed with the blue shrimp *L. stylirostris* [18] and the pink shrimp *F. duorarum* broodstock [62], BFT could enhance spawning performance as compared to the conventional pond and tank-reared system, respectively (i.e. high number of eggs per spawn and high spawning activity; Fig 4). Such superior performance might be caused by better control of water quality parameters and continuous availability of food (biofloc) in a form of fatty acids protected against oxidation, vitamins, phospholipids and highly diverse "native protein", rather than conventional systems which "young" breeders are often limited to pelletized feed. These nutrients are required to early gonad formation in young breeders and subsequent ovary development. The continuous availability of nutrients could promote high nutrient storage in hepatopancreas, transferred to hemolymph and directed to ovary, resulting in a better sexual tissue formation and reproduction activity [18].

Regarding to shrimp broodstock management, one of the most important management procedures is related to control of solids and stocking density. High levels of solids negatively affect shrimp health, particularly with shrimp weight higher than 15g [47]. Settling solids or "biofloc volume" should be managed below than 15mL/L (measured in Imhoff cones) [38, 47]. Excess of particulate organic matter covered breeder's gills and could limit oxygen exchange, might resulting in mortalities.

Stocking density has to be carefully managed, mainly in sub-adult/adult phase (i.e. >15g). High density or high biomass will lead to an increase in organic matter, TSS levels and N-compounds in tanks or in ponds [63]. Moreover, physical body damages are prevented at low density, improving breeder's health. For review, a suggested stocking density is well described in [64].

For fish, no literature is available regarding BFT and application in breeders. The same trend observed in penaeid shrimp might be observed in fish. The continuous consumption of diverse microbiota (biofloc) should improve nutrients transfer, gonad formation and reproduction performance in fish. Lipid is a well-known nutrient that plays a key role in reproduction of aquatic species. In tilapia, breeders fed with crude palm oil based-feed (n-6 fatty acid rich source) presented high concentration of acid arachidonic or "ARA" (C20:4 n-6) in gonads, eggs and larvae of tilapia as compared to fish oil or linseed oil-based feeds [65]. As a result, better reproductive performance was observed in terms of higher total number of eggs per fish, larger gonad sizes, shorter latency period, inter spawning interval and

higher spawning frequency. ARA is an essential fatty acid crucial in reproduction, acting as hormone precursor [66]. In the study [33] was found high ARA content in biofloc harvested in tilapia culture freshwater tanks. Bioflocs in freshwater bioreactors contained high ARA content using glucose and glycerol as a carbon source [67]. These findings suggested that biofloc (according its nutritional profile, for review see section 5.0) might positively influence the reproductive performance in fish, supplying nutrients for gonad development, possibly also enhancing larval quality *a posteriori.* BFT in tilapia broodstock could be an effective method to increase tilapia fry production and further research is need in this field.

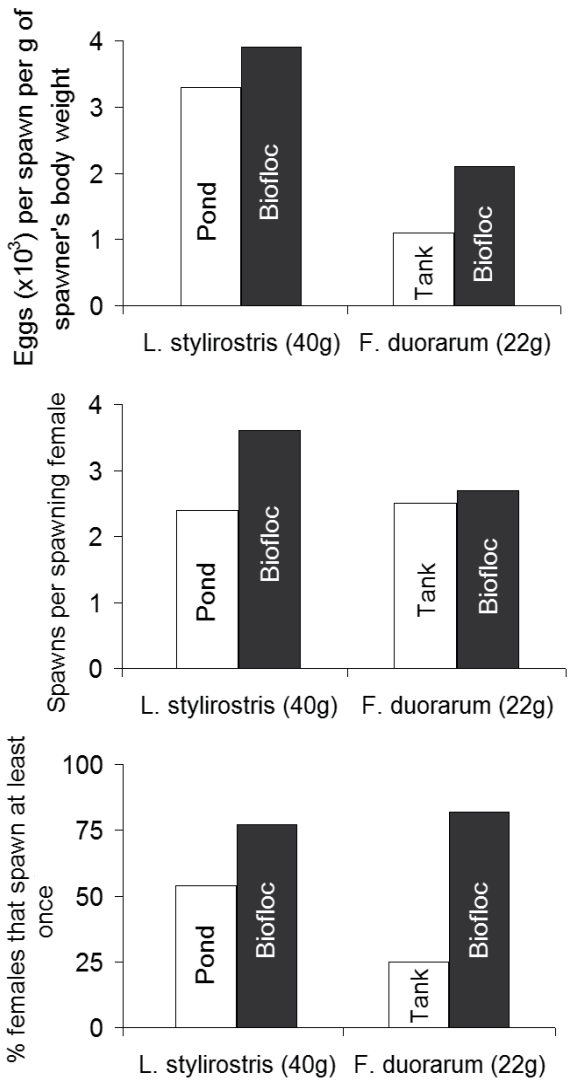


Figure 4. Spawning performance of *F. duorarum* (tank-reared vs biofloc) and *L. stylirostris* (pond-reared vs biofloc) performed in 45 and 30 days after ablation, respectively. Mean weights in parenthesis (more details in [18] and [62]).

4.3. The "natural probiotic" effect of biofloc

Biofloc can be a novel strategy for disease management in contrast to conventional approaches such as antibiotic, antifungal, probiotic and prebiotic application. The "natural probiotic" effect in BFT could act internally and/or externally against, i.e., to *Vibrio sp.* and ectoparasites, respectively. This effect is promoted by large groups of microorganisms, but mainly bacteria that is considered the first trophic level in the system.

Internally, bacteria and its synthesized compounds could act similar to organic acids and might be effective bio-control agents, also given beneficial host's microbial balance in the gut [68]. The regular addition of carbon in the water is known to select for polyhydroxyalkanoates (PHA) accumulating bacteria and other groups of bacteria that synthesize PHA granules. The microbial storage product poly-ß-hydroxybutyrate (PHB), a biodegradable polymer belonging to the polyesters class, is only one compound of a whole family of polyhydroxyalkanoates. PHB is produced by a widely variety of microorganisms such as *Bacillus sp., Alcaligenes sp., Pseudomonas sp.* from soluble organic carbon and is also involved in bacterial carbon metabolism and energy storage [68]. This polymer could comprise ~80% of the bacteria's cell dry matter and up to 16% on biofloc dry weight [69]. Different carbon sources or structures of carbon substrate will result in varying types of PHA [69].

Such granules are synthesized under conditions of physiological and nutrient stress, i.e., when an essential nutrient like nitrogen is limited in the presence of an excess carbon source [68]. When these polymers are degraded in the gut, they could have antibacterial activity similar to short chain fatty acids (SCFAs) or organic acids. The breakdown of PHA inside the gastrointestinal tract can be carried out via chemical and enzymatic hydrolysis [70].

Chemical hydrolysis can be carried out by treating the polymers with, i.e., NaOH, in which could significantly accelerate its digestibility [70]. On the other hand, enzyme hydrolysis is generally carried out by extracellular depolymerases activities which are widely distributed among bacteria and fungi, acting as a preventive or curative protector against *Vibrio sp.* infections and stimulate growth and survival of shrimp and fish larvae [69].

The working mechanism of PHAs with respect to their antibacterial activity is not well understood [68]. As they could act similarly to SCFA, some studies speculated the working mechanism by (i) reduction of pH, in which antibacterial activity increases with decreasing pH value [71]; (ii) inhibiting the growth of pathogenic bacteria by interference on cell membrane structure and membrane permeability, as well as instability of internal protons balance, lowering ATP and depletion of cellular energy [72]; and (iii) down-regulate virulence factor expression and positively influence the gut health of animals [73]. Further research is need to maximizing PHA content in bioflocs applied, i.e., for fish/shrimp feed, characterizing and analyzing their bio-control efficacy in different host-microbe systems [68].

Externally, the working mechanism of biofloc microorganisms against pathogens seems to be by competition of space, substrate and nutrients. Some essentials nutrients such as

nitrogen are required by both groups (i.e. heterotrophic bacteria *vs Vibrio sp.*) limiting their growth. Inhibiting compounds excreted by BFT microorganisms, light intensity and type of carbon source also could reduce pathogens growth. Unfortunately, limited information is available on this field. In a study with fish fingerlings [74] was reported that tilapia (initial weight 0.98 ± 0.1g) reared under BFT limited water-exchange condition (FLOC) presented less ectoparasites in gills and ectoderm's mucous as compared to conventional water-exchange system (CW) after 60 days (Fig 5).

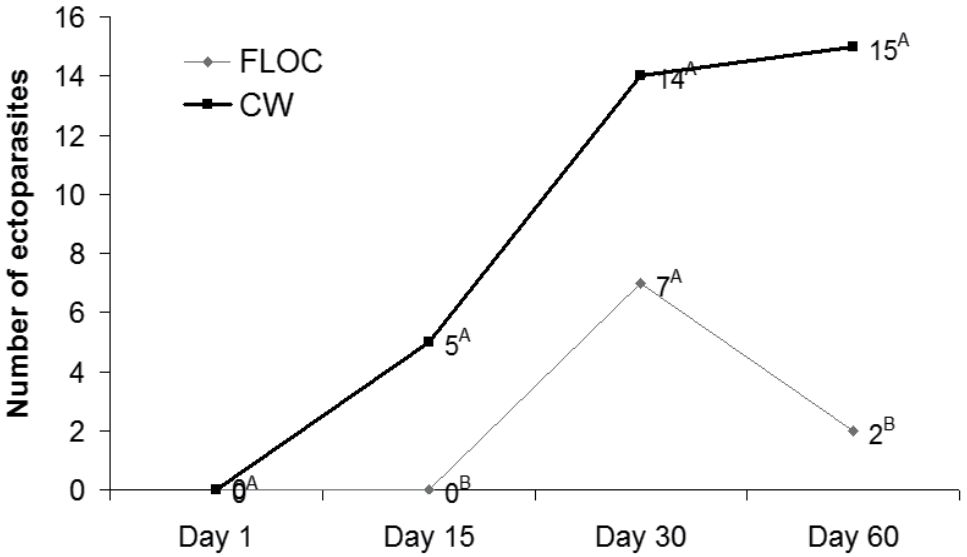


Figure 5. Number of total ectoparasites in gills and ectoderm's mucous of fry tilapia reared under BFT limited water-exchange condition (FLOC) and conventional water-exchange system (CW) after 60 days (more details in [74])

4.4. Aquaponics

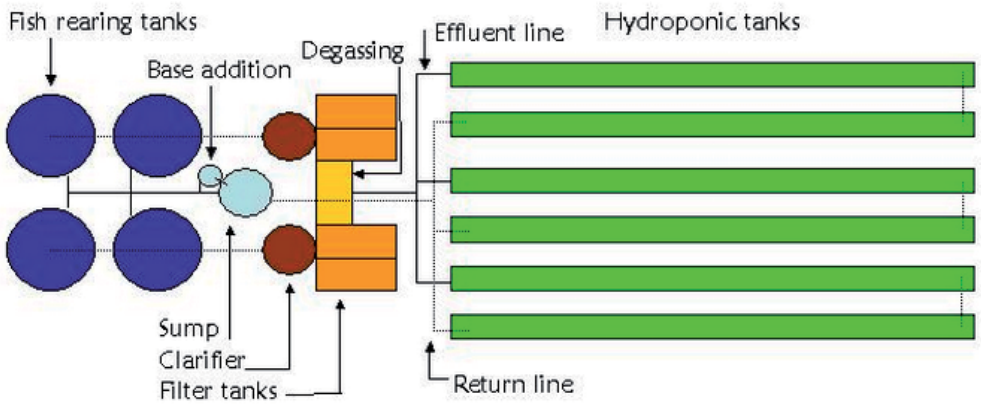
Aquaponics is a sustainable food production system that combines a traditional aquaculture with hydroponics in a symbiotic environment. The water is efficiently recirculated and reused for maximum benefits through natural biological filtration and recirculation. The waste that is excreted by aquatic species or uneaten feed is naturally converted into nitrate and other beneficial nutrients in the water. Those nutrients are then absorbed by the vegetables and fruits in a "natural fertilization way".

Aquaculture species including fish, crayfish, freshwater prawns or shrimp are usually reared in tanks and the water directed into separated race-ways of hydroponics vegetables. A worldwide well-known aquaponics system was successfully developed by University of Virgin Islands (Fig 6). Typical plants raised in aquaponics include lettuce, chard, tomato, fruits such as passion fruit, strawberry, water melon, etc.; and a large variety of spices. Size of aquaculture tanks varies according aquatic species/vegetables demand and usual shapes includes round, square or rectangular tanks.

(Source: UVI website www.uvi.edu)

Figure 6. Aquaponics system at University of Virgin Islands

Nowadays, BFT have been successfully applied in aquaponics. The presence of rich-biota (microorganisms of biofloc) and a variety of nutrients such as micro and macronutrients originated from un-eaten or non-digested feed seems to contribute in plant nutrition. A well-known example of biofloc and aquaponics interaction was also developed by UVI. However, the application of BFT in aquaponics needs particular attention, mainly on management of solid levels in water (for review, see [28]). High concentration of solids may cause excessive adhesion of microorganism on plants roots (biofilm), causing its damage, lowering oxygenation and poor growth. Filtering and settling devices are often needed (Fig 7).



(Source: UVI website www.uvi.edu)

Figure 7. Scheme of worldwide well-known UVI Aquaponics System

5. Microbial biomass application in animal food industry

The cost of diets in several animal cultures is predominantly due to the cost of protein component [75]. In the case of aquaculture, its massive expansion in the last decades has begun to face some important limitations like increasing prices of fishmeal, a raw material prime component of aquaculture diets. However, pressure caused in natural stocks (over-fishing) has depleted fishmeal production and, as a consequence, continuous increase in prices has been observed [76]. Moreover, growth of aquafeed industry (driven by an increase in fish/shrimp demand as the global population continues to growth), the competition with other animal cultures (such as swine and poultry) and differences in fishmeal quality also collaborated with increase in prices of fishmeal. The quality attributed to fishmeal includes high palatability, high content of digestible protein, highly unsaturated fatty acids (HUFA) and minerals.

In this context, alternatives should be evaluated opposing this non-optimism scenario. Aquaculture industry needs to investigate alternative source of proteins to replace less sustainable ones. Candidates of protein sources might have good digestibility, palatability, energy content, low ash content and present a well-balanced essential amino acids profile (EAA) [77].

In the past years, BFT has been emerged not only as promising alternative to grow-out system, but also as a method to obtain protein for compounds diets originated from its diverse microbiota. Collected in tanks/ponds [46, 62] or produced in bioreactors [17, 39, 67] biofloc (Fig 8) is a raw material to produce "biofloc meal". In bioreactors, biofloc production can clean up effluent waters from aquaculture facilities, converting dissolved nutrients into single-cell protein [78]. Usually, two types of bioreactors have been employed: sequencing batch reactors (SBRs) and membrane batch reactors (MBRs), both controlling ammonia, nitrite and suspended solids with great efficacy (for review of bioreactors and its employ, see Kuhn et al 2012). Moreover, excess of solids removed from culture tanks or ponds and/or concentrated into solid removal devices [28] could also be a recyclable source of biofloc for biofloc meal production. This sustainable approach of protein source is getting more attention in the aquaculture industry. The microbial particles can provide important nutrients such as protein [33, 46], lipids [10, 37], aminoacids [80] and fatty acids [33, 67, 81].

Biofloc meal (also called "single-celled" protein), added to compounded feed is currently focus of intensive research in nutrition fields [17, 78]. However, to produce this protein ingredient some processes are required such as drying, milling and storage. In this context, nutritional characteristics could be affected (by i.e. temperature during drying), which the "native" properties could be altered.

Nutritional composition of biofloc differs according to environmental condition, carbon source applied, TSS level, salinity, stocking density, light intensity, phytoplankton and bacteria communities and ratio, etc. Regarding to age of bioflocs, in "young" biofloc heterotrophic bacteria is mainly presented as compared to "old" biofloc dominated by fungi [79]. In biofloc particles, protein, lipid and ash content could vary substantially (12 to 49, 0.5 to 12.5 and 13 to 46%, respectively; Table 2). The same trend occurs with fatty acids (FA)

profile. Essential FA such as linoleic acid (C18:2 n-6 or LA), linolenic acid (C18:3 n-3 or ALA), arachidonic acid (C20:4 n-6 or ARA), eicosapentanoic acid (C20:5 n-3 or EPA) and docosahexaenoic acid (C22:6 n-3 or DHA), as well as sum of n-3 and sum of n-6 differ considerably between 1.5 to 28.2, 0.04 to 3.3, 0.06 to 3.55, 0.05 to 0.5, 0.05 to 0.77, 0.4 to 4.4 and 2.0 to 27.0% of total FA. Type of carbon source, freshwater or marine water and production of biofloc biomass (in bioreactors or culture tanks) definitely influence the FA profile (Table 3 and 4). Vitamin and amino acids profile from biofloc produced in large-scale commercial bioreactors [82] in given in Table 5.

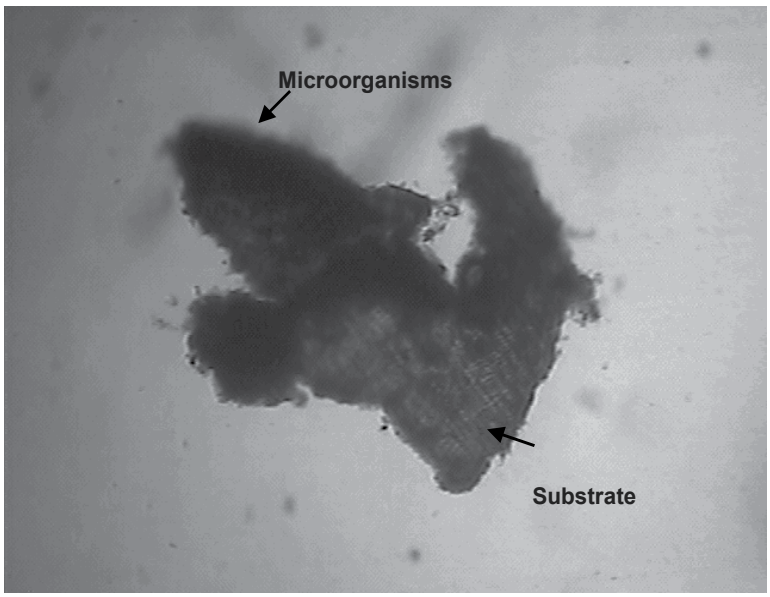


Figure 8. Biofloc particle (10x magnification) (Source: [54])

Information is still scarce about how microorganisms profile and its nutritional composition could impact animal growth. However, is already known that microorganisms in biofloc might partially replace protein content in shrimp diets, although were not always the case [10, 88]. Recent studies determined how reducing the protein content of diet would affect growth performance of shrimp reared in biofloc conditions. In the study [15] was found that at least 10% of protein content in pelletized feed can be reduced when *F. paulensis* postlarvae are raised in BFT conditions. In [89] was observed that shrimp fed with less than 25% crude protein under biofloc conditions performed similarly to shrimp raised under regular clear-water intensive culture with a 37%-protein diet. The biofloc system also delivered more consistent survival rates, especially at higher density. A low-protein biofloc meal-based pellet (25% CP) was evaluated as a replacement of conventional high-protein fishmeal diet (40% CP) for *L. vannamei* in a relatively low temperature (25°C) under biofloc conditions [35]. The results showed that is possible to replace 1/3 part of a conventional diet by alternative low-protein biofloc meal pellet without interfering survival and shrimp performance.

Crude protein (%)	Carbohydrates (%)	Lipids (%)	Crude fiber (%)	Ash (%)	Reference
43.0	-	12.5	-	26.5	[27]
31.2	-	2.6	-	28.2	[83]
12.0 - 42.0	-	2.0 - 8.0	-	22.0 - 46.0	[84]
31.1	23.6	0.5	-	44.8	[10]
26.0 - 41.9	-	1.2 - 2.3	-	18.3 - 40.7	[80]
30.4	-	1.9	12.4*	38.9	[85]
49.0	36.4	1.13	12.6	13.4	[17]
38.8	25.3	<0.1	16.2	24.7	[78]
28.8 - 43.1	-	2.1 - 3.6	8.7 - 10.4	22.1 - 42.9	[86]
30.4	29.1	0.5	0.8	39.2	[37]
18.2-29.3	22.8-29.9	0.4-0.7	1.5-3.5	43.7-51.8	[47]
18.4-26.3	20.2-35.7	0.3-0.7	2.1-3.4	34.5-41.5	[87]
28.0-30.4	18.1-22.7	0.5-0.6	3.1-3.2	35.8-39.6	[62]

*Lignin+cellulose

Table 2. Proximate analysis of biofloc particles in different studies.

Also, recent studies have been demonstrated that fishmeal in shrimp diets can be partially replaced by other protein sources under biofloc conditions or by biofloc meal. In [90] was evaluated two fishmeal replacement levels (40 and 100% of replacement) by other ingredients (soyabean meal and viscera meals) in diets for *Litopenaeus vannamei* reared in a biofloc system. The authors observed that fishmeal can be replaced in a level of 40.0% without interfering on growth performance and water quality. On the other hand, incorporating treated solids (microbial flocs) generated from tilapia effluent into shrimp feed, [91] demonstrated that shrimp performance was significantly increased as compared to untreated solids (settling basins of tilapia culture units). In [92] a trial performed in clear-water conditions detected that fishmeal can be completely replaced with soy protein concentrate and biofloc meal (obtained from super-intensive shrimp farm effluent) in 38% CP diets without adverse effects on *L. vannamei* performance. Moreover, [17] observed that biofloc produced in SBRs bioreactors using tilapia effluent and sugar as a growth media could offer an alternative protein source to shrimp feeds. Microbial floc-based diets significantly outperformed control fishmeal-based diets in terms of weight gain per week with no differences in survival.

Regarding to biofloc meal production, one bottleneck seems to be the large amount of wet biofloc biomass required to produce 1kg of dry biofloc meal. Estimative indicates that biofloc plug in 1L settling cones contained only 1.4% of dry matter [14]. The reference [17] indicated that 1 kg of microbial floc could be produced per 1.49 kg of sucrose in bioreactors. Certainly more research is needed on this field. On the other hand, other applications of

biofloc meal in animal industry should be evaluated, mainly considering its nutritional profile and relatively low costs as compared to other protein sources (i.e. fishmeal) [17]. In aquaculture, biofloc meal could be included into broodstock pelletized feed, prior or after eyestalk ablation. Further research is encouraged in this field.

Fatty Acid	**% of total fatty acid**							
C14:0	0.10	0.60	0.80	0.45	1.43	0.69	0.61	0.43
C15:0	0.15	0.25	0.25	0.30	0.31	0.31	0.17	0.26
C16:0	2.2	17.0	26.0	15.0	6.06	8.01	6.34	8.86
C16:1	4.0	3.7	3.0	5.0	6.61	2.61	1.61	1.54
C17:0	0.05	0.4	0.5	0.2	0.20	0.23	0.14	0.68
C18:0	0.5	4.0	7.1	6.0	2.37	4.82	3.94	6.27
C18:1 n-7	1.5	3.0	1.9	2.7	3.96	1.72	2.71	4.19
C18:1 n-9	1.8	19.0	30.0	18.0	3.34	7.26	8.12	12.05
C18:2 n-6 (LA)	5.0	19.0	28.2	11.0	1.91	17.24	11.95	21.87
C18:3 n-3 (ALA)	0.04	0.5	0.45	2.0	0.23	0.99	0.20	0.21
C20:0	-	0.10	0.20	0.20	0.06	0.34	0.33	0.49
C20:1 n-9	0.05	0.10	0.15	0.10	0.25	0.20	0.06	0.02
C20:3 n-6	0.15	0.10	0.06	0.07	0.55	0.36	0.15	0.04
C20:4 n-6 (ARA)	0.7	0.3	0.15	0.20	0.77	0.87	0.17	0.06
C20:5 n-3 (EPA)	0.10	0.11	0.05	0.25	0.15	0.15	0.19	0.12
C22:6 n-3 (DHA)	0.05	-	0.07	0.05	0.18	0.06	0.18	0.10
∑ Saturated	22.08	22.99	35.35	22.45	10.76	14.85	11.53	16.99
∑ Monounsaturated	8.16	26.22	35.45	27.15	16.51	14.21	12.5	17.8
∑ n-3	0.4	0.6	0.7	0.65	1.04	2.02	0.60	0.43
∑ n-6	7.0	20.0	27.0	12.0	4.03	19.03	12.27	21.97
Type of water	freshwater	freshwater	freshwater	freshwater	freshwater	freshwater	marine	marine
Carbon source	Acetate	Glycerol	(Glycerol+ *Bacillus*)	Glucose	Glucose	Glycerol	Glucose	Glycerol
Collection	bioreactors	bioreactors	bioreactors	bioreactors	bioreactors	bioreactors	bioreactors	bioreactors
Reference	[39]				[67]			

Table 3. Fatty acid profile of biofloc (produced in experimental bioreactors) using different carbon source in marine water and freshwater

Fatty Acid	% of total fatty acid		
C14:0	2.02-2.48	13.8-16.1	5.4-6.2
C15:0	0.70-0.77	1.1-1.5	1.1-1.3
C16:0	17.88-19.10	45.4-53.5	48.7-49.3
C16:1	7.15-7.74	9.9-15.3	16.5-21.6
C17:0	-	0.7	0.9-1.0
C18:0	6.24-7.27	3.4-3.5	3.7-4.5
C18:1 n-7	11.05-11.28	-	-
C18:1 n-9	8.51-10.08	8.8-9.2	7.7-10.8
C18:2 n-6 9 (LA)	15.38-16.68	1.5-2.5	2.2-2.6
C18:3 n-3 (ALA)	0.65-0.73	2.0-2.3	2.2-3.3
C20:0	0.87-1.44	0.2-0.4	0.4
C20:1 n-9	0.74-0.80	0.3-0.4	0.5
C20:3 n-6	0.40-0.46	0.2	0.2
C20:4 n-6 (ARA)	3.11-3.55	0.3-0.4	0.3-0.4
C20:5 n-3 (EPA)	0.39-0.46	0.3-0.5	0.5
C22:6 n-3 (DHA)	0.74-0.77	0.2-0.4	0.3-0.4
∑ Saturated	30.2-34.92	67.6-73.0	61.5-61.9
∑ Monounsaturated	28.10-29-38	19.7-25.0	28.3-30.5
∑ n-3	1.38-1.91	2.8-3.4	3.2-4.4
∑ n-6	23.5-25.81	2.0-3.0	2.7-3.1
Type of water	freshwater	marine	marine
Carbon source	Wheat flour	molasses	molasses
Collection	Tilapia tanks	shrimp tanks	shrimp tanks
Reference	[33]	[87]	[62]

Table 4. Fatty acid profile of biofloc (collected in tanks) using different carbon source in marine water and freshwater

Amino Acids	As Fed (%)
Alanine	3.82
Arginine	3.60
Aspartic acid	6.36
Glutamic acid	8.04
Glycine	2.81
Histidine	1.46
Isoleucine	3.38
Leucine	5.06
Lysine	4.34
Methionine	1.41
Cysteine	0.55
Phenylalanine	3.29
Proline	2.77
Serine	2.82
Taurine	0.25
Threonine	3.11
Tryptophan	0.98
Tyrosine	2.83
Valine	3.52
Total	**60.4**
Vitamins	
Niacin	83.3 mg/kg
Thiamine B1	7.7 mg/kg
Riboflavin	39.0 mg/kg
Vitamin B12	12.0 mg/kg
Vitamin E	29.8 IU/kg

Table 5. Example of vitamin and amino acids profile from biofloc produced in large-scale commercial bioreactors [82].

6. Conclusions and perspectives of BFT

Biosecurity is a priority in aquaculture industry. For example, in shrimp farming, considerable impact of disease outbreaks during the past two decades greatly affected the operational management of shrimp farms worldwide [10]. Infected PLs and incoming water seem to be the main pathway for pathogen introduction. This scenario forced farmers to look for more biosecure culture practices to minimize the risk associated with exposure to pathogens [2]. Biofloc technology brings an obvious advantage of minimizing consumption and release of water, recycling *in situ* nutrients and organic matter. Furthermore, pathogens introduction is reduced, improving the farm biosecurity.

Biofloc technology will enable aquaculture grow towards an environmental friendly approach. Consumption of microorganisms in BFT reduces FCR and consequently costs in feed. Also, microbial community is able to rapidly utilize dissolved nitrogen leached from shrimp faeces and uneaten food and convert it into microbial protein. These qualities make minimal-exchange BFT system an alternative to extensive aquaculture. Microorganisms in biofloc might partially replace protein content in diets or decrease its dependence of fishmeal.

Related to biofloc meal and its perspectives, the study [17] detected initial estimates of cost for producing a metric ton of biofloc meal is approximately $400 to $1000. The same authors cited that global soymeal market varied approximately from $375 to $550/metric ton from January 2008 through May 2009. During the same time period, fishmeal varied approximately from $1000 to $1225, suggesting feasibility on replacement of either soybean and/or fish meal by biofloc meal. Moreover, generated from a process that cleans aquaculture effluents [17, 39] biofloc meal production avoids discharge of waste water and excessive damage to natural habitats [4]. This ingredient seems to be free of deleterious levels of mycotoxins, antinutritional factors and other constituents that limit its use in aquafeeds [79]. Large-scale production of biofloc meal for use in aquaculture could result in environmental benefits to marine and coastal ecosystems, as the need for wild fish as an aquafeed ingredient is reduced [79, 92].

Sensorial quality of BFT products is also an important issue. BFT may bring higher profit if fresh non-frozen shrimp/fish is sold to near-by market, mainly at inland locations. These advantages certainly should be more explored and niche markets achieved, contributing to social sustainability.

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Chapter 13

Phytoplankton Biomass Impact on the Lake Water Quality

Ozden Fakioglu

Additional information is available at the end of the chapter

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1. Introduction

Pyhtoplankton is a plant plankton which cannot move actively and changes location depending on the movement of water. Phytoplankton communities are widely spreaded from aquatic to terresial lands. Plankton form the first ring of food chain in aquatic environment effecting the efficiency of this environment. Daily, seasonally and yearly changes are important for calculating efficiency in aquatic fields. Phytoplankton composition is a trophic indication of the water mass. In addition, phytoplankton species are used as an indicator for determining the nutrient level which is the basis for preparing and monitoring the strategies of the lake management in the lakes. Using phytoplankton communities or other aquatic organisms for evaluating water quality is based on very ancient times. Saprobik and trophic inducator types are used in many researches [1-3]. In addition, various numerical indices have been developed [1-2]. However, none of them have been accepted extensively. This is caused by several reasons. Those reasons are:

a. Differences in phytoplankton groups and group concept
b. Dynamic properties of phytoplankton groups
c. Habitat diversity of freshwater ecosystems
d. Phytogeographical differences [4].

Phytoplankton communities are influenced by significant changes every year. The competitive environment known as seasonal succession has been changing [5]. If the conditions don't change, this process results in the choice of communities dominated by one or more species. Phytoplankton responds rapidly to the condition chages. Conditional changes will result the formation of high compositional diversity [6].

The first approaches to classify algal communities don't have wide range of application in determining water quality. Pankin's approaches to classify algal communities in 1941 and 1945 weren't generally accepted [4]. Reynold's [7-8] applied a classic phytosociological

approach to phytoplankton data obtained from the lakes in northeast england and classified them into various communities. Sommer [5] found high similarities among the compositions of species and seasonal succession of Alpine lakes. Mason [9] reported that oligotrophic and eutrophic lakes have the communities of characteristic phytoplankton.

There are qualitative differences among phytoplankton communities in oligotrophic and eutrophic lakes. The compositon and the amoung of phytoplankton communities are affected by environmental conditions. For example, a numerical decrease is observed in *Anabeana* and *Aphanizomenon* species from heterosis blue-green algae found in mesotrophic lake layers with a decrease of nitrogen saturation in the lakes. In addition, there are differences among the environmental conditions preferred by *Diatoms*, *Dinoflagellate* or *Cosmarium*, *Pandorina* and *Gemellicystis* species, even though they can be found in the lakes with same level of nutrients [10].

In the works related with phytoplankton communities used for predicting the ecological structure of aquatic systems, it has been tried to develop functional groups by improving the systematic investigation. Furthermore, some indices was developed according to numerical and biovolume values of phytoplankton (Palmer Index (1969), Descy Index (1979), TDI Index (1995) etc.). HPLC pigment analysis is used for the diagnosis of phytoplankton species in recent years [11].

The methods we will mention for examination of plankton in aquatic environments are summarized by compiling researchers' methods and techniques. The main target of these suggested methods is to obtain values close to the actual volume and weight of plankton in freshwater and to calculate the volume and weight (biomass) of these organisms by this method.

Besides, the methods and techniques in this subject are sensitive, determining ecological parameters which are characterizing the aquatic environment are important for the researches. Sampling error in this review may cause errors far beyond the susceptibility of calculations. In addition, vertically and horizontally distributions of plankton may show big changes against the effects of wind and light. For this reason, evaluating various samples collected as verticaly and horizontaly causes to get more reliable results from each sample.

Many techniques have been developed depending on the number, volume and cell structures of fresh water phytoplankton. In this section, studies conducted by calculating biovolume of phytoplankton used for estimating ecological characteristics of freshwater ecosystems will be summarized.

2. Changes in biomass of phytoplankton in lakes

The composition and biomass of phytoplankton are very important parameters for understanding the structure and tropic level of aquatic systems. Phytoplankton cell size, carbon content and functional structure are investigated by many researchers. Phytoplankton communities can have cell size from a few microns to a few milimeters depending on the groups they belong to. Biovolume measurements are estimated by

automatic or semi-automatic methods. For example, morphometric methods, holographic method etc. In addition to these, the commonly used one is geometric method [12]. In addition to these methods, the most common method for calculationg biomass is measuring chlorophyl *a* value. Chlorophyll *a* is an important photosynthetic pigment for plant organisms. Environmental factors affect the amount of ambient phytoplankton and chlorophyll *a* value changes depending on the amount of phytoplankton [13].

Coulter Counter method is based on measuring electrical conductivity among cells. Electric current flowing through the cells placed in physiological saline varies depending on the cell size. Thus, the cell size is determined.

Morphometric methods is used in determinining the quantitative properties of cells. With this method, cell density is calculated depending on cell wall, chloroplast, vacuole, depot material and cytoplasmic content [14].

Holographic scanning tecnology which is used in conjunction with one curved mirror to passively correct focal plane position errors and spot size changes caused by the wavelength instability of laser diodies [15].

Geometric method is based on estimating biomass of phytoplankton via geometric shapes and mathematical equations. This model was found by Kovada and Larrance. 20 geometric models developed by Hillebrand *et al.* [16] are used for calculating biomass of algae. Each model was designed depending on cell structure with the shapes like sphere, cone, triangle etc. This method was applied to phytoplankton species found in sea waters in China and 31 geometric models were developed in this study [12].

Phytoplankton communities show vertical changes from time to time. Chlorophyll *a* is a pigment used for estimating biomass of phytoplankton. Seasonal changes cause variation in chlorophyll *a* value. For this reason, water affects the production of column light transmittance, hence, the value of chlorophyll *a* [18]. In determining chlorophyll *a*, fiber glass filter papers used for filtering water samples are waited for 3-4 hours then they are decomposed and kept in 10 ml 90% acetone one night, centrifuged and optical density of the extract is made by reading from spectrophotometer with 630, 645 and 665 nm wavelength [17].

The first step for calculating phytoplankton biomass is to store and protect the phytoplankton samples.

3. Storing and protecting phytoplankton samples

Before collecting phytoplankton samples from the lake, turbidity and temperature of the water were measured by Secchi disk. Phytoplankton samples have to be stored in 100-150 ml glass or polyethylene containers with 2% Lugol's solution or 4% Buffere formalin solution [32].

4. Identification of phytoplankton

Water samples taken by phytoplankton scoop were identified according to world literature [19-29].

5. Phytoplankton caunting

Water samples put into hydrobios plankton counting chambers depending on phytoplankton density, after standing overnight by dropping lugol's solution, counting phytoplankton were made by using inverted microscope [30-31].

Following formula was used to calculate the number of phytoplankton [31]:

$$\text{Number of phytoplankton (piece/ml)} = \frac{C \times TA}{F \times A \times V}$$

Here;

C= The number of organisms found by counting (number),
TA= Bottom area of the cell count (mm^2),
F= Counted field number (number),
A= Field of view of the microscope (mm^2),
V= Volume of precipitated sample (ml).

6. Estimation of phytoplankton biomass

Phytoplankton analysis is possible by a simple Kolkwitz chamber. Except deposited plankton in sample bottle, liquid at the top pour a few millilitres and centrifuged and their volumes are measured in sedimentation tubes then they are transferred to Kolkwitz chamber for analysis. In this method, phytoplankton analysis is the first, semi-field analysis of Kolkwitz chamber is the second and the third one is the analysis of the various fields.

Biovolume was estimated in the measurement of phytoplankton biomass. Phytoplankton were emulated to the geometric shapes like sphere, cylinder and cone and necessary measurements were taken from the phytoplankton while counting [32]. Geometric shapes and calculations used for calculating biovolume was done according to the formulas (Table 1) stated by [12] and [16]. After calculating average volume of every species, total volume were calculated by multiplying with the number of species. Following formula was used to calculate total cell volume of phytoplankton [31];

$$HH = \sum_{i=1}^{n} (HNixSHi)$$

Here;

HH= Total biovolume of plankton (mm/l),
HNi= the number of organisms belongs to i. species /l,
SHi= Average cell volume of i. species.

Biovolume is calculated by assuming cell volume is equal to 1mg age weight/m^3 algal biovolume for 1mm/ m^3 [33].

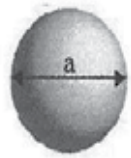
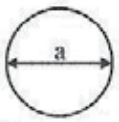
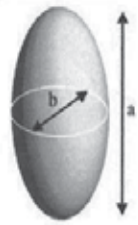
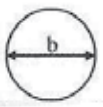
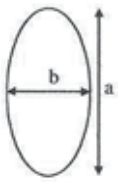
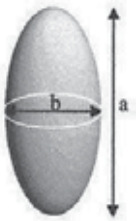
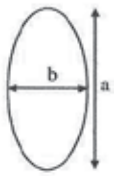
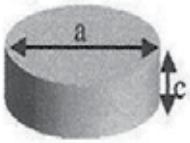
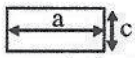
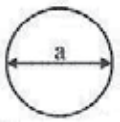
Shape	Biovolume	Samples
 	$V = \frac{\pi}{6} \cdot a^3$	*Crucigeniella apiculata* *Gomphosphaeria* sp. *Anabeana* sp.
  	$V = \frac{\pi}{6} \cdot b^2 \cdot a$	*Coelastrum microporum* *Actinastrum hantzschii* *Dinobryon divergens* *Cryptomonas* sp. *Pandorina* sp.
  	$V = \frac{\pi}{6} \cdot a \cdot b \cdot c$	*Trachelomonas caudata* *Peridinium* sp. *Botryococcus braunii* *Cocconeis placentula* *Phacus tortus*
  	$V = \frac{\pi}{4} \cdot a^2 \cdot c$	*Cyclotella* sp. *Mougeotia* sp.

Table 1. Geometrical shapes and formulas for calculating biovolume (continued)

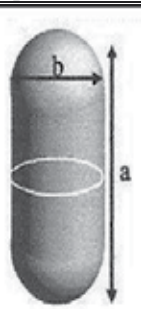
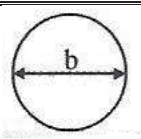
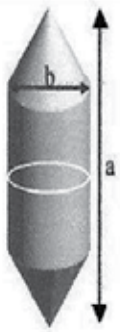
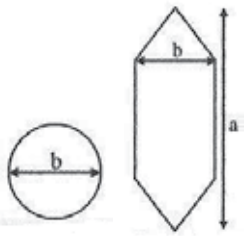
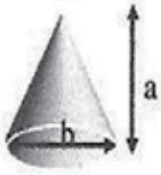
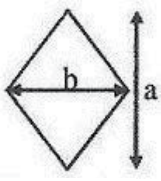
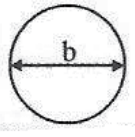
Shape	Biovolume	Samples
 	$V = \pi \cdot b^2 \cdot \left(\frac{a}{4} - \frac{b}{12}\right)$	*Stephanopyxis* sp.
 	$V = \frac{\pi}{4} \cdot b^2 \cdot \left(a - \frac{b}{3}\right)$	*Stephanopyxis*
 	$V = \frac{\pi}{12} \cdot a \cdot b^2$	*Monoraphidium contortum* *Actinastrum hantzschii*
 	$V = \frac{\pi}{12} \cdot a \cdot b^2$	*Spiraulax* sp.

Table 1. Geometrical shapes and formulas for calculating biovolume (continued)

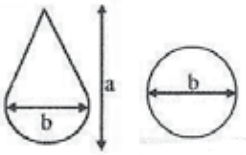
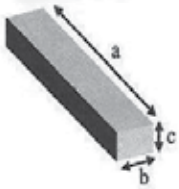
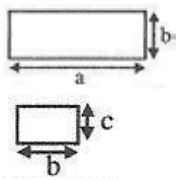
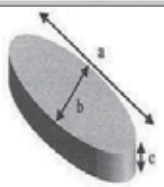
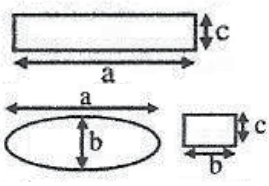
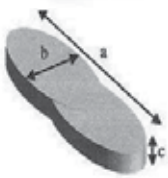
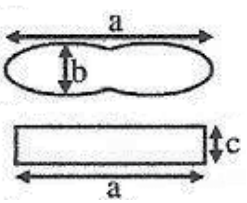
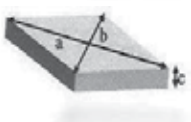
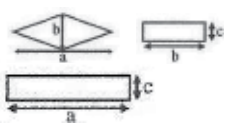
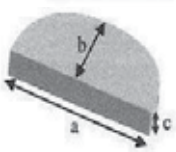
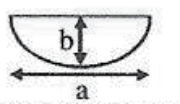
Shape	Biovolume	Samples
 	$V = \frac{\pi}{4} \cdot b^2 \cdot a$	*Chroomonas* sp.
 	$V = a \cdot b \cdot c$	*Asterionella* sp. *Synedra* sp. *Merismopedia* sp. *Epithemia zebra* var. *saxonica*
 	$V = \frac{\pi}{4} \cdot a \cdot b \cdot c$	*Pediastrum* sp. *Navicula* sp.
 	$V = \frac{\pi}{4} \cdot a \cdot b \cdot c$	*Cymatopleura* sp.
 	$V = \frac{1}{2} \cdot a \cdot b \cdot c$	*Nitzschia* sp.
 	$V = \frac{\pi}{4} \cdot a \cdot b \cdot c$	*Phaeodactylum* sp.

Table 1. Geometrical shapes and formulas for calculating biovolume (continued)

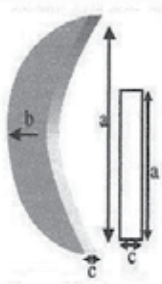
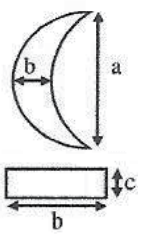
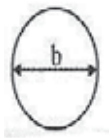
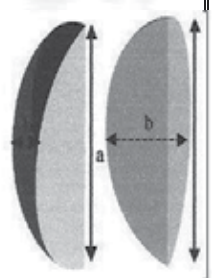
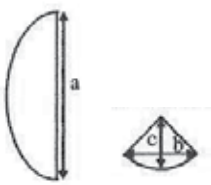
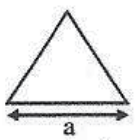
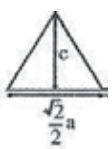
Shape	Biovolume	Samples
  	$V \cong \frac{\pi}{4} \cdot a \cdot b \cdot c$ $V \cong \frac{\pi}{6} \cdot a \cdot b^2$	*Monorophidium* sp. *Eunotia* sp.
 	$V = a \cdot c^2 \cdot a\sin\left(\frac{b}{2c}\right)$	*Cymbella* sp. *Amphora ovalis* *Epithemia* sp. *Rhopalodia gibba*
 	$V = \frac{\sqrt{3}}{4} \cdot c \cdot a^2$	*Hydrosera* sp.
 	$V = \frac{1}{6} \cdot a^2 \cdot c$	*Tetradinium*

Table 1. Geometrical shapes and formulas for calculating biovolume (continued)

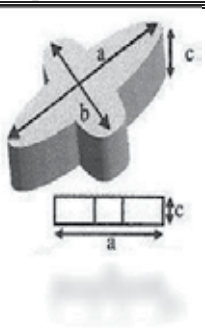
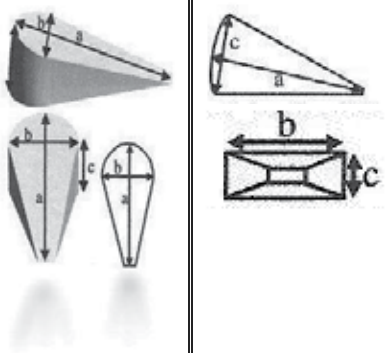
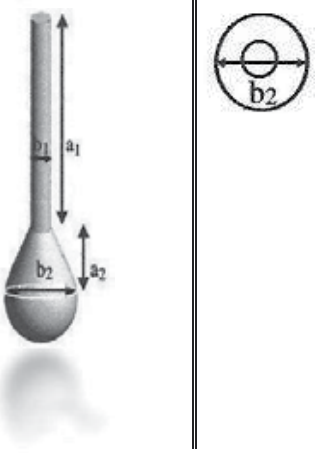
Shape	Biovolume	Samples
	$V \cong \frac{\pi}{4} \cdot a \cdot b \cdot c$	*Tabellaria* sp.
	$V \cong \frac{a \cdot b}{4} \cdot \left[a + \left(\frac{\pi}{4} - 1 \right) \cdot b \right] \cdot a \sin\left(\frac{c}{2a} \right)$	*Gomphonema constrictum*
	$V = \frac{\pi}{3} \cdot \left(a_1 + a_2 \right) \cdot b_1^2 + \frac{\pi}{4} \cdot \left(a_2 + b_2 \right) \cdot b_2^2 + \frac{\pi}{12} \cdot a_2 \cdot b_1 \cdot b_2$	*Euglena* sp.

Table 1. Geometrical shapes and formulas for calculating biovolume (continued)

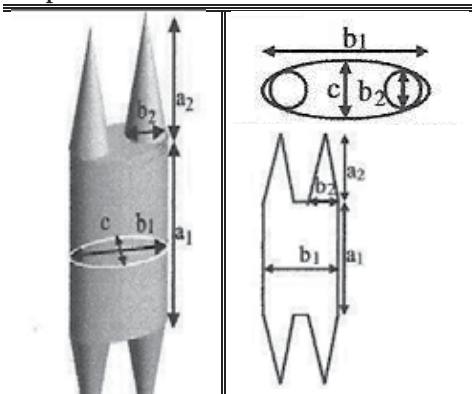
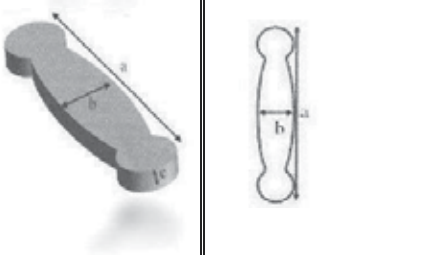
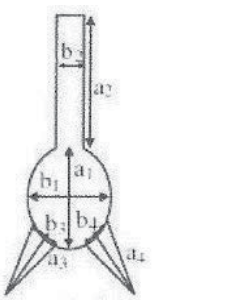
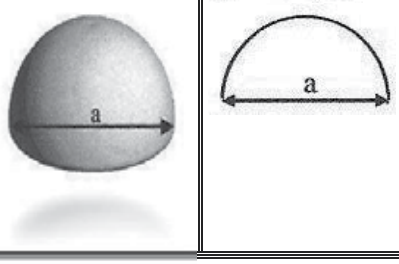
Shape	Biovolume	Samples
	$V = \frac{\pi}{4} \cdot a_1 \cdot b_1 \cdot c_1 + \frac{\pi}{3} \cdot a_2 \cdot b_2^2$	*Climacodium* sp.
	$V \cong \frac{\pi}{4} \cdot a \cdot b \cdot c$	*Caloneis* sp.
	$b_2 = b_3 = b_4$ $V = \frac{\pi}{4} \cdot a_2 \cdot b_2^2 \cdot + \frac{\pi}{12} \cdot$ $(a_3 + a_4) \cdot b_2^2 + \frac{\pi}{6} \cdot a_1 \cdot b_1 \cdot b_2$	*Staurastrum* sp.
	$V = \frac{\pi}{12} \cdot a^3$	*Cosmarium* sp.

Table 1. Geometrical shapes and formulas for calculating biovolume (continued)

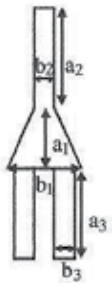
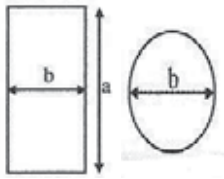
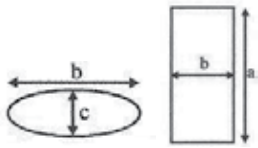
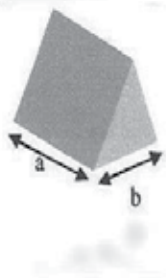
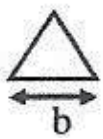
Shape	Biovolume	Samples
 	$V = \frac{\pi}{4} \cdot a_2 b_2^2 + \frac{\pi}{2} \cdot a_3 \cdot b_3^2 + \frac{\pi}{12} \cdot a_1 \cdot \left(b_1^2 + b_1 \cdot b_2 + b_2^2\right)$	*Ceratium hirundinella*
 	$V = \frac{\pi}{4} \cdot b^2 \cdot a$	*Pleurosira* sp.
 	$V = \frac{\pi}{4} \cdot a \cdot b \cdot c$	*Fragilaria crotonensis*
 	$V = \frac{\sqrt{3}}{4} \cdot a \cdot b^2$	*Ditylum* sp.

Table 1. Geometrical shapes and formulas for calculating biovolume (continued)

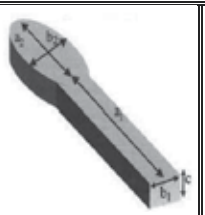
Shape	Biovolume	Samples
	 $V \cong c \cdot \left(a_1 \cdot b_1 + \frac{\pi}{4} \cdot a_2 \cdot b_2 \right)$	*Climacosphenia* sp.

Table 1. Geometrical shapes and formulas for calculating biovolume (continued)

7. Case studies conducted to phytoplankton biomass

There is a significant correlation between biomass of phytoplankton with the concentration of phosphorus. Changes are seen in phytoplankton biomass or production rate with the changes of the concentration of phosphorus in the lakes. It has been showed in the field studies that light and temperature play a significant role in the relationship between the extinction rate and biomass and carrying capacity of the composition of species [34].

In a study conducted in Lake Erie, samples have been collected from three different points in spring, summer and fall season for five years; 49 species were determined at the end of the study [35].

In a lake with 25000 km^2 surface area, phytoplankton biomass showed local changes and it was determined as 1.88 ± 0.12 g/m^3, 1.04 ± 0.07 g/m^3 and 0.63 ± 0.071 g/m^3 in west, mid and east part of the lake respectively. It was determined that algal biomass decreased and biomasses of *Aphanizomenon flos-aque*, *Stephanodiscus binderounus*, *S. niagarae*, *S. tenuis*, *Rhodomonas minuta* decreased in the rate of 70-98% from 1970 to 1983-1987 [35].

Phytoplankton communities and distributions were investigated from the samples taken weekly from two dam lake with different nutritient levels in Sicily. Lake Arancio is a shallow eutrophic lake and Lake Rosamarina is a deep mezotrofik lake. It was stated that the increment in the concentration of nutrients in Lake Arancio doesn't change the composition of phytoplankton but increase biomass of phytoplankton [36].

In a study examining 27 lakes in Russia; plankton biomass and total phosphorus concentration were investigated and it is stated that total phosphorus concentration changes between 10-137 mg/m^3 and biomass changes between 0.4-20 g/m^3. Total 160 phytoplankton species were identified and it was reported that most of those are belong to the blue-green algae and euglenophyceae classes. The lakes were determined to be hypertrophic and acidic [37].

In a study conducted with Lake Dorani, it was determined that the most common class in the lake was chlorophyceae followed by cyanophyceae. Total phytoplankton biomass is found similar to eutrophic lakes. While, nanoplankton biomass constitute 90% of total phytoplankton biomass in spring but it is 10% throughout the year. It is found that total biomass is high in summer, low in winter and changes between 0.43-30.30 mg/l [38].

Seasonal changes of phytoplankton communities in Lake Managua are investigated, it is reported that blue-green algae are dominant during the research period. Seasonal biomass are measured monthly for two years and the lowest phytoplankton biomass was found at the end of the rainy seasons (October, November). In short term studies (3-14 days), important changes in biomass were reported. Nutrient levels of the lake were estimated as hypertrophic according to chlorophyll *a* value (79 μg/l yearly average, 1987-1988) [39].

During the research conducted with Lake Beysehir, diatoms and green algae are found dominant. throughout the research *Aulacoseira granulata* and *Cyclotella meneghiniana* from centric diatom, *Asterionella formosa, Cocconeis placentula, Cymbella affinis* and *Ulnaria acus* from pennate diatoms, *Monoraphidium* spp., *Mougeotia* sp. and *Scenedesmus linearis* from Chlorophyta, *Dinobryon divergens* from Chrysophyta and *Cryptomonas marssonii, Rhodomonas lacustris* from Cryptophyta, *Merismopedia glauca* from Cyanophyta are commonly found and partly numerical increases are observed. Phytoplankton biomass in the lake changes between 0.40±0.11 and 6.43±1.00 mg/l. The lake is in mesotrophic nutrients level according to average phytoplankton biomass (1.98±0.2 mg/l) and it is in good ecological quality class [40].

8. Results and suggestions

When the comparison was made between geometrical and other models, geometrical model is used more. Trials for other models in computer environment still continue. Techniques used for calculating biomass have some advantages and disadvantages. For example, *Strombomonas gibberosa* is phytoplankton with complex shape. For this reason, some different opinions arise for choosing proper geometric shape for calculating biovolume.

Three problem stands out in the estimation of biomass.

1. The shapes of phytoplankton cells has irregular and complex structure which makes it hard to measure them under microscope
2. Cell dimensions changes in the study of dead cells. In addition, it makes hard to determine chloroplast and vacuollarin.
3. Physiological state of a cell (light, temperature, nutrient) may affect cell height and intracellular volume [14].

Calculating biomass is important for determining ecological status of aquatic ecosystems. There is a relationship between cell structure of phytoplankton communities and many Physico-chemical parameters. For this reason, physical and chemical changes of water have to be considered in biomass calculaions.

Since some species show physiological changes with the changes in environmental conditions, characteristics of phytoplankton groups should be well known. In some species in group of cyanobacteria, fringes observed depending on the increase of the value of nitrogen and phosphorus in the medium. This causes changes in cell dimensions. For this reason, it is suggested to support biomass usage for classifying freshwater ecological systems with physico-chemical parameters.

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Section 4

Novel Biomass Utilization

Chapter 14

Towards the Production of Second Generation Ethanol from Sugarcane Bagasse in Brazil

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Additional information is available at the end of the chapter

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1. Introduction

Brazil and the United States produce ethanol mainly from sugarcane and starch from corn and other grains, respectively, but neither resource are sufficient to make a major impact on world petroleum usage. The so-called first generation (1G) biofuel industry appears unsustainable in view of the potential stress that their production places on food commodities. On the other hand, second generation (2G) biofuels produced from cheaper and abundant plant biomass residues, has been viewed as one plausible solution to this problem [1]. Cellulose and hemicellulose fractions from lignocellulosic residues make up more than two-thirds of the typical biomass composition and their conversion into ethanol (or other chemicals) by an economical, environmental and feasible fermentation process would be possible due to the increasing power of modern biotechnology and (bio)-process engineering [2].

Brazil is the major sugar cane producer worldwide (ca. 600 million ton per year). After sugarcane milling for sucrose extraction, a lignocellulosic residue (sugarcane bagasse) is available at a proportion of ca. 125 kg of dried bagasse per ton of processed sugarcane. Therefore, sugarcane bagasse is a suitable feedstock for second generation ethanol coupled to the first generation plants already in operation, minimizing logistic and energetic costs.

State-owned energy group Petrobras is one of the Brazilian groups leading the development of second generation technologies, estimating that commercial production could begin by 2015. Other organizations making significant contributions to next generation biofuels in Brazil include the Brazilian Sugarcane Technology Centre (CTC), operating a pilot plant for the production of ethanol from bagasse in Piracicaba (Sao Paulo) [3]. Recently, GraalBio (Grupo Brasileiro Graal) has stated publically that will start production of bioethanol from sugarcane bagasse in one plant located at the Northeast region of the country.

Pretreatment, hydrolysis and fermentation can be performed by various approaches. According to a CTC protocol, the process of manufacturing ethanol from bagasse is divided into the following steps. First, the bagasse is pretreated via steam explosion (with or without a mild acid condition) to increase the enzyme accessibility to the cellulose and promoting the hemicellulose hydrolysis with a pentose stream. The lignin and cellulose solid fraction is subjected to cellulose hydrolysis, generating a hexose-rich stream (mainly composed of glucose, manose and galactose). The final solid residue (lignin and the remaining recalcitrant cellulose) is used for heating and steam generation. The hexose fraction is mixed with 1G cane molasses (as a source of minerals, vitamins and aminoacids) and fermented by regular *Saccharomyces cerevisiae* industrial strains (not genetically modified) using the same fermentation and distillation facilities of the Brazilian ethanol plants. The pentose fraction will be used as substrate for other biotechnological purposes, including ethanol fermentation.

Researchers are focusing on cutting the cost of the enzymes and the pretreatment process, as well as reducing energy input. Production of ethanol from sugarcane bagasse will have to compete with the use of bagasse for electricity cogeneration. Depending on the efficiency of the cogeneration plant, about half of the bagasse is required to produce captive energy in the form of steam and energy at the sugar and ethanol facility. It is estimated that the surplus bagasse could increase the Brazilian ethanol production by roughly 50% [3].

2. Lignocellulosic residues

Lignocellulosic residues are composed by three main components: cellulose, hemicellulose and lignin. Cellulose and hemicellulose are polyssacharydes composed by units of sugar molecules [4]. Sugarcane bagasse is composed of around 50% cellulose, 25% hemicellulose and 25% lignin. It has been proposed that because of its low ash content (around 2-3%), this product offers numerous advantages in comparison to other crop residues (such as rice straw and wheat straw) when used for bio-processessing purposes. Additionally, in comparison to other agricultural residues, bagasse is considered a richer solar energy reservoir due to its higher yields on mass/area of cultivation (about 80 t/ha in comparison to about 1, 2, and 20 t/ha for wheat, other grasses and trees, respectively) [5].

2.1. Cellulose

Cellulose is composed of microfibrils formed by glucose molecules linked by β-1,4, being each glucose molecule reversed in relation to each other. The union of microfibrils form a linear and semicrystalline structure. The linearity of the structure enables a strong bond between the microfibrils. The crystallinity confers resistance to hydrolysis due to absence of water in the structure and the strong bond between the glucose chains prevents hydrolases act on the links β -1,4 [1].

2.2. Hemicellulose

Hemicellulose is a polysaccharide made of polymers formed by units of xylose, arabinose, galactose, manose and other sugars, that present crosslinking with glycans. Hemicellulose

can bind to cellulose microfibrils by hydrogen bonds, forming a protection that prevents the contact between microfibrils to each other and yielding a cohesive network. Xyloglucan is the major hemicellulose in many primary cell walls. Nevertheless, in secondary cell wall, which predominate in the plant biomass, the hemicelluloses are typically more xylans and arabino-xylans. Typically, hemicellulose comprises between 20 to 50% of the lignocellulose polysaccharides, and therefore contributes significantly to the production of liquid biofuels [1]. Sugarcane bagasse contain approximately 25-30% hemicelluloses [13,14].

2.3. Lignin

Lignin is a phenolic polymer made of phenylpropanoid units [13], which has the function to seal the secondary cell wall of plants. Besides providing waterproofing and mechanical reinforcement to the cell wall, lignin forms a formidable barrier to microbial digestion. Lignin is undoubtedly the most important feature underlying plant biomass architecture. Sugarcane bagasse and leaves contain approximately 18-20% lignin [13]. The phenolic structure of this polymer confers a material that is highly resistant to enzymatic digestion. Its disruption represents the main target of pretreatments before enzymatic hydrolysis.

3. Pretreatment

The pretreatment process is performed in order to separate the carbohydrate fraction of bagasse and other residues from the lignin matrix. Another function is to minimize chemical destruction of the monomeric sugars [6]. During pretreatment the inner surface area of substrate particles is enlarged by partial solubilization of hemicellulose and lignin.. This is achieved by various physical and/or chemical methods [5]. However, it has been generally accepted that acid pretreatment is the method of choice in several model processes [7]. One of the most cost-effective pretreatments is the use of diluted acid (usually between 0.5 and 3% sulphuric acid) at moderate temperatures. Albeit lignin is not removed by this process, its disruption renders a significant increase in sugar yield when compared to other processes [1]. Regarding sugarcane bagasse several attempts have been made to optimize the release of the carbohydrate fraction from the lignin matrix, including dilute acid pretreatment, steam explosion, liquid hot water, alkali, peracetic acid and also the so called ammonia fiber expansion (AFEX).

4. Hydrolysis

Cellulose and hemicellulose fractions released from pretreatment has to be converted into glucose and other monomeric sugars. This can be achieved by both chemically- or enzymatically- oriented hydrolysis [7, 8].

4.1. Chemical hydrolysis

Whitin chemical hydrolysis, acid hydrolysis is the most used and it can be performed with several types of acids, including sulphurous, sulphuric, hydrochloric, hydrofluoric,

phosphoric, nitric and formic acid. While processes involving concentrated acids are usually operated at low temperatures, the large amount of acids required may result in problems associated with equipment corrosion and energy demand for acid recovery. These processes typically involve the use of 60-90% concentrated sulfuric acid. The primary advantage of the concentrated acid process in realtion to diluted acid hydrolysis is the high sugar recovery efficiency, which can be on the order of 90% for both xylose and glucose. Concentrated acid hydrolysis disrupts the hydrogen bonds between cellulose chains, converting it into a completely amorphous state [8].

On the other hand, during dilute acid hydrolysis temperatures of 200–240°C at 1.5% acid concentrations are required to hydrolyze the crystalline cellulose. Besides that, pressures of 15 psi to 75 psi, and reaction time in the range of 30 min to 2 h are employed. During this conditions degradation of monomeric sugars into toxic compounds and other non-desired products are inevitable [9]. The main advantage of dilute acid hydrolysis in comparison to concentrated acid hydrolysis is the relatively low acid consumption. However, high temperatures required to achieve acceptable rates of conversion of cellulose to glucose results in equipment corrosion [4].

4.2. Enzymatic hydrolysis

Differently from acid hydrolysis, biodegradation of sugarcane bagasse by cellulolytic enzymes can be performed at much lower temperatures (around 50°C or even lower). Moreover conversion of cellulose and hemicellulose polymers into their constituent sugars is very specific and toxic degradation products are unlikely to be formed. However, a pretreatment step is required for enzymatic hydrolysis, since the native cellulose structure is well protected by the matrix compound of hemicellulose and lignin [4].

Cellulase is the general term for the enzymatic complex able to degrade cellulose into glucose molecules. The mechanism action accepted for hydrolysis of cellulose are based on synergistic activity between endoglucanase (EC 3.2.1.4), exoglucanase (or cellobiohydrolase (EC 3.2.1.91)), and β-glucosidase (EC 3.2.1.21). The first enzyme cleaves the bounds β-1,4-glucosidic of cellulose chains to produce shorter cello-dextrins. Exoglucanase release cellobiose or glucose from cellulose and cello-dextrin chains and, finally β-glucosidases hydrolyze cellobiose to glucose. The intramolecular β-1,4-glucosidic linkages are cleaved by endoglucanases randomly. Endoglucanases and exoglucanases have different modes of action. While endoglucanase hydrolyze intramolecular cleavages, exoglucanases hydrolyze long chains from the ends. More specifically, exoglucanases or cellobiohydrolases have action on the reducing (CBH I) and non-reducing (CBH II) cellulose chain ends to liberate glucose and cellobiose. These enzymes acts on insoluble cellulose, then their activity are often measured using microcrystalline cellulose. Lastly, β-glucosidases or cellobioase hydrolyze cellobiose to glucose. They are important to the process of hydrolysis because they removed cellobiose to the aqueous phase that is an inhibitor to the action of endoglucanases and exoglucanases [10].

The multi-complex enzymatic cocktail known as cellulase and hemicellulase can be produced by a variety of saprophytic microorganisms. *Trichoderma* and *Aspergillus* are the genera most used to produce cellulases. Among them, one of the most productive of biomass degrading enzymes is the filamentous fungus *Trichoderma reesei*. It cellulolytic arsenal is composed by a mixture of endoglucanases and exoglucanases that act synergistically to break down cellulose to cellobiose. Two β-glucosidases have been identified that are implicated in hydrolyzing cellobiose to glucose. An additional protein, swollenin, has been described that disrupts crystalline cellulose structures, presumably making polysaccharides more accessible to hydrolysis. The four most abundant components of *T. reesei* cellulase together constitute more than 50% of the protein produced by the cell under inducing conditions [9, 15]. Cellulases are essential for the biorefinery concept. In order to reduce the costs and increase production of commercial enzymes, the use of cheaper raw materials as substrate for enzyme production and focus on a product with a high stability and specific activity are mandatory. Apart from bioethanol, there are several applications to these enzymes, such as in textile, detergent, food, and in the pulp and paper industries.

5. Enzyme production using sugarcane bagasse

Cultivation of microorganisms in agroindustrial residues (such as bagasse) aiming the production of enzymes can be divided into two types: processes based on liquid fermentation or submerged fermentation (SmF), and processes based on solid-state fermentation (SSF) [5]. In several SSF processes bagasse has been used as the solid substrate. In the majority of the processes bagasse has been used as the carbon (energy) source, but in some others it has been used as the solid inert support. Cellulases have been extensively studied in SSF using sugarcane bagasse. It has been reported the production of cellulases from different fungal strains [5].

Several processes have been reported for the production of enzymes using bagasse in SmF. One of the most widely studied aspects of bagasse application has been on cellulolytic enzyme production. Generally basidiomycetes have been employed for this purpose, in view of their high extracellular cellulase production. A recent example was the use of Trichoderma reesei QM-9414 for cellulase and biomass production from bagasse. Additionally, white-rot fungi were successfully used for the degradation of long-fiber bagasse. Most of the strains caused an increase in the relative concentration of residual cellulose, indicating that hemicellulose was the preferred carbon source [5].

6. Processes for ethanol production

Ethanol production from lignocellulosic residues is performed by fermentation of a mixture of sugars in the presence of inhibiting compounds, such as low molecular weight organic acids, furan derivatives, phenolics, and inorganic compounds released and formed during pretreatment and/or hydrolysis of the raw material. Ethanol fermentation of pentose sugars (xylose, arabinose) constitutes a challenge for efficient ethanol production from these

residues, because only a limited number of bacteria, yeasts, and fungi can convert pentose (xylose, arabinose), as wells as other monomers released from hemicelluloses (mannose, galactose) into ethanol with a satisfactory yield and productivity [8]. Hydrolysis and fermentation processes can be designed in various configurations, being performed separately, known as separate hydrolysis and fermentation (SHF) or simultaneously, known as simultaneous saccharification and fermentation (SSF) processes.

When hydrolysis of pretreated cellulosic biomass is performed with enzymes, these biocatalysts (endoglucanase, exoglucanase, and β-glucosidase) can be strongly inhibited by hydrolysis products, such as glucose, xylose, cellobiose, and other oligosaccharides. Therefore, SSF plays an important role to circumvent enzyme inhibition by accumulation of these sugars. Moreover, because accumulation of ethanol in the fermenters does not inhibit cellulases as much as high concentrations of sugars, SSF stands out as an important strategy for increasing the overall rate of cellulose to ethanol conversion. Some inhibitors present in the liquid fraction of the pretreated lignocellulosic biomass also have a significant and negative impact on enzymatic hydrolysis. Due to the decrease in sugar inhibition during enzymatic hydrolysis in SSF, the detoxifying effect of fermentation, and the positive effect of some inhibitors present in the pretreatment hydrolysate (e.g. acetic acid) on the fermentation, SSF can be an advantageous process when compared to SHF [8]. Another important advantage is a reduction in the sensitivity to infection in SSF when compared to SHF. However, this was demonstrated by Stenberg and co-authors [12] that this is not always the case. It was observed that SSF was more sensitive to infections than SHF. A major disadvantage of SSF is the difficulty in recycling and reusing the yeast since it will be mixed with the lignin residue and recalcitrant cellulose.

In SHF, hydrolysis of cellulosic biomass and the fermentation step are carried out in different units [8], and the solid fraction of pretreated lignocellulosic material undergoes hydrolysis (saccharification) in a separate tank by addition of acids/alkali or enzymes. Once hydrolysis is completed, the resulting cellulose hydrolysate is fermented and the sugars converted to ethanol. *S. cerevisiae* is the most employed microorganism for fermenting the hydrolysates of lignocellulosic biomass. This yeast ferments the hexoses contained in the hydrolysate but not the pentoses. Several strategies (screening biodiversity, metabolic and evolutionary engineering of microorganisms) have been attempted to overcome this metabolic limitation.

One of the main features of SHF process is that each step can be performed at its optimal operating conditions (especially temperature and pH) as opposed to SSF [7].Therefore, in SHF each step can be carried out under optimal conditions, i.e. enzymatic hydrolysis at 45-50°C and fermentation at 30-32°C. Additionally, it is possible to run fermentation in continuous mode with cell recycling. The major drawback of SHF, as mentioned before, is that the sugars released during hydrolysis might inhibit the enzymes. It must be stressed out that ethanol produced can also act as an inhibitor in hydrolysis but not as strongly as the sugars. A second advantage of SSF over SHF is the process integration obtained when hydrolysis and fermentation are performed in one single reactor, which reduces the number of reactors needed.

Experimental data from ethanol output using sugarcane bagasse as the substrate is being released in the literature. Vásquez et al. (2007) [13] described a process in which 30 g/L of ethanol was produced in 10h fermentation by *S. cerevisiae* (baker's yeast) at an initial cell concentration of 4 g/L (dry weight basis) using non-supplemented bagasse hydrolysate at 37°C. The hydrolysate was obtained by a combination of acid/alkali pre-treatment, followed by enzymatic hydrolysis. Krishnan et al. (2010) [14] reported 34 and 36 g/L of ethanol on AFEX-treated bagasse and cane leaf residue, respectively, using recombinant S. cerevisiae and 6% (w/w) glucan loading during enzymatic hydrolysis. Overall the whole process produced around 20kg of ethanol per 100kg of each bagasse or cane leaf, and was performed during 250h including pretreatment, hydrolysis and fermentation. According to the authors this is the first complete mass balance on bagasse and cane leaf.

In view of the growing concern over climate change and energy supply, biofuels have received positive support from the public opinion. However, growing concern over first generation biofuels in terms of their impact on food prices and land usage has led to an increasing bad reputation towards biofuels lately. The struggle of 'land vs fuel' will be driven by the predicted 10 times increase in biofuels until 2050. The result is that biofuels are starting to generate resistance, particularly in poor countries, and from a number of activist non-governmental organizations with environmental agendas. This is highly unfortunate as it is clear that liquid biofuels hold the potential to provide a more sustainable source of energy for the transportation sector, if produced sensibly. Since replacement of fossil fuels will take place soon, a way to avoid these negative effects from first generation biofuels (mainly produced from potential food sources) is to make lignocellulosic derived fuels available within the shortest possible time. It is known that this process involves an unprecedented challenge, as the technology to produce these replacement fuels is still being developed [1]. Fuels derived from cellulosic biomass are essential in order to overcome our excessive dependence on petroleum for liquid fuels and also address the build-up of greenhouse gases that cause global climate change [2].

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Chapter 15

Sugarcane Biomass Production and Renewable Energy

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Additional information is available at the end of the chapter

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1. Introduction

Bio-fuel production is rooting in Uganda amidst problems of malnutrition and looming food insecurity "in [1,2]". The use of food for energy is a Worldwide concern as competition for resources between bio-fuel feedstocks and food crop production is inevitable. This is especially true for the category of primary feedstocks that double as food crops. Controversy surrounds the sustainability of bio-fuels as a source of energy in Uganda.

Given the above circumstances, adequate studies are required to determine the amount of feedstock or energy the agricultural sector can sustainably provide, the adequacy of land resources of Uganda to produce the quantity of biomass needed to meet demands for food, feed, and energy provision. Sugarcane is one of the major bio-fuel feed-stocks grown in Uganda.

Growth in sugarcane cultivation in Uganda is driven by the increased demand for sugar and related by-products. Annual sugar consumption in Uganda is estimated at 9 kg per capita with a predicted per capita annual consumption increase by 1 % over the next 15 years "in [3]".

This growth has resulted in increased demand for land to produce staple foods for households and thus encroaching on fragile ecosystems like wetlands, forests and shallow stoney hills and, a threat to food security " in [4]". The situation is likely to worsen with the advent of technology advancements in the conversion of biomass into various forms of energy like electricity and biofuels. A development that has attracted government and investors into the development of policies "in [5, 6]" that will support the promotion of bio-fuels in Uganda "in [7]".

Competition for land resources and conflicts in land use is imminent with the advent of developments in the use of agricultural crop resources as feedstocks for renewable energy

production. Sugarcane is one such crop for which production is linked to various issues including the sustainability of households in relation to food availability, income and environmental integrity. The plans for government to diversify on altnertive sources of energy with focus on biofuels and electricity generation has aggravated the situation. This chapter aims at demonstrating how sugarcane biomass can sustainably be produced to support fuel and electrical energy demands while conserving the environment and ensuring increased household income and food security.

This study was conducted with a major objective of assessing sustainable production of bio-fuels and electricity from sugarcane biomass in the frame of household poverty alleviation, food security and environmental integrity.

2. Research methods

The assessment of sugarcane production potential is done for the whole country. The rest of the studies were done at the Sugar estate and the outgrower farmers.

2.1. Assessment of sugarcane production potential

The overall suitability assessment involved the use of the partial suitability maps of temperature, rainfall and soil productivity ratings (Figures 1 and 2). An overlay of the three maps gave suitability ratings for sugarcane bio-fuel feedstock.

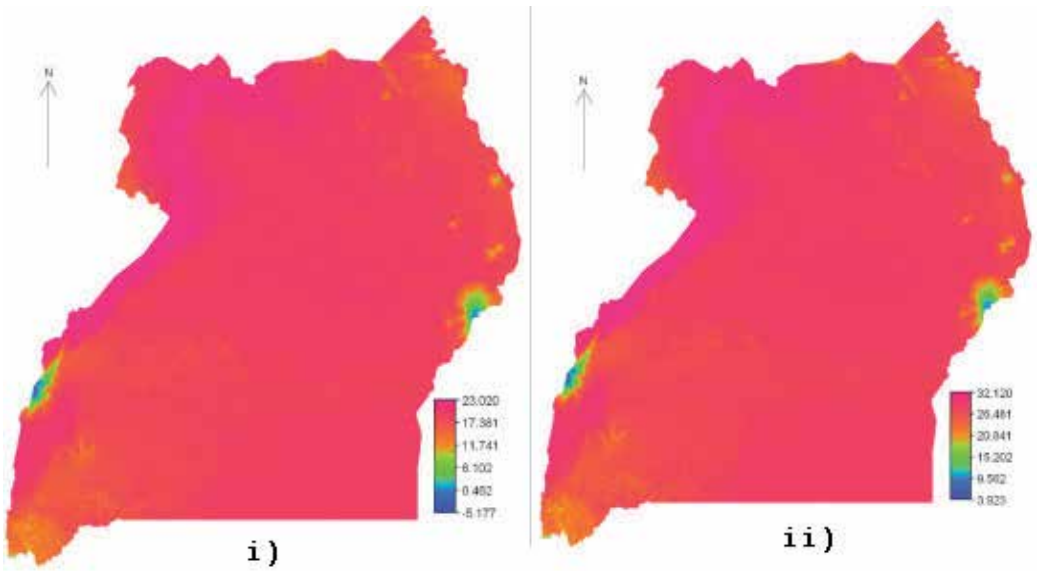

Figure 1. i) Minimum temperatures and maximum temperatures ii) " in [8] ".

Subtraction of gazetted areas, wetlands and water bodies produced final suitability maps and tables presented in the results. Steep areas have not been excluded since they are associated with highlands which are densely populated areas. It is hoped that soil

conservation practices will be practiced where such areas are considered for production of sugarcane feedstock. Urban areas, though expanding, are negligible and have not been considered in the calculations.

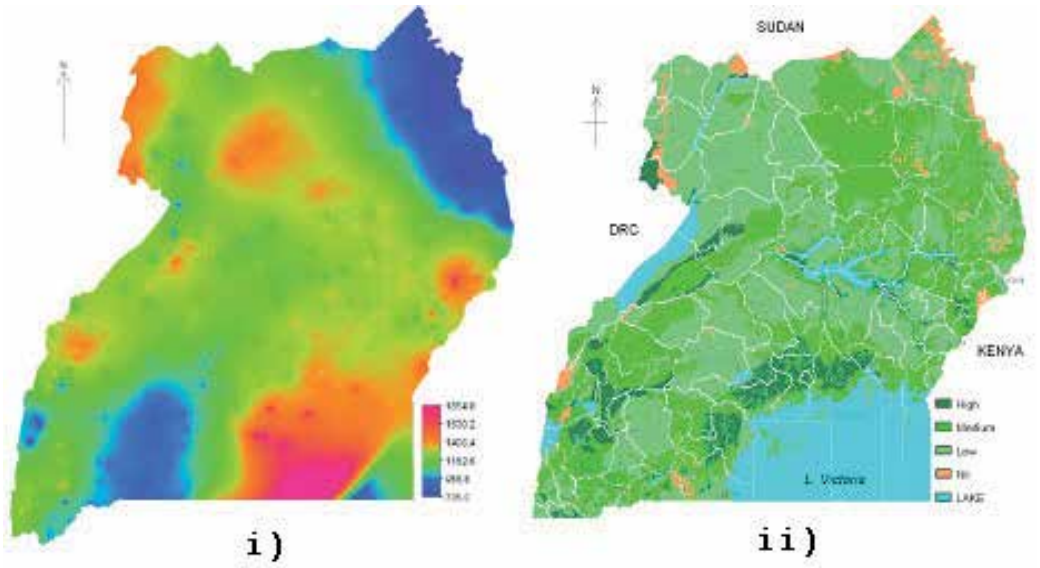

Figure 2. i) Mean annual rainfall " in [8] "; ii) Soil productivity ratings " in [9] ".

The suitability of the land resource quality for sugarcane was based on sets of values which indicate how well each cane requirement is satisfied by each land quality say: mean annual rainfall, minimum and maximum temperatures and soil productivity. The four suitability classes (rating), assessed in terms of reduced yields, and were defined according to " in [10] ". Potential land-use conflict visualization also gives an indication of land available for the production of sugarcane bio-fuel feedstocks. Conflict visualization for food versus sugarcane was done by an overlay of suitability maps of maize with sugarcane. Land-use conflict with gazetted areas was assessed by overlaying gazetted area maps with sugarcane suitability map.

2.2. Sustaining sugarcane biomass productivity

The total biomass production of five commercial sugarcane varieties grown on the estate across all crop cycles (plant and three ratoons) was developed.

The data on cane yield and cane productivity of plant and ratoon crops between 1995-1996 and 2009-2010 were collected from annual reports and compared to experimental data. The total bio-mass (cane, trash and tops) production in plant and two ratoons were recorded at harvest age i.e. 18 and 17 months for plant and ratoon crops respectively. Data was collected from three locations of plot size 54 m^2 (4 rows of 10m length). Nutrient status of crop residues on oven dry basis was adopted as suggested by " see [11] " for calculation of nutrient return to the soil and nutrients available to succeeding crop.

A replicated trial with four replications was established during 2009-2010 with different levels of chemical fertilizers and factory by-products (filter mud and boiler ash) to test their influence on cane and sugar yields.

Semi-commercial trials were established on the estate to study the influence of green manuring with sunn hemp against no green manured blocks (control), aggressive tillage against reduced tillage, and intercropping with legumes on cane yield and juice quality parameters.

Field studies were conducted during 2002-2004 and 2007-2009 to evaluate the influence of different levels of Nitrogen (N), Phosphorus (P), Potassium (K) and sulphur (S) on cane yield and juice quality of plant crop of sugarcane.

The cane yield data on green cane vs burnt cane harvesting systems, and aggressive vs reduced tillage operations were collected and analysed for biomass yield.

2.3. Assessment of potential biofuel productivity and cane biomass electricity generation

Ethanol yield estimates from sugarcane is based on yield per ton of sugarcane. In addition, the production of bagasse from the cane stalk available for electricity generation were collected and analysed as per the following bagasse-steam-electrical power norms at Kakira sugar estate:

i. Bagasse production is 40 % of sugarcane production
ii. Moisture % in bagasse is 50%
iii. 1.0 ton of bagasse produces 2.0 tons of steam
iv. 5.0 tons of steam produces 1.0 Mwh electrical power

Therefore 2.5 tons of bagasse produces 5.0 tons of steam which will generate 1.0 Mwh electrical power. The electric power used in Kakira is hence generated from a renewable biomass energy source.

In 2005, Kakira had two 20 bar steam-driven turbo-generators (3 MW + 1.5 MW) in addition to 5 diesel standby generators. Thereafter, two new boilers of 50 tonnes per hour steam capacity at 45 bar-gauge pressure, with all necessary ancillaries such as an ash handling system, a feed water system and air pollution controls (such as wet scrubbers and a 40m 30 high chimney) were installed

2.4. Contribution to household income and food security

Indicative economic assessments included the use of gross sales for the raw material (farm gate) and ethanol. Annualized sugarcane net sales were compared to household annual expenditures to allow assessment of cane contribution to household income. Integration of commodity prices gives insight on the potential contribution of bio-fuels to household poverty alleviation and overall development of rural areas.

3. Results

3.1. Suitability

The agro-ecological settings favor the growing of sugarcane with a potential 10,212,757 ha (49.6%) at a marginal level of production with 2,558,698 ha (12.4%) land area potentially not suitable for cane production. Although the current production is far below the potential production "in [12]", the related cane production is 908,935,330 and 60,769,069 tons respectively. It is also evident that there is possibility of increasing production through expansion of land area under sugarcane.

1. Suitability of sugarcane production and conflict visualization between food crops and gazetted areas in Uganda

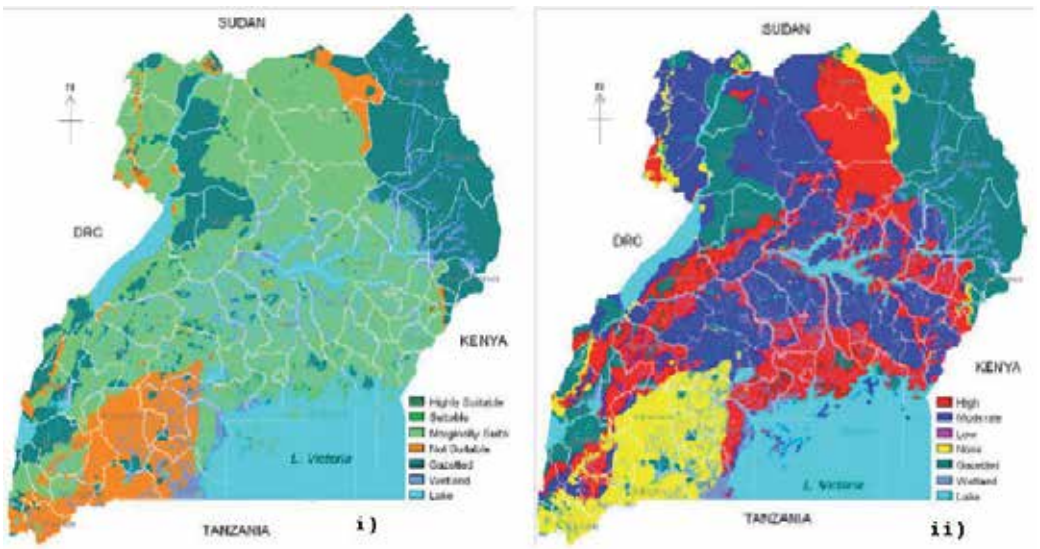

Figure 3. Sugar cane suitability ratings (i) and conflict visualization between food crops and gazetted areas

The marginal productivity of cane in Uganda is a function of Rainfall amount and the atmospheric temperature. Nevertheless the average optimum yields (89 ton / ha) at marginal level of productivity are comparable to yields of 85 ton / ha in a commercialized production in Brazil "in [13] ".

Expanding acrage under sugarcane is likely to increase pressure on gazetted biodiversity rich areas including wetlands with consequent potential loss of bio-diversity.

Sugarcane and maize (food crop) have similar ecological requirements, presenting a situation of high potential land-use conflict as 49.6 % of arable land can be grown with both sugarcane and food crops (figure 3 i). Figure 3 ii), shows 14 % of the land where sugarcane has potential conflict with gazetted areas of which 4.3 % has potential conflict with forest reserves.

Sugarcane, given its energy balance advantage, is likely to be beneficial if promoted as bio-fuel feedstock as this is likely to increase sugarcane prices to the benefit of the small scale farmer.

3.2. Agronomy

The beneficial effects of integrated agronomic practices like reduced tillage operations, balanced fertilization; organic recycling of mill by-products (filter mud and boiler ash); intercropping with legumes; green manuring with sunn hemp; crop residue recycling through cane trash blanketing in ratoons by green cane harvesting to sustain soil fertility and cane productivity in monoculture sugarcane based cropping system are presented and discussed. Partitioning of dry matter between plant and ratoon crops of cane grown on the estate and Outgrowers fields were quantified and also presented in this chapter.

3.2.1. Influence of agronomic practices on cane yield and cane productivity

3.2.1.1. Green manuring

There was considerable increase in cane yield (7.92 tc/ha) and cane productivity (0.62 tc/ha/m) in plant and ratoon crops due to green manuring as compared to blocks without green manuring (Table 1).

Crop cycle	Green manuring		No green manuring		Variance	
	Yield tc/ha	Productivity tc/ha/m	Yield tc/ha	Productivity tc/ha/m	Yield tc/ha	Productivity tc/ha/m
Plant	125.3	5.9	112.2	5.2	13.1	0.8
Ratoon 1	95.3	5.8	92.4	5.0	2.8	0.8
Ratoon 2	92.4	5.0	84.6	4.7	7.8	0.3
# average	104.3	5.6	96.4	5.0	7.9	0.6

Table 1. Cane yield and cane productivity variance due to green manuring

Growing sunn hemp (Crotolaria juncia) during fallow period for in-situ cultivation has been a common practice to improve soil health on the estate since 2004. Sunn hemp at 50% flowering on average produces 27.4 t/ha and 5.9 t/ha of fresh and dry weights respectively. It contains 2.5% N on oven dry basis and adds about 147kg N/ha to the soil. Of this amount, 30% (44kg N/ha) is presumed to be available to the succeeding sugarcane plant crop. "[11]" reported that N available to sugarcane ranges between 30 - 60% of total N added to soils in South Africa.

3.2.1.2. Balanced fertilization

The results indicated that application of N to plant crop at 100kg /ha, phosphorus at 160kg P_2O_5 /ha, potassium at 100 K_2O /ha and sulphur at 40kg /ha significantly increased the cane yields by 23.3 tc/ha; 22.25 tc/ha; 12.07 tc/ha and 8.71 tc/ha respectively over no application of

N, P, K and S. Sugar yields were also improved due to N, P, K and S application by 2.91 ts/ha; 2.17 ts/ha; 2.88 ts/ha and 0.44 ts/ha respectively as compared to no application (Table 2).

Nutrient levels (kg/ha)	Attributes	
	Cane yield (tc/ha)	Sugar yield (ts/ha)
N levels: 0	112.68	15.69
50	124.38	17.08
100	135.98	18.60
P_2O_5 levels: 0	108.70	14.51
80	120.40	16.34
160	130.95	18.01
K_2O levels: 0	108.06	15.58
50	124.86	17.32
100	134.13	18.46
Sulphur levels: 0	113.90	11.92
40	122.61	12.36
CD at 5%: N	10.69	1.20
P_2O_5	9.80	1.18
K_2O	9.06	1.06
S	6.94	0.32

Table 2. Influence of N, P, K and S nutrition on cane and sugar yields

Balanced fertilizer application is very vital for crop growth. Adequate amounts of especially the major nutrients need to be supplied for proper crop growth. Excessive application of N in cane plant crop has been shown to inhibit the activity of free living N-fixing bacteria and chloride ions from Muriate of potash adversely affecting soil microbial populations "in [14]".

3.2.1.3. Mill by-products

Millable stalk population at harvest was significantly higher due to application of filter mud and boiler ash + 100% recommended dose of fertilizers (RDF) than all other treatment combinations. However, there was no significant effect on stalk length, number of internodes and stalk weight due to different treatments. Cane and sugar yields were significantly higher by 30.2 tc/ha and 3.8 ts/ha respectively due to application of filter mud and boiler ash + 100% RDF. The data are presented in Table 3.

Cane-mill by-products (filter mud and boiler ash) do contain valuable amounts of N, P, K, Ca, and several micronutrients "in [15]". These in addition to the inorganic fertilizers applied considerably increased cane yields as compared to treatments which received only inorganic fertilizers. In [11] it is also showed that organic wastes-including filter mud and bolier ash could be used as an alternative source of nutrients in cane cultivation.

Treatment	Yield (T/ha)	
	Cane	Sugar (Estimated)
1. 100% recommended dose of fertilizers (RDF)	131.6	16.6
2. Filter mud + Boiler ash @ 32 + 8 T/ha alone	122.1	16.2
3. 100% RDF+ Filter mud + Boiler ash @ 32 + 8 T/ha	161.7	20.4
4. 75% RDF+ Filter mud + Boiler ash @ 32 + 8 T/ha	143.8	17.5
5. 50% RDF+ Filter mud + Boiler ash @ 32 + 8 T/ha	142.1	17.3
6. 25% RDF+ Filter mud + Boiler ash @ 32 + 8 T/ha	135.0	16.6
CD @ 5%	12.3	1.9

Table 3. Influence of mill by-products on cane and sugar yields

3.2.1.4. Partitioning of bio-mass

Among the crop cycles, plant crop recorded higher bio-mass production than succeeding ratoons. The production of crop residues were also higher (50.7 t/ha) in plant crop than 1st (45.7 t/ha and 2nd (36.6 t/ha) ratoons. The data are presented in the Table 4 below.

Crop cycle	Cane weight (Tc/ha)	Tops weight (T/ha)	Trash weight (T/ha)	Total bio-mass (T/ha)	Tops + Trash weight T/ha	% over total
Plant	137.5	30.3	20.4	188.2	50.7	27.0
Ratoon 1	124.9	26.8	18.9	170.59	45.7	27.0
Ratoon 2	107.5	19.7	16.8	144.02	36.6	25.4
# average	123.3	Fresh: 25.6 Dry: 9.0	Fresh: 18.7 Dry: 16.9	- -	Fresh: 44.3 Dry: 25.8	26.4

Table 4. Crop-wise partitioning of bio-mass

Results indicate that on average, 25.8 tons of dry matter (cane trash and tops) is produced from each crop cycle at harvest. In burnt cane harvesting system, all this dry matter is lost unlike in green cane harvesting. This explains the gradual decline in cane yield in such harvesting systems. The decline is presumed to be due to deteriorating organic matter and other physical and chemical properties of the soil " in [11] " .

After decomposition of cane trash, 139kg N, 59kg P_2O_5, 745kg K_2O, 41kg Ca, 46kg Mg and 34kg S /ha were added to the soils and these added nutrients would be available to the succeeding crop at 30% of the total nutrients " in [11] " . The nutrient concentration of crop residues (trash + tops) were taken into account for computing nutrient additions to the soil and their availability to the succeeding ratoon crops and the data are presented in Table 5.

Nutrient status of crop residues (%)	Total dry matter (T/ha)	Total nutrients	
		Added to soil (kg/ha)	Available to crops @ 30% (kg/ha)
N : 0.54	25.8	139	42
P_2O_5 : 0.23	25.8	59	18
K_2O : 2.89	25.8	745	223
Ca : 0.16	25.8	41	12
Mg : 0.18	25.8	46	14
S : 0.13	25.8	34	10

** Adopted from [11]

Table 5. Nutrient status of crop residues and their availability to the succeeding crops

3.3. Renewable energy potential

3.3.1. Ethanol productivity

Sugarcane, given its energy balance advantage, is likely to be beneficial if promoted as bio-fuel feedstock as this is likely to increase sugarcane prices to the benefit of the small scale farmer.

Promoting sugarcane as a feedstock for ethanol is likely to improve rural livelihood and also minimize on forest encroachment since energy output per unit land area is very high for sugarcane.

In Brazil for example the production of sugar cane for ethanol only uses 1% of the available land and the recent increase in sugar cane production for bio-fuels is not large enough to explain the displacement of small farmers or soy production into deforested zones " in [13] ".

To minimize competition over land, it is advisable to grow sugarcane that has high yields with higher energy output compared to other biofuel crops. High yielding bio-fuels are preferable as they are less likely to compete over land "in [16]".

3.3.2. Bioelectricity generation

Hydropower contributes about 90 per cent of electricity generated in Uganda with sugarcane based bagasse bioelectricity, fossil fuel and solar energy among other sources of power. Although the current generation of 800 MW "in [18] ". has boosted industrial growth, the capacity is still lagging behind the demand that is driven by the robust growth of the economy.

The low pressure boilers of 45 bar currently generate 22 MW of which 10 MW is connected to the grid. However, Kakira sugar estate has a target of generating 50 MW of electricity with the installation of higher pressure boilers of 68 bar in 2013. This target can be surpassed given the abundance of the bagasse (Table 6 and 7).

Particulars	Plant	Ratoon 1	Ratoon 2	Ratoon 3	Total/ average
Cane harvest area (ha)	1,461.1	1,541.7	1,535.2	392.8	4,930.8
Total cane supply (tons)	155,207.3	162,132.3	137,436.4	40,138.9	494,915.9
Average cane yield (tc/ha)	106.23	105.16	89.52	87.28	100.37
Average harvest age (months)	19.20	18.15	17.98	16.50	17.96
Cane productivity (tc/ha/m)	5.53	5.79	4.97	5.29	5.40
Bagasse production /ha	42.49	42.06	35.80	34.90	40.14
Steam generation (tons /ha)	84.98	84.12	71.60	69.80	80.28
Electric power generation (Mwh/ha)	17.00	16.82	14.32	13.96	16.05

*This electric power generation is calculated based on using low pressure boilers of 45 bar at Kakira estate

Table 6. Mean estate cane production/productivity, electrical generation* norms (2008 – 2012)

Particulars	Plant	Ratoon 1	Ratoon 2	Ratoon 3	Total/ average
Cane harvest area (ha)	3,429.3	3,206.9	2,334.7	1,431.3	10,402.2
Total cane supply (tons)	320,100.20	290,073.29	188,274.65	113,837.25	912,285.49
Average cane yield (tc/ha)	93.34	90.45	80.64	79.53	87.70
Average harvest age (months)	18.50	17.50	18.00	16.00	17.50
Cane productivity (tc/ha/m)	5.05	5.17	4.48	4.97	5.01
Bagasse production /ha	37.30	36.18	32.25	31.81	35.08
Steam generation (tons /ha)	74.60	72.36	64.50	63.62	70.16
Electric power generation (Mwh/ha)	14.90	14.50	12.90.	12.70	14.00

*This electric power generation is calculated based on using low pressure boilers of 45 bar at Kakira estate

Tc = Tons of cane

Table 7. Mean outgrowers cane production/productivity, electrical generation* norms (mean for 2008 – 2012)

Putting into consideration the productivity norms at Kakira estate and outgrowers (Table 8), with a potential of producing 908.9 m tons of sugarcane, Uganda has a potential of producing bio-electricity that surpasses the nation's demand by far. Much of this electrical power can be exported to the region, greatly expanding on Uganda's export base.

Particulars	Plant	Ratoon 1	Ratoon 2	Ratoon 3	Total/ average
Cane harvest area (ha)	4,890.4	4,748.6	3,869.9	1,824.1	15,333.0
Total cane supply (tons)	475,307.50	452,205.60	325,711.05	153,976.15	1,407,200.4
Average cane yield (tc/ha)	97.19	95.22	84.17	84.41	91.78
Average harvest age (months)	18.75	17.75	18.00	16.00	17.65
Cane productivity (tc/ha/m)	5.18	5.36	4.67	5.27	5.20
Bagasse production /ha	38.88	38.09	33.67	33.76	36.71
Steam generation (tons /ha)	77.76	76.18	67.34	67.52	73.42
Electric power generation (Mwh/ha)	15.55	15.24	13.47	13.50	14.68

*This electric power generation is calculated based on using low pressure boilers of 45 bar at Kakira estate

Table 8. Combined (Estate + Outgrowers cane production/productivity, Electrical power generation* norms (mean for 2008 – 2012)

3.4. Household income and food security

The competition for resources between sugarcane and food crops is apparent with foreseen consequent increased food insecurity. Fifty percent of the arable land area good for food crop production is equally good for sugarcane.

A farm household that allocates all of its one hectare of land to sugarcane is expected to earn 359 $ at high input level, 338 $ at intermediate level and 261 $ at low input level " in [4] ". The 391 $ required to purchase maize meal is well above the net margins from one ha. This shows that proceeds from one hectare cannot sustain a household of 5. It is further revealed that maize produced from 0.63 ha can sustain a household nutritionally; however considering the annual household expenditure (760.8 $; "in [17] "), about three hectares of land under sugarcane are required at low input level to support a household " in [4]".

However, this study reveals that sugarcane sales accrued from ethanol under a scenario of a flourishing bio-fuel industry is associated with increased income that is likely to support households (Table 9). An ethanol gross sale per person per day is 1.6 dollars; an indication that the cultivation of sugarcane based biofuel is likely to contribute to alleviation of household poverty. A trickle-down effect on household income is expected from a foreseen expansion of bagasse-based electricity generation beyond the estate into the national electricity grid.

Production / year		Gross sales			Conflict		
Cane production	Billion	Farm	Ethanol	Capita	Food	Gazetted	Forest
ton	litres	/ha/year			%		
908.9 m	75.4	1869	22161	1.6	50.0	14.0	4.3
Sugarcane= USD 21/ton: projected population of 33 m in 2009 is used							

Table 9. Sugarcane productivity, sales and potential land-use conflict

4. Further research

The expansion of cane production is largely driven by market forces oblivious to the detrimental impact the industry is likely to have on food, livelihood security and the status of biodiversity. In addition to lack of appropriate policies to support the small-scale cane farmer, the policies are largely sectoral with no linkages with other relevant policies. Information is required to support the sustainable development of the cane industry with minimal negative impact on food and livelihood security and the status of biodiversity.

5. Relevant questions to explore among others include

Can food crop productivity be improved in the context of a sugarcane-based farming system?

Can the understanding of the dimensions of food and livelihood security in sugarcane-based farming systems inform the synergistic development and review of relevant policies in the food, agriculture, health, energy, trade and environment sectors? What are the social impacts of the industry in light of the various agro-ecological zones of the country? What is the gender based livelihood strategies with special emphasis on labor exploitations- child labor etc?

What do people consider as possible options for improving food and livelihood security in a sugarcane-based farming system? Do these options differ between different actors (local women and men, NGOs and government)? How do families cope with food inadequacy, inaccessibility and malnutrition?

Can the study inform the carbon credit market initiative for farming systems in Uganda through the climate smart agriculture concept? Are the proposed assessment tools appropriate for Ugandan situations and the cane-based systems in particular?

6. Conclusion

Driven by the need to meet the increasing local and regional sugar demand, and fossil fuel import substitution, cane expansion has potential negative impact on food security and biodiversity. However, this negative impact parallels the benefits related to cane cultivation. Cane biomass yield can be improved and sustained through the integrated use of various

practices reported in this study. Consequently this reduces the need to expand land acreage under cane while releasing land for use in food crop productivity. The high biomass returned to the ground sequesters carbon thereby offering the opportunity for sugarcane based farmers to earn extra income through the sale of carbon credits. Trickle down effects are expected to increase household income through the production and marketing of cane based biofuel and electricity.

These developments are expected to improve the farmers purchasing power, making households to be less dependent on the land and more food secure financially.

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Chapter 16

Paenibacillus curdlanolyticus Strain B-6 Multienzyme Complex: A Novel System for Biomass Utilization

Khanok Ratanakhanokchai, Rattiya Waeonukul, Patthra Pason, Chakrit Tachaapaikoon, Khin Lay Kyu, Kazuo Sakka, Akihiko Kosugi and Yutaka Mori

Additional information is available at the end of the chapter

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1. Introduction

To develop a bio-based economy for sustainable economic growth, it is necessary to produce chemicals and fuels from renewable resources, such as plant biomass. Plant biomass contains a complex mixture of polysaccharides, mainly cellulose and hemicellulose (mainly xylan), and other polysaccharides (Aspinall, 1980). The hemicelluloses, as well as the aromatic polymer lignin, interact with the cellulose fibrils, creating a rigid structure strengthening the plant cell wall. Therefore, complete and rapid hydrolysis of these polysaccharides requires not only cellulolytic enzymes but also the cooperation of xylanolytic enzymes (Thomson, 1993). Many microorganisms that produce enzymes capable of degrading cellulose and hemicellulose have been reported and characterized. Two enzyme systems are known for their degradation of lignocellulose by microorganisms. In many aerobic fungi and bacteria, endoglucanase, exoglucanase, and ancillary enzymes are secreted individually and can act synergistically on lignocellulose. The most thoroughly studied enzymes are the glycosyl hydrolases of *Trichoderma reesei* (Dashtban et al., 2009). On the other hand, several anaerobic cellulolytic microorganisms such as *Clostridium thermocellum* (Lamed & Bayer, 1988), *C. cellulovorans* (Doi et al., 2003), *C. josui* (Kakiuchi et al., 1998) and *C. cellulolyticum* (Gal et al., 1997) are known to produce a cell-associated, large extracellular polysaccharolytic multicomponent complex called the cellulosome, in which several cellulolytic and xylanolytic enzymes are tightly bound to a scaffolding protein (core protein). Thus, the cellulosome provides for a large variety of enzymes and attractive enzymatic properties for the degradation of recalcitrant plant biomass. So far, anaerobic microorganisms have been identified as producing the multienzyme complex, cellulosome

(Doi & Kosugi, 2004; Demain et al., 2005). However, when compared with aerobic enzymes, production of those enzymes by anaerobic culture presents a high cost because of the high price of medium, slow rate of growth and low yield of enzyme, while only a little information has been reported on cellulosome-like multienzyme complex produced by aerobic bacteria (Kim & Kim, 1993; Jiang et al,, 2004; van Dyk et al., 2009). Therefore, the multienzyme complexes, cellulosomes, produced by aerobic bacteria show great potential for improving plant biomass degradation. A facultatively anaerobic bacterium, *P. curdlanolyticus* strain B-6, is unique in that it produces extracellular xylanolytic-cellulolytic multienzyme complex under aerobic conditions (Pason et al., 2006a, 2006b; Waeonukul et al., 2009b). In the following years, the characteristics, function, genetics and mechanism of the xylanolytic-cellulolytic enzymes system of this bacterium has been the subject of considerable research. In light of new findings in this field, this review will describe the state of knowledge about the multienzyme complex of strain B-6 and its potential biotechnological exploitations.

1.1. Composition of lignocellulosic biomass

Lignocellulosic biomass is composed mainly of plant cell walls, with the structural carbohydrates, cellulose and hemicelluloses and heterogeneous phenolic polymer lignin as its primary components (Fig. 1). However, their proportions vary substantially, depending on the type, the species, and even the source of the biomass (Aspinall et al., 1980; Pérez et al., 2002; Pauly et al., 2008).

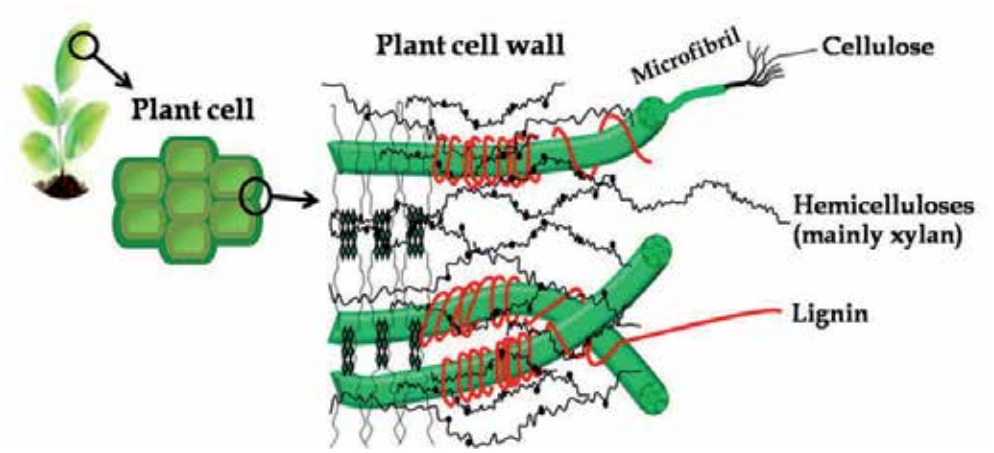

Figure 1. Structure of lignocellulosic plant biomass. (This figure is adapted from Tomme et al., 1995).

Cellulose: Cellulose, the main constituent of the plant cell wall, is a polysaccharide composed of linear glucan chains linked together by β-1,4-glycosidic bonds with cellobiose residues as the repeating unit at different degrees of polymerization, depending on resources. The cellulose chains are grouped together to form microfibrils, which are bundled together to form cellulose fibers. The cellulose microfibrils are mostly independent but the ultrastructure of cellulose is largely due to the presence of covalent bonds, hydrogen bonds

and Van der Waals forces. Hydrogen bonding within a cellulose microfibril determines 'straightness' of the chain but inter-chain hydrogen bonds might introduce order (crystalline) or disorder (amorphous) into the structure of the cellulose (Klemm et al., 2005). In the latter conformation, cellulose is more susceptible to enzymatic degradation (Pérez et al., 2002). In nature, cellulose appears to be associated with other plant compounds and this association may affect its biodegradation.

Hemicelluloses: Hemicelluloses are the second most abundant polymers and differ from cellulose in that they are not chemically homogeneous. Hemicelluloses are branched, heterogenous polymers of pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose) and acetylated sugars. They have lower molecular weight compared to cellulose and branches with short lateral chains that are easily hydrolysed (Saha, 2003; Scheller & Ulvskov, 2010). Hemicelluloses differ in composition. Hemicelluloses in agricultural biomass like straws and grasses are composed mainly of xylan, while softwood hemicelluloses contain mainly glucomannan. In many plants, xylans are heteropolysaccharides with backbone chains of 1,4-linked β-D-xylopyranose units. In addition to xylose, xylan may contain arabinose, glucuronic acid, or its 4-*O*-methyl ether, acetic acid, ferulic and *p*-coumaric acids. Hemicelluloses are bound via hydrogen bonds to the cellulose microfibrils in the plant cell wall, crosslinking them into a robust network. Hemicelluloses are also covalently attached to lignin, forming together with cellulose to form a highly complex structure.

Lignin: Lignin is the third most abundant polymer in nature. It is present in plant cell walls and confers a rigid, impermeable, resistance to microbial attack and oxidative stress. Lignin is a complex network formed by polymerization of phenyl propane units and constitutes the most abundant non-polysaccharide fraction in lignocelluloses (Pérez et al., 2002; Sánchez, 2009). The three monomers in lignin are *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol; they are joined through alkyl–aryl, alkyl–alkyl and aryl–aryl ether bonds. Lignin embeds the cellulose thereby offering protection against microbial and enzymatic degradation. Furthermore, lignin is able to form covalent bonds to some hemicelluloses, e.g. benzyl ester bonds with the carboxyl group of 4-*O*-methyl-D-glucuronic acid in xylan. More stable ether bonds, also known as lignin carbohydrate complexes, can be formed between lignin and arabinose, or between galactose side groups in xylans and mannans.

1.2. Biodegradation of lignocellulosic biomass

Several biological methods for lignocellulose recycling based on the enzymology of cellulose, hemicelluloses, and lignin degradation have been developed. To date, processes that use lignocellulolytic enzymes or microorganisms could lead to promising, environmentally friendly technologies. The relationship between cellulose and hemicellulose in the cell walls of higher plants is much more intimate than was previously thought. It is possible that molecules at the cellulose-hemicellulose boundaries, and those within the crystalline cellulose, require different enzymes for efficient hydrolysis.

Cellulase: Cellulases responsible for the hydrolysis of cellulose are composed of a complex mixture of enzymes with different specificities to hydrolyze the β-1,4-glycosidic linkages (Fig. 2A). Cellulases can be divided into three major enzyme activity classes (Goyal et al., 1991; Rabinovich et al., 2002). These are endoglucanases or endo-1-4-β-glucanase (EC 3.2.1.4), exoglucanase or cellobiohydrolase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21). Endoglucanases, are thought to initiate attack randomly at multiple internal sites in the amorphous regions of the cellulose fiber, which opens-up sites for subsequent attack by the cellobiohydrolases. Cellobiohydrolases remove cellobiose from the ends of both sides of the glucan chain. Moreover, cellobiohydrolase can hydrolyze highly crystalline cellulose. β-glucosidase hydrolyzes cellobiose and in some cases short chain cellooligosaccharides to glucose.

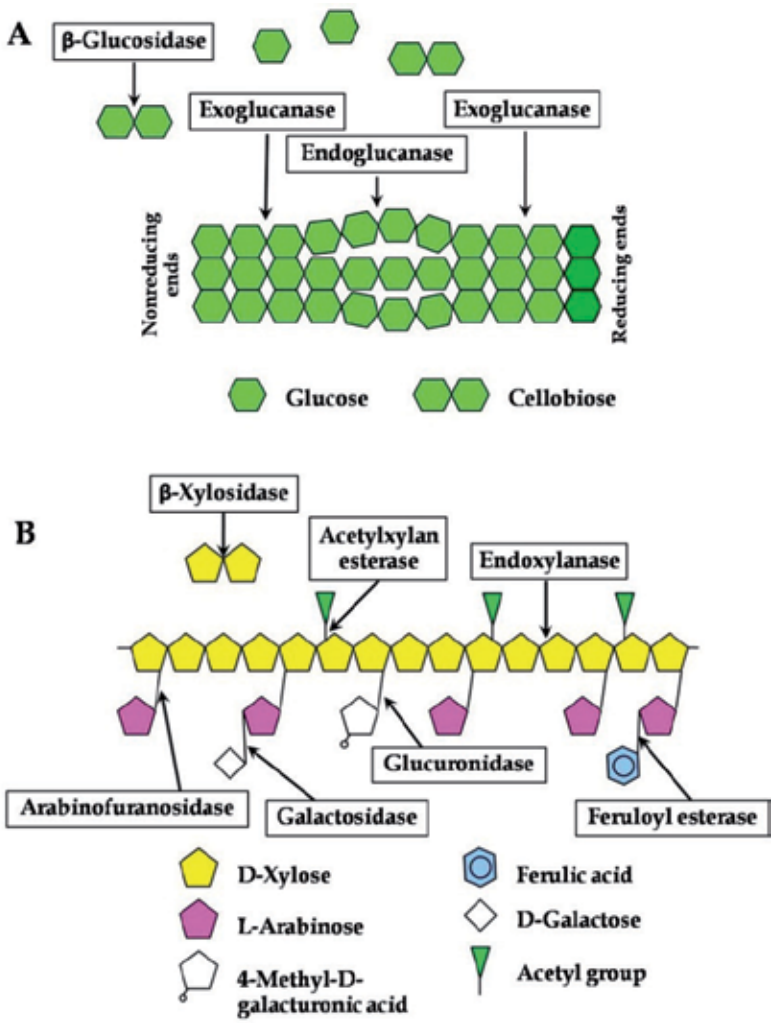

Figure 2. Enzyme systems involved in the degradation of cellulose (A) and xylan (B). (This figure is adapted from Aro et al., 2005).

Hemicellulase: Xylan is the main carbohydrate found in hemicelluloses. Its complete degradation requires the cooperative action of a variety of hydrolytic enzymes (Fig. 2B). Xylanases are frequently classified according to their action on distinct substrates: endo-1,4-β-xylanase (endoxylanase) (EC 3.2.1.8) generates xylooligosaccharides from the cleavage of xylan while 1,4-β-xylosidase (EC 3.2.1.37) produces xylose from xylobiose and short chain xylooligosaccharides. In addition, xylan degradation needs accessory enzymes, such as α-L-arabinofuranosidase (EC 3.2.1.55), α-4-*O*-methyl-D-glucuronidase (EC 3.2.1.39), acetyl xylan esterase (EC 3.1.1.72), ferulic acid esterase (EC 3.1.1.73), and *p*-coumaric acid esterase (EC 3.1.1.-), acting synergistically, to efficiently hydrolyze wood xylans. In the case of acetyl-4-*O*-methylglucuronoxylan, which is one of the most common hemicelluloses, four different enzymes are required for degradation: endo-1,4-β-xylanase, acetyl esterase (EC 3.1.1.6), α-glucuronidase, and β-xylosidase. The degradation of *O*-acetyl galactoglucomannan starts with the rupture of the polymer by endomannanase (EC 3.2.1.78). Acetylglucomannan esterase (EC 3.1.1.-) removes acetyl groups, and α-galactosidase (EC 3.2.1.22) eliminates galactose residues. Finally, β-mannosidase (EC 3.2.1.85) and β-glucosidase break down the endomannanase-generated oligomeric β-1,4 bonds (Thomson, 1993; Li et al., 2000; Pérez et al., 2002).

1.3. Multienzyme complex cellulosome

The enzyme systems for the lignocellulose degradation by microorganisms can be generally regarded as non-complexed or complexed enzymes (Lynd et al., 2002). In the case of aerobic fungi and bacteria, the cellulase enzymes are free and mostly secreted. In such organisms, by the very nature of the growth of the organisms, they are able to reach and penetrate the cellulosic substrate and, hence, the secreted cellulases are capable of hydrolyzing the substrate. The enzymes in these cases are not organized into high molecular weight complexes and are called non-complexed (Fig. 3A). The polysaccharide hydrolases of the aerobic fungi are largely described based on the examples from *Trichoderma, Penicillum, Fusarium, Humicola, Phanerochaete,* etc., where a large number of the cellulases are encountered (Dashtban et al., 2009; Sánchez, 2009). In contrast, various cellulases and hemicellulases from several anaerobic cellulolytic microorganisms, are tightly bound to a scaffolding protein, as core protein and organized to form structures on the cell surfaces; these systems are called complexed enzymes or cellulosomes (Fig. 3B). The cellulosome is thought to allow concerted enzyme activities in close proximity to the bacterial cell, enabling optimum synergism between the enzymes presented on the cellulosome. Concomitantly, the cellulosome also minimizes the distance over which hydrolysis products must diffuse, allowing efficient uptake of these oligosaccharides by the host cells (Bayer et al., 1994; Schwarz, 2001; Lynd et al., 2002).

Biotechnological applications in terms of hydrolysis efficiency for complexed enzyme systems might have an advantage over non-complexed enzyme systems. The high efficiency of the cellulosome has been attributed to (i) the correct ratio between catalytic domains that optimize synergism between them, (ii) appropriate spacing between the individual

components to further favor synergism, (iii) the presence of different enzymatic activities (cellulolytic or hemicellulolytic enzymes) in the cellulosome that can remove "physical hindrances" of other polysaccharides in heterogeneous plant cell materials (Lynd et al., 2002), and (iv) the presence of carbohydrate-binding modules (CBMs) that can increase the rate of hydrolysis by bringing the cellulosome into intimate and prolonged association with its recalcitrant substrate (Shoseyov et al., 2006). Thus, the complexed enzyme system, cellulosome, may provide great potential for the degradation of plant biomass.

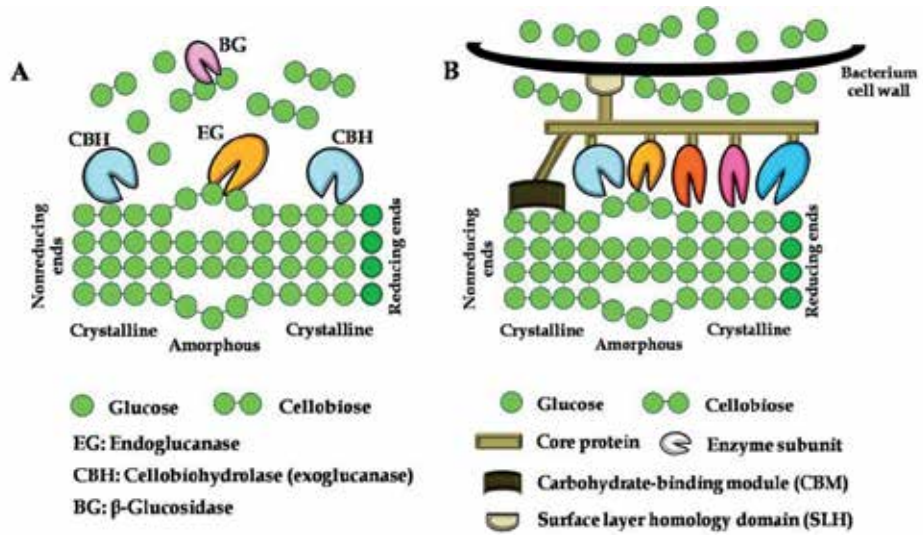

Figure 3. Simplified schematic of the hydrolysis of amorphous and microcrystalline celluloses by non-complexed (A) and complexed (B) cellulase systems. (This figure is adapted from Lynd et al., 2002).

The cellulosome was first identified in 1983 from the anaerobic, thermophilic, spore-forming *Clostridium thermocellum* (Lamed et al., 1983). The cellulosome of *C. thermocellum i*s commonly studied along with cellulosomes from the anaerobic mesophiles, *C. cellulovorans* (Doi et al., 2003), *C. josui* (Kakiuchi et al., 1998) and *C. cellulolyticum* (Gal et al., 1997). All cellulosomes share similar characteristics, they all contain a large distinct protein, referred to as the scaffoldin which allows binding of the whole complex to microcrystalline cellulose via CBM. Also, the cellulosome scaffoldin expresses type I cohesins which allow binding of a wide variety of cellulolytic and hemicellulolytic enzymes within the complex via the expression of complementary type I dockerins on enzymes. Similarly, at the C-terminal the scaffoldin expresses type II cohesins, which allow the binding of the cellulosome to the cell through type II dockerins on surface layer homology proteins (SLH) (Fig. 4).

Cellulosomes are produced mainly by anaerobic bacteria, mostly from the class clostridia, and some anaerobic fungi such as genus *Neocallimastix* (Dalrymple et al., 1997), *Piromyces* (Teunissen et al., 1991) and *Orpinomyces* (Li et al., 1997). However, evidence suggests the presence of cellulosomes or cellulosome-like multienzyme complexes in a few aerobic microorganisms (Table 1). It is speculated that several other cellulolytic bacteria may also produce cellulosomes not yet described.

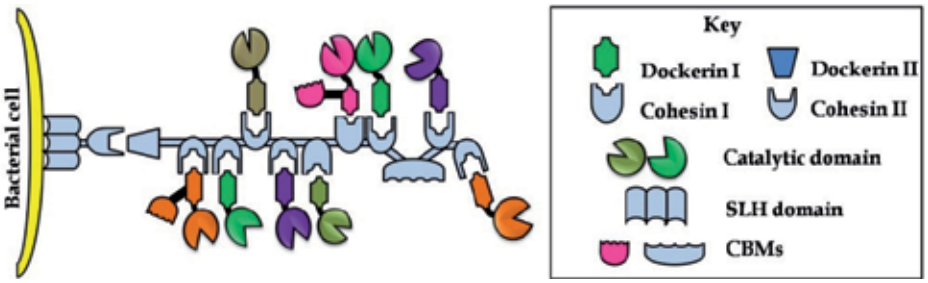

Figure 4. Simplified schematic of general cellulosome components and connection with cell surface based on knowledge of *Clostridium* cellulosome. (This figure is adapted from Bayer et al., 1994).

Anaerobic			Aerobic		
Microorganism	Source	Ref.	Microorganism	Source	Ref.
Bacteria			***Bacteria***		
Acetivibrio cellulolyticus	Sewage	Ding et al., 1999	*Bacillus circulans* F-2	Potato starch granules	Kim and Kim, 1993
Amorocellulobacter alkalithermophilum	Soil	Watthanalarm-lort et al., 2012	*Bacillus licheniformis* SVD1	Bioreactor	van Dyk et al., 2009
Bacteroides cellulosolvens	Sewage	Ding et al., 2000	*Paenibacillus curdlanolyticus* B-6	Anaerobic digester	Pason et al., 2006b
Bacteroides sp. strain P-1	Anaerobic digester	Ponpium et al., 2000	*Sorangium cellulosum*	Soil	Hou, et al., 2006
Butyrivibrio fibrisolvens	Rumen	Berger et al., 1990			
Clostridium acetobutylicum	Soil	Sabathé et al.,2002			
Clostridium cellobioparum	Rumen	Lamed et al., 1987	***Actinomycetes***		
Clostridium cellulolyticum	Compost	Pagès et al., 1997	*Streptomyces olivaceoviridis* E-86	Soil	Jiang et al., 2004
Clostridium cellulovorans	Fermenter	Sleat et al., 1984			
Clostridium josui	Compost	Kakiuchi et al., 1998	***Fungi***		
Clostridium papyrosolvens	Paper mill	Pohlschröder et al., 1994	*Chaetomium* sp. Nov. MS-017	Rotted wood	Ohtsuki et al., 2005
Clostridium thermocellum	Sewage soil	Lamed et al., 1983			
Eubacterium cellulosolvens	Rumen	Blair and Anderson, 1999b			
Ruminococcus albus	Rumen	Ohara et al., 2000			
Ruminococcus flavefaciens	Rumen	Ding et al., 2001			
Tepidimicrobium xylanilyticum BT14	Soil	Phitsuwan et al., 2010			

Anaerobic			Aerobic		
Microorganism	Source	Ref.	Microorganism	Source	Ref.
Thermoanaerobacterium thermosaccharolyticum NOI-1	Soil	Chimtong et al., 2011			
Fungi					
Neocallimastix patriciarum	Rumen	Dalrymple et al., 1997			
Orpinomyces joyonii	Rumen	Qiu et al., 2000			
Orpinomyces PC-2	Rumen	Borneman et al., 1989			
Piromyces equi	Rumen	Teunissen et al., 1991			
Piromyces E2	Faeces	Teunissen et al., 1991			

Table 1. Cellulosome and cellulosome-like multienzyme complexes from anaerobic and aerobic microorganisms. (This table is adapted from Doi & Kosugi, 2004).

2. Novel multienzyme complex system from *P. curdlanolyticus* strain B-6

Efficient enzymatic degradation of lignocellulosic biomass requires a tight interaction between the enzymes and their substrates, and the cooperation of multiple enzymes to enhance the hydrolysis due to the complex structure. Multienzyme complexes, cellulosomes from anaerobic cellulolytic microorganisms, are dedicated to hydrolyzing lignocellulosic substances efficiently because of a large variety of cellulases and hemicellulases in complexes, useful enzymatic properties, and binding ability to insoluble cellulose and/or xylan via CBMs (Bayer et al., 2004; Doi and Kosugi, 2004; Schwarz et al., 2001; Shoham et al., 1999). When compared with aerobic enzymes, they produce several individual enzymes, but microorganisms are not binding to insoluble substrates. However, *P. curdlanolyticus* B-6 was found to produce a multienzyme complex under aerobic conditions (Pason et al., 2006a, 2006b). Little information has been reported on cellulosome-like multienzyme complexes produced by aerobic bacterium (Kim & Kim, 1993; Jiang et al., 2004; van Dyk et al., 2009). Therefore, the multienzyme complex produced by strain B-6 is critical for improving plant biomass degradation.

2.1. Selection of multienzyme complex-producing bacteria under aerobic cultivation

Among several *Bacillus* strains, isolated from various sources and cultivated under aerobic conditions, *P. curdlanolyticus* strain B-6 shows important evidences for multienzyme complex producing bacterium (Pason et al., 2006a) as follows: high production of cellulase and xylanase, presence of CBMs that have ability to bind to insoluble substances, adhesion of bacterial cells to insoluble substances, and production of multiple cellulases and

xylanases in the form of a high molecular weight complex. Thus, strain B-6 exhibits great promise bacterium in the production of multienzyme complex under aerobic conditions. Some properties of bacterial cells and cellulase and xylanase from strain B-6 compared with other *Bacillus* spp. are shown in Table 2.

Strain (*Bacillus* sp.) and growth condition	Specific activity (U/mg protein)		Enzyme binding ability to insoluble substances (%)		Adhesion of cells to insoluble substances (%)		Zymogram analysis	
	CMCase	Xylanase	Avicel	Xylan	Avicel	Xylan	CMCase band	Xylanase band
1. Strain B-6								
Avicel grown	0.16	1.12	57.1	64.3	28.0	39.9	11	13
Xylan grown	0.12	7.19	39.1	51.5	13.6	74.7	9	15
2. Strain H-4								
Avicel grown	0.15	1.10	50.0	50.0	0	0	2	2
Xylan grown	0.09	4.23	31.1	38.5	0	0	1	3
3. Strain S-1								
Avicel grown	0.15	0.90	43.4	49.1	0	0	3	2
Xylan grown	0.09	4.49	37.9	45.8	0	0	2	3
4. Strain X-11								
Avicel grown	0	0	0	0	0	0	0	0
Xylan grown	0.05	3.29	29.2	45.0	0	0	0	2
5. Strain X-24								
Avicel grown	0	0	0	0	0	0	0	0
Xylan grown	0.06	3.19	29.6	36.1	0	0	0	2
6. Stain X-26								
Avicel grown	0	0	0	0	0	0	0	0
Xylan grown	0.04	3.10	28.2	38.2	0	0	0	2

Table 2. Production of carboxymethyl cellulase (CMCase) and xylanase by *Bacillus* strains; binding ability of enzymes to insoluble substances; adherence of bacterial cells to insoluble substances; and zymograms analysis in culture supernatant.

P. curdlanolyticus strain B-6 was a facultative, spore-forming, Gram-positive, motile, rod-shaped organism and produced catalase. Thus, this bacterium was identified as a member of the genus *Bacillus* according to Bergey's Manual of Systematic Bacteriology (Sneath, 1986). The bacterium was also identified by 16S rRNA gene sequence analysis. The use of a specific PCR primer designed for differentiating the genus *Paenibacillus* from other members of the *Bacillaceae* showed that this strain had the same amplified 16S rRNA gene fragment as a member of the genus *Paenibacillus*. Based on these observations, it is reckoned that this strain was transferred to the genus *Paenibacillus* (Shida et al., 1997). The 16S rDNA sequence of this strain had 1,424 base pairs and 97% similarity with *Paenibacillus curdlanolyticus* (Innis & Gelfand, 1990).

2.2. Characteristics of *P. curdlanolyticus* B-6 multienzyme complex

During growth of *P. curdlanolyticus* B-6 on Berg's mineral salt medium containing 0.5% xylan as carbon sources, the protein concentration in the medium was low up to the late stationary growth phase. CMCase and xylanase activities could be detected in the culture medium after the late exponential phase (Pason et al., 2006b). At the declining growth phase, the extracellular xylanase and CMCase rapidly increased due to the release of enzymes from the cell surfaces into the culture medium. These phenomena were different from the growth patterns of other aerobic bacteria, which grew and produced extracellular enzymes into culture supernatant immediately, but similar to those of the anaerobic bacteria which produced multienzyme complexes (cellulosomes) around the cell surfaces and adhered to these substrates and secreted into culture supernatant later (Bayer & Lamed, 1986; Lamed & Bayer, 1988). The observation of cell surfaces at the late exponential growth phase by scanning electron microscopy (SEM) revealed that the cells adhered to xylan (Fig. 5A), similar to the cells of the cellulosome producing anaerobic bacterium, *C. thermocellum*, which is a cell associated entity that mediates the adhesion of the bacterium to cellulose (Lamed et al., 1987; Mayer et al., 1987), whereas the surface of the cells of strain B-6 at the late stationary growth phase lacked such structures because the multienzyme complex was released into the medium from the cell surfaces (Fig. 5B). In addition, the pattern of multienzyme complex in the culture medium at the late stationary growth phase was determined. Native-polyacrylamide gel electrophoresis (native-PAGE) exhibited a high molecular weight band at the top of the gel (Fig. 6, lane 1). This protein band was dissociated into major and minor components through treatment by boiling in sodium dodecyl sulphate (SDS) solution, showing at least 18 proteins with molecular masses in the range of 29 to 280 kDa (Fig. 6, lane 2). Among those protein bands, at least 15 bands showed xylanase activities (Fig. 6, lane 3) and at least 9 bands showed CMCase activities (Fig. 6, lane 4) on zymograms. These multiple cellulases and xylanases are assembled into the high molecular weight complexes and released from the cell surfaces into medium at the late stationary growth phase. In *C. thermocellum*, the cellulosome consisted of many different types of glycosyl hydrolases, including cellulases, hemicellulases, and carbohydrate esterases, which served to promote their synergistic action (Lamed et al., 1983). These evidences confirm that the strain B-6 can produce xylanolytic-cellulolytic enzyme system that exists as multienzyme complex under aerobic conditions.

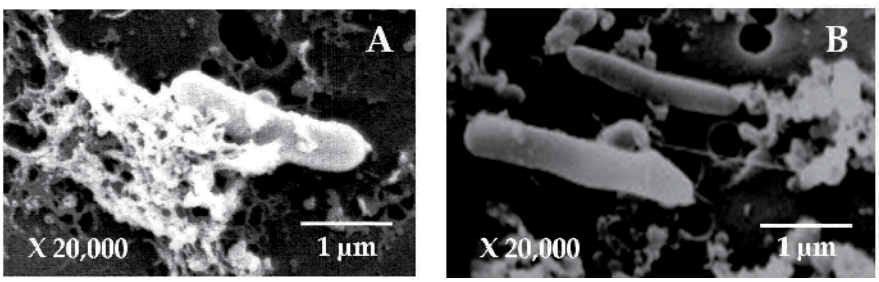

Figure 5. SEM of the cell surfaces of *P. curdlanolyticus* B-6 harvested at the late exponential growth phase showing adhesion of cell to xylan (A) and the cell harvested at the late stationary growth phase showing no adhesion of cell to xylan (B).

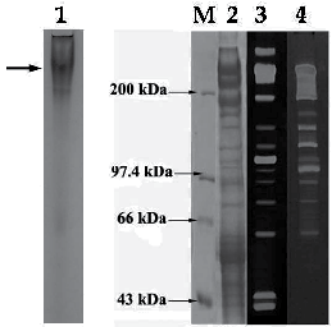

Figure 6. Proteins and enzymes patterns of multienzyme complex in culture supernatant at the late stationary growth phase; Native-PAGE (lane 1), SDS-PAGE (lane 2), and zymograms analysis of xylanase activity (lane 3), and CMCase activity (lane 4).

2.3. Effect of carbon sources on the induction of multienzyme complex in *P. curdlanolyticus* B-6

The effect of polymeric substances such as cellulose, xylan, corn hull, and sugarcane bagasse, and of soluble sugars such as L-arabinose, D-galactose, D-glucose, D-xylose, and cellobiose on the induction of multienzyme complexes in a facultatively anaerobic bacterium, *P. curdlanolyticus* B-6, was investigated under aerobic conditions (Waeonukul et al., 2008; 2009b). Cells grown on each carbon source adhered to cellulose. Hence strain B-6 cells from all carbon sources must have an essential component responsible for anchoring the cells to the substrate surfaces. Native–PAGE, SDS–PAGE, zymograms analysis, and enzymatic assays revealed that many proteins having xylanolytic and cellulolytic activities from *P. curdlanolyticus* B-6 grown on each carbon source were produced as multienzyme complex into the culture supernatants. These results indicated that strain B-6 produced multienzyme complexes when grown on both polymeric substances and soluble sugars. However, the subunits expressed in the multienzyme complex of strain B-6 depended on the carbon sources. These observations are consistent with previous reports that the enzymatic activities and enzyme compositions of the cellulosomes of *C. thermocellum* (Bayer et al., 1985; Bhat et al., 1993; Nochur et al., 1993), *C. cellulolyticum* (Mohand-Oussaid et al., 1999), and *C. cellulovorans* (Kosugi et al., 2001; Han et al., 2004; 2005) and the xylanosome of *S. olivaceoviridis* E-86 (Jiang et al., 2004) were affected by carbon sources in the media.

Many investigators have reported that the synthesis of cellulosome assemblies requires the presence of crystalline cellulose under anaerobic conditions, and that synthesis hardly occurs in growth on glucose or other soluble carbohydrates (Nochur et al., 1992; Blair & Anderson; 1999a; Bayer 2004; Doi & Kosugi, 2004). Some strains of *C. thermocellum* (Bayer et al., 1985; Bhat et al., 1993), however, can induce cellulosome synthesis when grown on cellobiose. *P. curdlanolyticus* B-6 differs from most cellulosome-producing microorganisms in that it produces multienzyme complex when grown on both polymeric substances and soluble sugars under aerobic conditions. Therefore, the mechanism of multienzyme complex formation by strain B-6 must be different from that of other microorganisms.

3. The feature of *P. curdlanolyticus* B-6 multienzyme complex

Recently, the structures and mechanisms for assembly of multienzyme complexes, cellulosomes, in anaerobic cellulolytic microorganisms are clear (Bayer et al., 2004, 2007; Doi & Kosugi, 2004). Generally, the key feature of the cellulosome is a scaffoldin that integrates the various catalytic subunits into the complex by self-assembly by cohesion-dockerin interaction. However, the structure and mechanism of the multienzyme complex produced by a facultatively anaerobic bacterium, such as *P. curdlanolyticus* B-6 is still unknown. In order to describe features of the multienzyme complex system produced by strain B-6, the multienzyme complex was purified by four kinds of chromatography (cellulose affinity, gel filtration, anion-exchange and hydrophobic-interaction chromatographys) (Fig. 7).

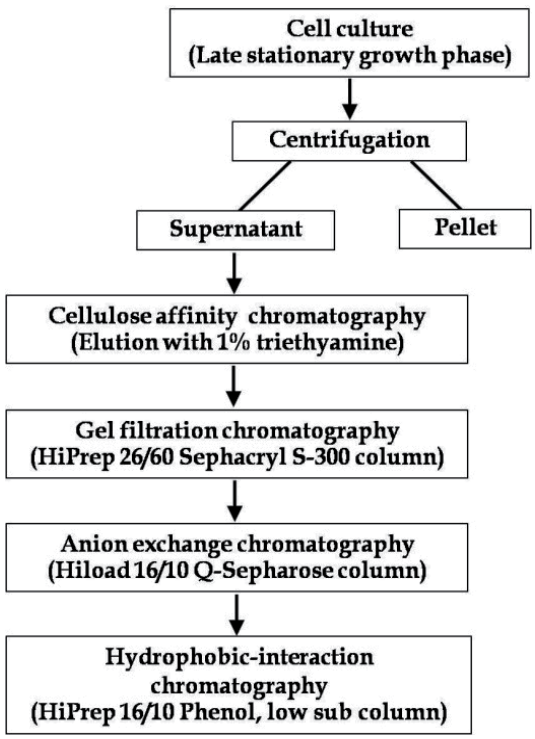

Figure 7. Isolation and purification of multienzyme complex of *P. curdlanolyticus* strain B-6.

The multienzyme complex of *P. curdlanolyticus* strain B-6 with molecular mass of 1,450 (G1) was isolated from culture supernatant at the late stationary growth phase through cellulose affinity and Sephacryl S-300 gel filtration chromatographys (Pason et al., 2006b). Basically, the individual cellulosomes from anaerobic bacteria show 600 kDa to 2.1 MDa complexes size and show cohesion-dockerin domain as a signature protein (Bayer et al., 2004; Doi & Kosugi, 2004). While, multienzyme complexes from aerobic microorganisms, were range in mass from about 468 kDa to 2 MDa (with contained 5-12 protein subunits) (Table 3) and has no report of cohesion-dockerin domain. Here, the multienzyme complex produced by strain B-6 under aerobic conditions was the first report on characterization.

Multienzyme complex	Mol. Mass (kDa)	Protein subunits	Ref.
Aerobic microorganisms			
Paenibacillus curdlanolyticus B-6	1450	11	Pason et al.,2006b
Bacillus circulans F-2	669	7	Kim and Kim, 1993
Bacillus licheniformis SVD1	2000	12	van Dyk et al., 2009
Sorangium cellulosum	1000-2000	10	Hou et al., 2006
Streptomyces olivaceoviridis E-86	1200	5	Jiang et al., 2004
Chaetomium sp. Nov. MS-017	468	12	Ohtsuki et al., 2005
Anaerobic microorganisms			
Clostridium acetobutylicum	665	11	Sabathé et al.,2002
Clostridium cellulolyticum	600	14	Gal et al., 1997
Clostridium cellulovorans	900	10	Shoseyov & Doi 1990
Clostridium josui	700	14	Kakiuchi et al., 1998
Clostridium popyrosolvens	600	15	Pohlschröder et al., 1994
Clostridium thermocellum	2100	14	Lamed et al., 1983
Ruminococcus albus	1500	15	Ohara et al., 2000

Table 3. Molecular weights and protein subunits of multienzyme complexes from aerobic and anaerobic microorganisms.

Elucidation of the purified multienzyme feature of *P. curdlanolyticus* strain B-6 was followed by anion-exchange and hydrophobic-interaction chromatographys (Pason et al., 2010). The complex G1 from gel filtration chromatography (1,450 kDa) was purified by anion-exchange chromatography and showed at least five large protein complexes or aggregates, namely F1-F5. Among the fractions obtained from anion-exchange chromatography, F1 was apparently the most suited fraction to study on the organization and function of the multienzyme system of strain B-6 because F1 formed one clear band on the top of native PAGE, had the highest xylanase activity, and its subunit composition was clearly shown on SDS-PAGE. In the final step, complex F1 was separated to one major complex (H1) and two minor protein components (H2 and H3) by hydrophobic-interaction chromatography. The multienzyme complex (H1) was composed of a 280 kDa protein with xylanase activity, a 260 kDa protein that is a truncated form on the C-terminal side of the 280 kDa protein, two xylanases of 40 and 48 kDa, and 60 and 65 kDa proteins having both xylanase and CMCase activities (Fig. 8). The two components (280 and 40 kDa) of the multienzyme complex has characteristics similar to the cellulosome of *C. thermocellum* in that it is composed of a scaffolding protein and a catalytic subunit (Bayer et al., 1998; Demain et al., 2005). The 280 kDa protein resembled the scaffolding proteins of the multienzyme complex based on its migratory behavior in polyacrylamide gels and as a glycoprotein. The 280 kDa protein and a 40 kDa major xylanase subunit are the key components of multienzyme complex of the strain B-6.

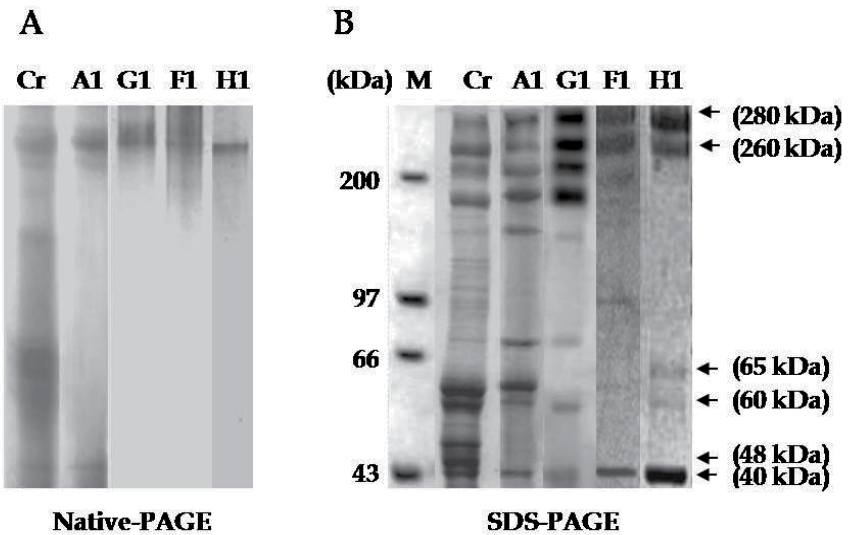

Figure 8. Native-PAGE (A) and SDS-PAGE (B) in isolated complex from culture supernatant at the late stationary growth phase (lane Cr), affinity column (lane A1), gel filtration column (lane G1), anion-exchange column (lane F1) and hydrophobic-interaction column (lane H1). All samples contained 200 μg of protein.

These apparently propose that *P. curdlanolyticus* B-6 produced multienzyme complex, which consisted of many subunit compositions. The large protein (280 kDa) may function as a scaffoldin-like protein that allowed the enzyme subunits, majority is 40 kDa, binding to form a multienzyme complex. The key components, 280 and 40 kDa, are identified in the next topic.

4. Molecular structure of important xylanases

P. curdlanolyticus B-6 produces an extracellular xylanolytic-cellulolytic multienzyme complex mainly comprised of xylanases under aerobic conditions. To understand the xylanase system, a genomic library of the strain B-6 was constructed and screened for high xylanase activity. Recently, six xylanase genes, *S1* (Pason et al., 2010), *xyn10A* (Waeonukul et al., 2009a), *xyn10B* (Sudo et al., 2010), *xyn10C* (unpublished data), *xyn10D* (Sakka et al., 2011) and *xyn11A* (Pason et al., 2010) were cloned, and the translated products were characterized (Table 4).

Enzyme	Modular structure	GH family	Mol. Mass (kDa)	GenBank accession No.
S1		U	91	-
Xyn10A		10	142	EU418764
Xyn10B		10	40	AB570291
Xyn10C		10	35	AB688987
Xyn10D		10	61	AB600191
Xyn11A		11	40	FJ956758

Abbreviations: CBM, carbohydrate-binding module; Fn, fibronectin homology module; GH, glycosyl hydrolase; SLH, surface layer homology domain; U, unhomology sequence

Table 4. Modular structure xylanases of *P. curdlanolyticus* strain B-6.

S1 protein: From the early research, the 280 kDa subunit (S1) plays a role of scaffoldin in assembling the enzyme complex and shows xylanase activity (Pason et al., 2010). The *S1* gene consists of 2,589 nucleotides and encodes 863 amino acids with a molecular weight of 91,000 Da, indicating that the 280 kDa subunit is highly glycosylated. Sequence analysis revealed that S1 did not have significant homology with any proteins in the databases except for two surface layer homology (SLH) domains in its N-terminal region. Surprisingly, the recombinant S1 exhibits xylanase activity, and cellulose- and xylan-binding ability, suggesting that the S1 should be a novel xylanase and CBM(s) with new functions (unpublished data).

Xylanase Xyn10A: The *xyn10A* gene consists of 3,828 nucleotides encoding a protein of 1,276 amino acids with a predicted molecular weight of 142,726 Da. Xyn10A is a multidomain enzyme comprised of nine domains in the following order: three family-22 CBMs, a family-10 catalytic domain of glycosyl hydrolases (GH), a family-9 CBM, a glycine-rich region, and three SLH domains. Xyn10A can effectively hydrolyze insoluble xylan and natural biomass without pretreatment such as sugarcane bagasse, corn hull, rice bran, rice husk and rice straw. Xyn10A binds to various insoluble polysaccharides such as cellulose, xylan and chitin. The SLH domains functioned in Xyn10A by anchoring this enzyme to the cell surfaces of *P. curdlanolyticus* B-6. Removal of the CBMs from Xyn10A strongly reduced the ability of binding and plant cell wall hydrolysis. Therefore, the CBMs of Xyn10A play an important role in the hydrolysis of native biomass materials (Waeonukul et al., 2009a).

Xylanase Xyn10B: The *xyn10B* gene consists of 1,047 nucleotides encoding a protein of 349 amino acids with a predicted molecular weight of 40,480 Da. Xyn10B consists of only a family-10 catalytic of GH. Xyn10B is an intracellular endoxylanase (Sudo et al., 2010).

Xylanase Xyn10C: The *xyn10C* gene consists of 957 nucleotides and encodes 318 amino acid residues with a predicted molecular weight of 35,123 Da. Xyn10C is a single module enzyme consisting of a signal peptide and a family-10 catalytic module of GH (unpublished data).

Xylanase Xyn10D: The *xyn10D* gene consists of 1,734 nucleotides and encodes 577 amino acid residues with a calculated molecular weight of 61,811 Da. Xylanase Xyn10D is a modular enzyme consisting of a family-10 catalytic module of the GH, a fibronectin type-3 homology (Fn3) module, and family-3 CBM, in that order, from the N terminus. The CBM3 in Xyn10D has an affinity for cellulose and xylan, and plays an important role in hydrolysis of arabinoxylan and native biomass materials (Sakka et al., 2011).

Xylanase Xyn11A: The *xyn11A* gene consists of 1,150 bp and encodes a protein of 385 amino acids with a molecular weight of 40,000 Da. Xyn11A is composed of two major functional domains, a catalytic domain belonging to family-11 GH and a CBM classified as family-36. A glycine- and asparagine-repeated sequences existed between the two domains. Xyn11A has been identified to be one of the major xylanase subunit in the multienzyme complex of strain B-6 (Pason et al., 2010).

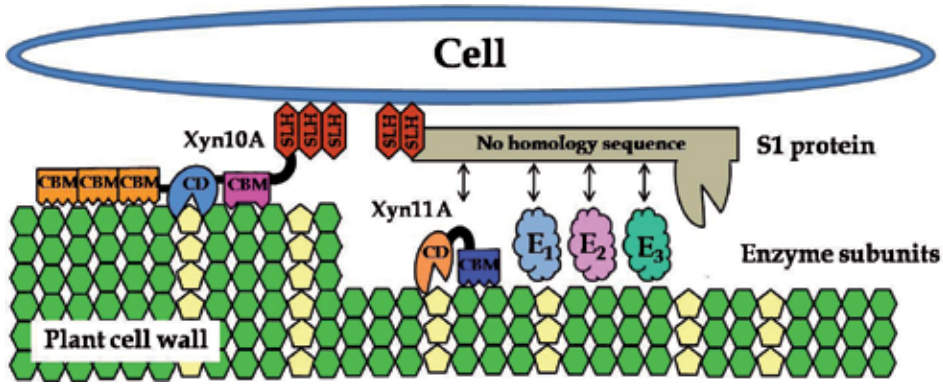

Figure 9. Simplified schematic view of the interaction between the *P. curdlanolyticus* B-6 multienzyme complex system and its substrate, and its connection to the cell surface via an associated anchoring protein. (Abbreviations: CBM, carbohydrate-binding module; CD, catalytic domain, E_n, enzyme subunit; SLH, surface layer homology domain).

Based on both biochemical and molecular biological findings, a simplistic schematic view of the enzyme system from *P. curdlanolyticus* B-6 and its interaction with substrate and cell surface was created and presented in Fig. 9. In this assessment, the S1 protein did not have significant homology with any proteins in the databases except for two S-layer homology domains in its N-terminal region. However, the S1 protein that exhibits xylanase activity and cellulose- and xylan-binding ability, and contains cell anchoring function, seems remarkable. The multifunctional protein S1 is also responsible for forming the enzyme subunits into the complex and anchoring the complex into cell surface via the SLH domain. The interaction between S1 protein and enzyme subunits should be a mechanism distinct from the cohesion-dockerin interaction known in cellulosome of anaerobic microorganisms, since cohesion- or dockerin– like sequences were not observed in the S1 protein or the major

xylanase subunit, Xyn11A. In addition, strain B-6 also produces cell bound multimodular xylanase Xyn10A that contains the numerous CBMs and SLH domains. Xyn10A can bind to the plant cell wall through CBM, whereas the catalytic module (GH10) is able to access its target substrate. Thus, the CBM greatly increases the concentration of the enzyme in the vicinity of the substrate, leading to the observed increase in polysaccharide hydrolysis. Besides, the presence of the functional CBMs and SLH domains in Xyn10A allows the cells to attach to substrate. Although, the overall structure of the enzyme complex system of the strain B-6 is not entirely clear, the enzyme complex has unique characteristics distinct from multienzyme complex cellulosome of anaerobic microorganisms. However, the mechanism for complex formation, interaction between the S1 protein as scaffoldin and enzyme subunits, needs to be further investigated.

5. Biotechnological uses of *P. curdlanolyticus* B-6 multienzyme complex

Biological conversion of lignocellulosic materials has been proposed as a renewable and sustainable route for the production of value-added products (Bayer et al., 2007, Doi et al., 2003). There is much interest in exploiting the properties of multienzyme complexes for practical purposes. The facultative bacterium, *P. curdlanolyticus* strain B-6 produces a unique extracellular multienzyme system under aerobic conditions that effectively degrade cellulose and hemicellulose by gaining access through the protective matrix surrounding the cellulose microfibrils of plant cell walls. Therefore, the multienzyme complex from strain B-6 is a promising enzyme which can potentially be used in many applications, such as enhancing extraction and production of value-added bioproducts by saccharification of cell wall components and application for construction of the modular enzymes creation (Fig. 10).

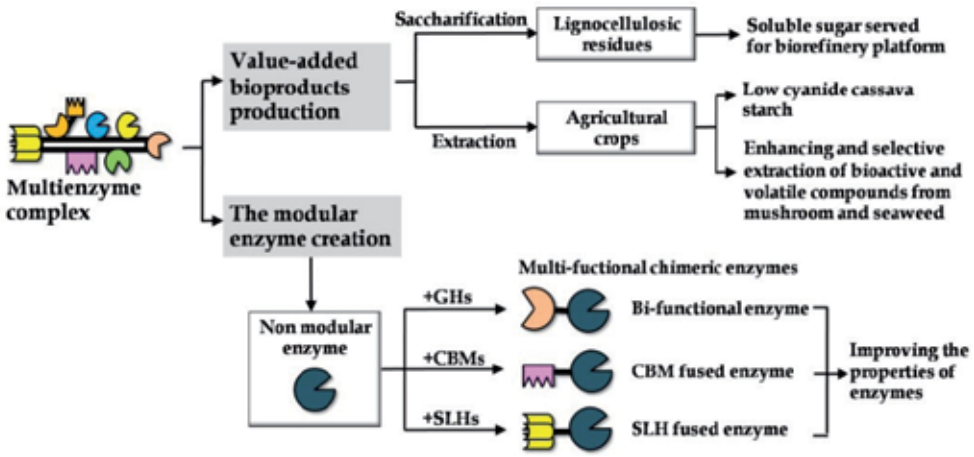

Figure 10. The multienzyme complex of *P. curdlanolyticus* strain B-6 for biotechnological applications.

Biological treatment and saccharification using microorganisms and their enzymes selectively for degradation of lignocellulosic residues has the advantages of low energy consumption, minimal waste production, and environmental friendliness (Schwarz, 2001). The catalytic components of the multienzyme complex release soluble sugars, simple 5- and 6-carbon, from lignocellulose providing the primary carbon substrates, which can be subsequently converted into fuels by microorganisms. For enzyme saccharification, the close proximity between cellulolytic and xylanolytic enzymes is key to concerted degradation of the substrate, whereby the activities of the different enzymes facilitate the activities of their counterparts by promoting access to appropriated portions of the rigid insoluble substrates, since the release of sugar products was high. The synergistic action of the combination of enzymes by different modes of actions (xylanases and cellulases) and the presence of xylan- or cellulose-binding ability on lignocellulose enhanced soluble sugars released from the plant cell walls. In practicality, the multienzyme complex produced from *P. curdlanolyticus* B-6 allows access to lignocellulosic substrate and produces reducing sugar more than non-complexed enzymes from fungi (*T. viride* and *Aspergillus niger*) when the same cellulase activity (0.1 unit) was applied for degradation of corn hull and rice straw residues (unpublished data). In addition, the multienzyme complex of strain B-6 has been used to improve the extraction of plant food such as making low-cyanide-cassava starch by using multienzyme complex to enhance linamarin released by allowing more contact between linamarase and linamarin (Sornyotha et. al., 2009). Also, extraction of volatile compounds such as sea food-like flavor from seaweed, served for food supplement. Consequently, enzymatic treatment has advantages for the preparation of β-glucan and acidic α-glucan-protein complex from the fruiting body of mushroom, *Pleurotus sajor-caju* because the specificity of the multienzyme complex and gentle conditions allow for the recovery of high purity glucans in their native forms with minimal degradation (Satitmanwiwat et al., 2012a,b).

Typically, most plant cell wall degrading enzymes are composed of a series of separate modules (modular enzymes). These domains may fold and function in an independent manner and are normally separated by short linker. *P. curdlanolyticus* B-6, produces a number of glycosyl hydrolase (GHs) families and CBM families which have different substrates recognition affinity and increase amorphous regions of cellulose by H-bond elimination. Interestingly, modular architecture created by chimeric proteins creation with various tandem CBMs, GHs, and SLH-specific, should make it possible to construct effective lignocellulosic degrading enzymes, strongly binding, targeting enzyme to their substrates and bacterial cell surfaces for enhancing a variety of substrates hydrolysis. The strong carbohydrate-binding property of the cellulose-binding domain and xylan-binding domain, specific degradative activities exhibit important properties of the lignocellulosic material degrading enzymes that can be used in biotechnology.

6. Conclusion

A facultatively anaerobic bacterium *P. curdlanolyticus* strain B-6, isolated from an anaerobic digester fed with pineapple wastes, is unique in that it produces extracellular xylanolytic-

cellulolytic multienzyme complex capable of efficient degradation of plant biomass materials under aerobic conditions. The production of strain B-6 multienzyme complex under aerobic conditions has several advantages: (i) a simple process, (ii) low price of medium, (iii) high growth rate, (iv) large quantities of extracellular enzymes yields, and (v) safe use with regard to health and environmental aspects. Thus, strain B-6 and its multienzyme complex is a promising tool for an industrial process employing direct hydrolysis for the bioconversion of cellulose as well as hemicellulose in biomass. This review shows that strain B-6 multienzyme complex is a novel enzymatic system known at the biochemical, genetic, and mechanism level. It also stresses that some points still need to be further investigated, mainly (i) the elucidation of scaffolding protein functions, (ii) the characterization of others key enzyme subunits, (iii) the assembly mechanism of the multienzyme complex, (iv) improvement of the efficiency in degradation of biomass of the multienzyme complex, and (v) improvement of the production of the multienzyme complex. The latter will certainly represent a challenge for future research.

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Chapter 17

Methanogenic System of a Small Lowland Stream Sitka, Czech Republic

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Additional information is available at the end of the chapter

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1. Introduction

Methane (CH_4) is an atmospheric trace gas present at concentration of about 1.8 ppmv, that represents about 15% of the anthropogenic greenhouse effect (Forster et al. 2007). The atmospheric CH_4 concentration has increased steadily since the beginning of the industrial revolution (~ 0.7 ppmv) and is stabilized at ~1.8 ppmv from 1999 to 2005 (Forster et al. 2007). An unexpected increase in the atmospheric growth of CH_4 during the year 2007 has been recently reported (Rigby et al. 2008), indicating that the sources and sinks of atmospheric CH_4 are dynamics, evolving, and not well understood. Freshwater sediments, including wetlands, rice paddies and lakes, are thought to contribute 40 to 50 % of the annual atmospheric methane flux (Cicerone & Oremland 1988; Conrad 2009).

The river hyporheic zone, volume of saturated sediment beneath and beside streams containing some proportion of water from surface channel, plays a very important role in the processes of self-purification because the river bed sediments are metabolically active and are responsible for retention, storage and mineralization of organic matter transported by the surface water (Hendricks 1993; Jones & Holmes 1996, Baker et al. 1999, Storey et al. 1999, Fischer et al. 2005). The seemingly well-oxygenated hyporheic zone contains anoxic and hypoxic pockets („anaerobic microzones") associated with irregularities in sediment surfaces, small pore spaces or local deposits of organic matter, creating a 'mosaic' structure of various environments, where different microbial populations can live and different microbially mediated processes can occur simultaneously (Baker et al. 1999, Morrice et al. 2000, Fischer et al. 2005). Moreover, hyporheic-surface exchange and subsurface hydrologic flow patterns result in solute gradients that are important in microbial metabolism. Oxidation processes may occur more readily where oxygen is replenished by surface water infiltration, while reduction processes may prevail where surface-water exchange of oxygen

is less, and the reducing potential of the environment is greater (Hendricks 1993). As water moves through the hyporheic zone, decomposition of the organic matter consumes oxygen, creating oxygen gradients along the flow path. Thus,compared to marine or lake surface sediments, where numerous studies on O_2 profiles have showed that O_2 concentrations become zero within less than 3 mm from the surface, the hyporheic sediment might be well-oxygenated habitats even up to the depth of 80 cm (e.g. Bretschko 1981, Holmes et al. 1994) . The extent of the oxygen gradient is determined by the interplay between flow path lengh, water velocity, the ratio of surface to ground water, and the amount and quality of organic matter. Organic matter decomposition in sediments is an important process in global and local carbon budgets as it ultimately recycles complex organic compounds from terrestrial and aquatic environments to carbon dioxide and methane. Methane is a major component in the carbon cycle of anaerobic aquatic systems, particularly those with low sulphate concentrations. Since a relatively high production of methane has been measured in river sediments (e.g. Schindler & Krabbenhoft 1998, Hlaváčová et al. 2005, Sanders et al. 2007, Wilcock & Sorrell 2008, Sanz et al. 2011), we proposed that river sediments may act as a considerable source of this greenhouse gas which is important in global warming (Hlaváčová et al. 2006).

Breakdown of organic matter and gas production are both results of well functioned river self-purification. This degrading capacity, however, requires intensive contact of the water with biologically active surfaces. Flow over various morphological features ranging in size from ripples and dunes to meanders and pool-riffle sequences controls such surface-subsurface fluxes. Highly permeable streambeds create opportunities for subsurface retention and long-term storage, and exchange with the surface water is frequent. Thus, study of the methane production within hyporheic zone and its subsequent emission to the atmosphere can be considered as a measure of mineralization of organic matter in the freshwater ecosystem and might be used in evaluation of both the health and environmental quality of the rivers studied.

Methane (CH_4) is mostly produced by methanogenic archaea (Garcia et al. 2000, Chaban et al. 2006) as a final product of anaerobic respiration and fermentation, but there is also aerobic methane formation (e.g. Karl et al. 2008). Methanogenic archaea are ubiquitous in anoxic environments and require an extremely low redox potential to grow. They can be found both in moderate habitats such as rice paddies (Grosskopf et al. 1998a,b), lakes (Jürgens et al. 2000, Keough et al. 2003) and lake sediments (Chan et al. 2005), as well as in the gastrointestinal tract of animals (Lin et al. 1997) and in extreme habitats such as hydrothermal vents (Jeanthon et al. 1999), hypersaline habitats (Mathrani & Boone 1995) and permafrost soils (Kobabe et al. 2004, Ganzert et al. 2006). Rates of methane production and consumption in sediments are controlled by the relative availability of substrates for methanogenesis (especially acetate or hydrogen and carbon dioxide). The most important immediate precursors of methanogenesis are acetate and H_2/CO_2. The acetotrophic methanogens convert acetic acid to CH_4 and CO_2 while the hydrogenotrophic methanogens convert CO_2 with H_2 to CH_4 (Conrad 2007).

Methane oxidation can occur in both aerobic and anaerobic environments; however, these are completely different processes involving different groups of prokaryotes. Aerobic methane oxidation is carried out by aerobic methane oxidizing bacteria (methanotrophs, MOB), while anaerobic methane oxidizers, discovered recently, thrive under anaerobic conditions and use sulphate or nitrate as electron donors for methane oxidation (e.g. Strous & Jetten 2004). MOB are a physiologically specialized group of methylotrophic bacteria capable of utilizing methane as a sole source of carbon and energy, and they have been recognized as major players in local and global elemental cycling in aerobic environments (Hanson & Hanson 1996, Murrell et al. 1998, Costelo & Lidstrom 1999, Costelo et al. 2002, McDonald et al. 2008). Aerobic MOB have been detected in a variety of environments, and in some they represent significant fractions of total microbial communities (e.g. Henckel et al. 1999; Carini et al, 2005, Trotsenko & Khmelenina 2005, Kalyuzhnaya et al. 2006). However, the data on the diversity and activity of methanotrophic communities from the river ecosystems are yet fragmentary. Methanotrophs play an important role in the oxidation of methane in the natural environment, oxidizing methane biologically produced in anaerobic environments by the methanogenic archaea and thereby reducing the amount of methane released into the atmosphere.

The present investigation is a part of a long-term study focused on organic carbon and methane dynamics and microbial communities in hyporheic zone of a Sitka, small lowland stream in Czech Republic. The overall purpose of this research was to characterize spatial distribution of both methanogens and methanotrophs within hyporheic sediments and elucidate the differences in methane pathways and methane production/consumption as well as methane fluxes and atmospheric emissions at different sites along a longitudinal profile of the stream.

2. Material and methods

2.1. Study site

The sampling sites are located on the Sitka stream, Czech Republic (Fig. 1). The Sitka is an undisturbed, third-order, 35 km long lowland stream originating in the Hrubý Jeseník mountains at 650 m above sea level. The catchment area is 118.81km^2, geology being composed mainly of Plio-Pleistocene clastic sediments of lake origin covered by quaternary sediments. The mean annual precipitation of the downstream part of the catchment area varies from 500 to 600 mm. Mean annual discharge is 0.81 m^3.s^{-1}. The Sitka stream flows in its upper reach till Šternberk through a forested area with a low intensity of anthropogenic effects, while the lower course of the stream naturally meanders through an intensively managed agricultural landscape. Except for short stretches, the Sitka stream is unregulated with well-established riparian vegetation. River bed sediments are composed of gravels in the upper parts of the stream (median grain size 13 mm) while the lower part, several kilometres away from the confluence, is characterised by finer sediment with a median grain size of 2.8 mm. The Sitka stream confluences with the Oskava stream about 5 km north of Olomouc. More detailed characteristics of the geology, gravel bar, longitudinal

physicochemical (e.g. temperature, pH, redox, conductivity, O_2, CH_4, NO_3, SO_4) patterns in the sediments and a schematic view of the site with sampling point positions have been published previously (Rulík et al. 2000, Rulík & Spáčil 2004). Earlier measurements of a relatively high production of methane, as well as potential methanogenesis, confirmed the suitability of the field sites for the study of methane cycling (Rulík et al. 2000, Hlaváčová et al. 2005, 2006).

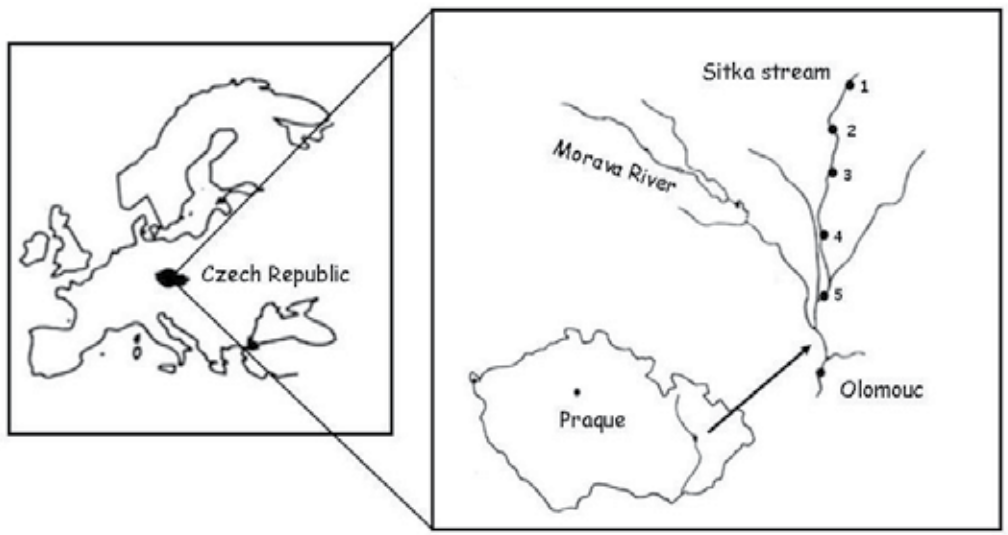

Figure 1. A map showing the location of the Sitka stream. Black circles represents the study sites (1-5)

2.2. Sediment sample collecting and sample processing

Five localities alongside stream profile were chosen for sampling sediment and interstitial water samples based on previous investigations (Figure 2, Table 1). Hyporheic sediments were collected with a freeze-core using N_2 as a coolant (Bretschko & Klemens 1986) throughout summer period 2009-2011. At each locality, three cores were taken for subsequent analyses. After sampling, surface 0-25 cm sediment layer and layer of 25-50 cm in depth were immediately separated and were stored at a low temperature whilst being transported to the laboratory. Just after thawing, wet sediment of each layer was sieved and only particles < 1 mm were considered for the following microbial measurements and for all microbial activity measurements since most of the biofilm is associated with this fraction (Leichtfried 1988).

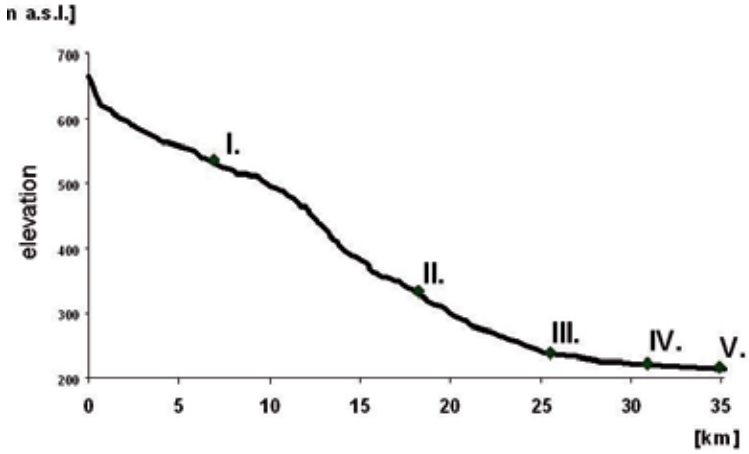

Figure 2. Graphic depiction of the thalweg of the Sitka stream with sampling localities. The main source of pollution is an effluent from Šternberk city sewage water plant, located just in the middle between stretch II and III.

Variable/ Locality	I.	II.	III.	IV.	V.
elevation above sea-level [m]	535	330	240	225	215
distance from the spring [km]	6,9	18,2	25,6	30,9	34,9
channel width [cm]	523	793	672	444	523
average flow velocity [m.s^{-1}]	0,18	0,21	0,46	0,42	0,18
stretch longitude [km]	12,6	9,3	6,3	4,7	2,3
stretch surface area [km^2]	0,043	0,06	0,043	0,024	0,012
stretch surface area (%)	24	32	24	13	7
dominant substrate composition	gravel	gravel	gravel	silt-clay	gravel-sand
grain median size [mm]	12,4	12,9	13,2	0,2	5,4
surface water PO_4^{3-} [mg L^{-1}]	0,15	0,24	7,0	2,6	1,8
surface water N - NO_3^- [mg L^{-1}]	0,01	0,21	1,2	0,5	0,18
surface water N - NH_4^+ [mg L^{-1}]	0,39	0,26	0,66	0,72	0,61
surface dissolved oxygen saturation [%]	101,7	110,0	105,8	108,5	103,5
surface water conductivity [μS.cm^{-1}]	107,5	127,5	404,8	394,0	397,7
hyporheic water conductivity [μS.cm^{-1}]	115,3	138,3	414,5	506,5	416,2
surface water temperature [°C]	8,1	9,7	10,7	11,1	8,9
surface water DOC [mg L^{-1}]	2,47	0,81	2,62	2,69	3,74
hyporheic water DOC [mg L^{-1}]	2,05	1,31	2,71	5,76	2,62

Table 1. Longitudinal physicochemical patterns of the Sitka stream (annual means). Hyporheic water means mix of interstitial water taken from the depth 10 up to 50 cm of the sediment depth

A few randomly selected subsamples (1 mL) were used for extraction of bacterial cells and, consequently, for estimations of bacterial numbers; other sub-samples were used for

measurement of microbial activity and respiration, organic matter content determination, etc. Sediment organic matter content was determined by oven-drying at 105 °C to constant weight and subsequent combustion at 550 °C for 5 hours to obtain ash-free dry weight (AFDW). Organic matter values were then converted to carbon equivalents assuming 45 % carbon content of organic matter (Meyer et al. 1981). Sediment from another freeze-core was oven-dried at 105 °C and subjected to granulometric analysis. Grain size distribution and descriptive sediment parameters were computed using the database SeDi (Schönbauer & Lewandowski 1999).

2.3. Water samples and analysis of methane

Surface water was collected from running water at a depth of 10 cm below the surface level in autumn 2009 at each study site. Interstitial water samples were collected using a set of 5–6 minipiezometers (Trulleyová et al. 2003) placed at a depth of about 20-50 cm randomly in sediments at each study site. The initial 50–100 mL of water was used as a rinse and discarded. As usual, two subsamples of interstitial water from each minipiezometer were collected from a continuous column of water with a 100 mL polypropylene syringe connected to a hard PVC tube, drawn from a minipiezometer and injected into sterile, clear vials (40 mL) with screw-tops, covered by a polypropylene cap with PTFE silicone septa (for analysis of dissolved gasses) and stored before returning to the laboratory. All samples were taken in the morning between 9 a.m. and 12 noon. All measurements were done during the normal discharge levels (i.e. no spates or high flood levels were included). Interstitial water temperature, dissolved oxygen (mg L^{-1} and percent saturation) and conductivity were measured in the field with a portable Hanna HI 9828 pH/ORP/EC/DO meter. Dissolved organic carbon (DOC) was measured by Pt-catalysed high temperature combustion on a TOC FORMACS^{HT} analyser. Long term observation of interstitial water temperature was carried out using temperature dataloggers Minikin (EMS Brno, Czech Republic) buried in the sediment depth of 25-30 cm for a period of one year. Dissolved ferrous iron (Fe^{2+}) concentration was measured using absorption spectrophotometry after reaction with 1,10-phenanthroline. Concentrations of organic acids were meausred using capilary electrophoresis equipped with diode array detector HP 3D CE Agilent (Waldbron, Germany). Limits of detection for particular organic acids were set as following: LOD (acetate) = 6,2 μmol L^{-1}; LOD (propionate) = 4,8 μmol L^{-1}; LOD (butyrate) = 2,9 μmol L^{-1}; LOD 32 (valerate) = 1,8 μmol L^{-1}.

Concentrations of dissolved methane in the stream and interstitial water were measured directly using a headspace equilibration technique. Dissolved methane was extracted from the water by replacing 10 mL of water with N_2 and then vigorously shaking the vials for 15 seconds (to release the supersaturated gas from the water to facilitate equilibration between the water and gas phases). All samples were equilibrated with air at laboratory temperature. Methane was analysed from the headspace of the vials by injecting 2ml of air sub-sample with a gas-tight syringe into a CHROM 5 gas chromatograph, equipped with the flame ionization detector (CH4 detection limit = 1μg L^{-1}) and with the 1.2m PORAPAK Q column (i.d. 3 mm), with nitrogen as a carrier gas. Gas concentration in water was calculated using

Henry's law. The saturation ratio (R) was calculated as the measured concentration of gas divided by the concentration in equilibrium with the atmosphere at the temperature of the water sample using the solubility data of Wiesenburg & Guinasso (1979).

2.4. Methanogenic potential and methanotrophic activity

The rate of methane production (methanogenesis) was measured using the PMP method (Segers 1998). C-amended solutions (flushed for 5 minutes with N_2) with acetate $Ca(CH_3COO)_2$ (100 mg C in the incubation flask) were used for the examination of methanogenic potential. All laboratory sediment incubations were performed in 250-mL dark glass flasks, capped with rubber stoppers, using approximately 100 g (wet mass) of sediment (grain size < 1 mm) and 180 mL of amended solution or distilled water. The headspace was maintained at 20 mL. Typically, triplicate live and dead (methanogenesis was inhibited by addition of 1.0 mM chloroform) samples from each depth were stored at 20°C in the dark and the incubation time was 72 hours; however, subsamples from the headspace atmosphere were taken every 24 hours. Gas production was calculated from the difference between final and initial headspace concentration and volume of the flask; results are expressed per volume unit of wet sediment ($CH4\ mL^{-1}$ WW $hour^{-1}$) or per unit dry weight of sediment per one day ($\mu g\ CH_4\ kg^{-1}$ DW day^{-1}). Rate of potential methane oxidation (methanotrophy) was measured using modified method of methane oxidation in soil samples from Hanson (1998). Briefly, 50 mL of methane was added by syringe to the closed incubation flask with the sieved sediment and then the pressure was balanced to atmospheric pressure. All laboratory sediment incubations were performed in 250-mL dark glass flasks, capped with rubber stoppers, using approximately 100 g (wet mass) of sediment (grain size < 2 mm). Typically, triplicate live and dead (samples killed by $HgCl_2$ to arrest all biological activity) samples from each depth were stored at 20°C in the dark, and incubation time was 72 hours; however, subsamples from the headspace atmosphere were taken every 24 hours. Potential CH_4 oxidation rates at the different concentrations were obtained from the slope of the CH_4 decrease with time ($r^2 > 0.90$; methane oxidation was calculated from the difference between final and initial headspace concentration and volume of the flask; results are expressed per volume unit of wet sediment ($CH4\ mL^{-1}$ WW $hour^{-1}$) or per unit dry weight of sediment per one day (mg $CH_4\ kg^{-1}$ DW day^{-1}).

2.5. Fluxes of methane across the sediment-water interface

Fluxes of methane across the sediment-water interface were estimated either by direct measurement with benthic chambers or calculated by applying Fick´s first law.

Benthic fluxes

The methane fluxes across the sediment-water interface were measured using the method of benthic chambers (e.g. Sansone et al. 1998). Fluxes were measured during the summer months (VII, VIII, IX). The plexiglas chamber (2.6 dm^3) covered an area 0.0154 m^2. The chambers (n = 7) were installed randomly and gently anchored on the substrate without

disturbing the sediment. Samples to determine of initial concentration of CH_4 were collected from each chamber before the beginning of incubation. Incubation time was 24 hours. Samples of water were stored in 40 ml glass vials closed by cap with PTFE/silicone septum until analysis.

Diffusive fluxes

Fluxes of methane between the sediment and overlying water were calculated from Fick´s first law as described by Berner (1980):

$$J = -D_S \times \Phi \times \left(\Delta C / \Delta x\right) \quad (1)$$

where J is the diffusive flux in μg m^{-2} s^{-1}, Φ is the porosity of the sediment, D_S is the bulk sediment diffusion coefficient in cm^{-2} s^{-1}, $\Delta C/\Delta x$ is the methane concetrations gradient in μg cm^{-3} cm^{-1}. Bulk sediment diffusion coefficient (D_S) is based on diffusion coefficient for methane in the water (D_0) and tortuosity (θ) according to the formula:

$$D_S = D_0 \theta^{-2} \quad (2)$$

Tortuosity (θ) is possible calculate from porosity according to equation (Boudreau 1996):

$$\theta^{-2} = 1 - ln\left(\Phi^2\right) \quad (3)$$

Diffusive fluxes of CH_4 were determined at all five study sites along the longitudinal profile of the Sitka stream.

2.6. Measurement of emissions

Gas flux across the air-water interface was determined by the floating chamber method four times during the year period in 2005 – 2006. The open-bottom floating PE chambers (5L domes with an area of 0.03 m^2) were maintained on the water's surface by a floating body (Styrene) attached to the outside. The chambers (n = 4 – 5) were allowed to float on the water's surface for a period of 3 hours. Previous measurements confirmed that time to be quite enough to establish linear dependence of concentration change inside the chambers on time for the gas samples collected every 30 min over a 3 hour period. Due to trees on the banks, the chambers at all study sites were continuously in the shade. On each sampling occasion, ambient air samples were collected for determining the initial background concentrations. Samples of headspace gas were collected through the rubber stopper inserted at the chamber's top, and stored in 100mL PE gas-tight syringes until analysis. Emissions were calculated as the difference between initial background and final concentration in the chamber headspace, and expressed on the 1m^2 area of the surface level per day according to the formula:

$$F = \left[\left(c_I - c_R\right) * V * 24 / t * 1000\right] / p \quad (4)$$

where F is a gas flux in mg $m^{-2}day^{-1}$; c_I is a concentration of particular gas in the chamber headspace in $\mu g\ L^{-1}$; c_R is a concentration of particular gas in background air; V is volume of the chamber in L; t is time of incubation in hr; p is an area of chamber expressed in m^2 . For each chamber, the fluxes were calculated using linear regression based on the concentration change as a function of time, regardless of the value of the coefficient of determination (cf. Duchemin et al. 1999, Silvenoinen et al. 2008).

In order to assess emissions produced from a total stream area, the stream was divided into five stretches according to the channel width, water velocity and substrate composition. For each stretch we have then chosen one representative sampling site (locality I-V) where samples of both stream and interstitial waters and sediments, respectively, were repeatedly taken. Localities were chosen in respect to their character and availability by car and measuring equipments. For calculation of whole-stream gases emissions into the atmosphere, the total stream area was derived from summing of 14 partial stretches. The area of these stretches was caculated from known lenght and mean channel width (measured by a metal measuring type). Longitudinal distance among the stretches was evaluated by using ArcGIS software and GPS coordinates that have been obtained during the field measurement and from digitalised map of the Sitka stream. The total area of the Sitka stream was estimated to be 181 380 m^2 or 0.18 km^2. Stretches have differed in their percentual contribution to this total area and also by their total lenght (Table 1).

The total annual methane emissions to the atmosphere from the five segments of the Sitka stream, E_a (kg yr^{-1}) were derived from seasonal average, maximum or minimum emissions measured on every locality and extrapolated to the total area of the particular segment. The total methane emissions produced by the Sitka stream annualy were then calculated according to the following formula:

$$E_a = \left(\sum p_i * F_i * 365\right) / 1000000 \quad (5)$$

where E_a is average, maximal or minimal assess of emission of methane from the total stream area in kilograms per year; p_i is an area of stretch (in m^2) representing given locality; F_i is average, maximal or minimal assess of the methane from a given locality expressed in mg $m^{-2}day^{-1}$.

2.7. Carbon isotopic composition of dissolved methane and carbon dioxide in sediments

Interstitial water samples for carbon isotopic analysis of methane and carbon dioxide were collected in 2010 - 2011 through three courses at study site. Sampling was performed by set of minipiezometers placed in a depth of 20 to 60 cm randomly in a sediment. After sampling, refrigerated samples were transported (within 72 hours) in 250 mL bottles to laboratory at the Department of Plant Physiology, Faculty of Science University of South Bohemia in Ceske Budejovice, which are equipped with mass spectrometry for carbon isotopes measurements. Firstly both water samples, for methane and for carbon dioxide, were extracted to helium headspace. After relaxation time isotopic equilibrium was

achieved and four subsamples of gas were determined by GasBanch (ThermoScientific) and IRMS Delta^{plus}XL equiped by TC/EA (ThermoFinnigan) for analysis of $\delta^{13}CO_2$. Afterwards $\delta^{13}CO_2$ of water samples were calculated from gaseous $\delta^{13}CO_2$ by fractionation factor from a linear equation (Szaran 1997):

$$\varepsilon_{\infty}{}^{13}C = -\left(0.0954 \pm 0.0027\right) T[^{\circ}C] + (10.41 \pm 0.12) \quad (6)$$

Stable isotope analysis of $^{13}C/^{12}C$ in gas samples was performed using preconcentration, kryoseparation of CO_2 and gas chromatograph combustion of CH_4 in PreCon (ThermoFinnigan) coupled to isotope ratio mass spectrometer (IRMS, Delta Plus XL, ThermoFinnigan, Brehmen, Germany). After conversion of CH_4 to CO_2 in the Finningan standard GC Combustion interface CO_2 will be tranfered into IRMS. The obtained $^{13}C/^{12}C$ ratios (R) will be referenced to $^{13}C/^{12}C$ of standard V-PDB (Vienna-Pee-Dee Belemnite)(Rs), and expressed as $\delta^{13}C = (R_{sample}/R_{standard} - 1) \times 1000$ in ‰. The standard deviation of $\delta^{13}C$ determination in standard samples is lower than 0.1‰ with our instrumentation. From our data, we also calculated an apparent fractionation factor α_C that is defined by the measured δCH_4 and δCO_2 (Whiticar et al. 1986):

$$\alpha_C = \left(\delta CO_2 + 10^3\right) / \left(\delta CH_4 + 10^3\right) \quad (7)$$

This fractionation factor gives rough idea of magnitude of acetoclastic and hydrogenotrophic methanogenesis.

2.8. Abundance of microbial cells and microbial community composition

For measuring of microbial parameters, formaldehyde fixed samples (2 % final conc.) were first mildly sonicated for 30 seconds at the 15 % power (sonotroda MS 73, Sonopuls HD2200, Sonorex, Germany), followed by incubation for 3 hours under mild agitation with 10 mL of detergent mixture (Tween 20 0.5%, vol/vol, tetrasodium pyrophosphate 0.1 M and distilled water) and density centrifugation (Santos Furtado & Casper 2000, Amalfitano & Fazi 2008). For density centrifugaton, the non-ionic medium Nycodenz (1.31 g mL^{-1}; Axis- Shield, Oslo, Norway) was used at 4600 G for 60 minutes (Rotofix 32A, Hettich, Germany). After the preparation processes, a 1 mL of Nycodenz was placed underneath 2 ml of treated slurry using a syringe needle (Fazi et al. 2005). 1 ml of supernatant was then taken for subsequent analysis.

2.9. Total cell numbers (TCN)

The supernatant was filtered onto membrane filters (0.2 μm GTTP; Millipore Germany), stained for 10 minutes in cold and in the dark with DAPI solution (1 mg/ ml; wt/ vol; Sigma, Germany) and gently rinsed in distilled water and 80 % ethanol. Filters were air-dried and fixed in immersion oil. Stained cells were enumerated on an epifluorescence microscope (Olympus BX 60) equipped with a camera (Olympus DP 12) and image analysis software (NIS Elements; Laboratory Imaging, Prague, Czech Republic). At least 200 cells within at

least 20 microscopic fields were counted in three replicates from each locality. TCN was expressed as bacterial numbers per 1 mL of wet sediments.

2.10. Procaryotic community composition

The methanogenic archaea, three selected methanogen families (*Methanobacteriaceae*, *Methanosetaceae* and *Methanosarcinaceae*) and methanotrophic bacteria belonging to groups I and II were detected using FISH (Fluorescence in situ hybridization) with 16S rRNA-targeted oligonucleotide probe labelled with indocarbocyanine dye Cy3. The prokaryotes were hybridized according to the protocol by Pernthaler et al. (2001). Briefly, the supernatants which were used also for TCN were filtered onto polycarbonate membrane filters (0.2 μm GTTP; Millipore), filters were cut into sections and placed on glass slides. For the hybridization mixtures, 2 μL of probe-working solution was added to 16 μL of hybridization buffer in a microfuge tube. Hybridization mix was added to the samples and the slides with filter sections were incubated at 46 °C for 3 hours. After incubation, the sections were transferred into preheated washing buffer (48 °C) and incubated for 15 minutes in a water bath at the same temperature. The filter sections were washed and air-dried. The DAPI staining procedure followed as previously described. Finally, the samples were mounted in a 4:1 mix of Citifluor and Vecta Shield. The methanogens and methanotrophs were counted in three replicates from each locality and the relative proportion of bacteria, archaea, methanogens and methanotrophs to the total number of DAPI stained cells was then calculated.

2.11. Nucleic acid extraction and Denaturing gradient gel electrophoresis (DGGE)

Nucleic acids were extracted from 0,3 g of sieved sediment with a Power Soil DNA isolation kit (MoBio, Carlsbad, USA) according to the manufacturer's instructions. 16S rRNA gene fragments (~350 bp) were amplified by PCR using primer pair specific for methanogens. Primer sequences are as follows, 0357 F-GC 5'-CCC TAC GGG GCG CAG CAG-3' (GC clamp at 5'-end CGC CCG CCG CGC GCG GCG GGCGGG GCG GGG GCA CGG GGG G) and 0691 R 5'- GGA TTA CAR GAT TTC AC -3' (Watanabe et al. 2004). PCR amplification was carried out in 50 μL reaction mixture contained within 0.2 mL, thin walled micro-tubes. Amplification was performed in a TC-XP thermal cycler (Bioer Technology, Hangzhou, China). The reaction mixture contained 5 μL of 10 × PCR amplification buffer, 200 μM of each dNTP, 0,8 μM of each primer, 8 μL of template DNA and 5.0 U of FastStart Taq DNA polymerase (Polymerase dNTPack; Roche, Germany). The initial enzyme activation and DNA denaturation were performed for 6 min at 95°C, followed by 35 cycles of 1 min at 95°C, 1 min at 55°C and 2 min at 69°C and a final extension at 69°C for 8 min (protocol by Watanabe et al. 2004). PCR products were visualised by electrophoresis in ethidium bromide stained, 1.5% (w/v) agarose gel.

DGGE was performed with an INGENYphorU System (Ingeny, Netherlands). PCR products were loaded onto a 7% (w/v) polyacrylamide gel (acrylamide: bisacrylamide, 37.5:1). The

polyacrylamide gels were made of 0.05% (v/v) TEMED (N,N,N,N-tetramethylenediamine), 0.06% (w/v) ammonium persulfate, 7 M (w/v) urea and 40 % (v/v) formamide. Denaturing gradients ranged from 45 to 60%. Electrophoresis was performed in 1×TAE buffer (40 mM Tris, 1 mM acetic acid, 1 mM EDTA, pH 7.45) and run initially at 110V for 10 min at 60°C, afterwatds for 16 h at 85 V. After electrophoresis, the gels were stained for 60 min with SYBR Green I nucleic acid gel stain (1:10 000 dilution) (Lonza, Rockland USA) DGGE gel was then photographed under UV transilluminator (Molecular Dynamics). Images were arranged by Image analysis (NIS Elements, Czech Republic). A binary matrix was created from the gel image by scoring of the presence or absence of each bend and then the cluster tree was constructed (programme GEL2k; Svein Norland, Dept. Of Biology, University of Bergen).

2.12. PCR amplification, cloning and sequencing of methyl coenzyme M reductase (mcrA) gene

Fragments of the methanogen DNA (~470 bp) were amplified by PCR using *mcrA* gene specific primers. Primer sequences for mcrA gene are as follows, mcrA F 5′-GGTGGTGTACGGATTCACACAAGTACTGCATACAGC-3′,mcrA R 5′-TTCATTGCAGTAGTTATGGAGTAGTT-3′. PCR amplification was carried out in 50 μl reaction mixture contained within 0.2 mL thin walled micro-tubes. Amplification was performed in a TC-XP thermal cycler (Bioer Technology, Hangzhou, China). The reaction mixture contained 5 μL of 10 x PCR amplification buffer, 200 μM of each dNTP, 0.8 μM of each primer, 2 μL of template DNA and 2.5 U of FastStart Taq DNA polymerase (Polymerase dNTPack; Roche, Mannheim, Germany). The initial enzyme activation and DNA denaturation were performed for 6 min at 95°C, followed by 5 cycles of 30s at 95°C, 30s at 55°C and 30s at 72°C, and the temperature ramp rate between the annealing and extension segment was set to 0.1°C/s because of the degeneracy of the primers. After this, the ramp rate was set to 1°C/s, and 30 cycles were performed with the following conditions: 30 s at 95°C, 30 s at 55°C, 30s at 72°C and a final extension at 72°C for 8 min. PCR products were visualised by electrophoresis in ethidium bromide stained, 1.5% (w/v) agarose gel.

Purified PCR amplicons (PCR purification kit; Qiagen, Venlo, Netherlands) were ligated into TOPO TA cloning vectors and transformed into chemically competent *Escherichia coli* TOP10F′ cells according to the manufacturer's instructions (Invitrogen, Carlsbad, USA). Positive colonies were screened by PCR amplification with the primer set and PCR conditions described above. Plasmids were extracted using UltraClean 6 Minute Plasmid Prep Kit (MoBio, Carlsbad, USA), and nucleotide sequences of cloned genes were determined by sequencing with M13 primers in Macrogen company (Seoul, Korea). Raw sequences obtained after sequencing were BLAST analysed to search for the sequence identity between other methanogen sequences available in the GenBank database. Then these sequences were aligned by using CLUSTAL W in order to remove any similar sequences. The most appropriate substitution model for maximum likelihood analysis was identified by Bayesian Information Criterion implemented in MEGA 5.05 software. The

phylogenetic tree was constructed by the maximum likelihood method (Kimura 2-parameter model). The tree topology was statistically evaluated by 1000 bootstrap replicates (maximum likelihood) and 2000 bootstrap replicates (neighbour joining).

3. Results

3.1. Sediment and interstitial water

The physicochemical sediment and interstitial water properties of the investigated sites showed large horizontal and vertical gradients. Sediment grain median size decreased along a longitudinal profile while organic carbon content in a sediment fraction < 1 mm remained unchaged (Table 2). Generally, interstitial water revealed relatively high dissolved oxygen saturation with the exceptions of localities IV and V where concentration of dissolved oxygen sharply decreased with the depth, however, never dropped below ~ 10%. Vice versa, these two localities were characterized by much higher concentrations of ferrous iron and dissolved methane (Table 2) compared to those sites located upstream. Concentration of the ferrous iron reflects anaerobic conditions of the sediment and showed the highest concentration to occur in the deepest sediment layers (40-50cm). Average annual temperatures of interstitial water at localities in downstream part of the Sitka stream were about 2.5 °C higher compared to localities upstream and may probably promote higher methane production occuring here. Precursors of methanogenesis, acetate, propionate and butyrate were found to be present in the interstitial water at all study sites, however, only acetate was measured regularly at higher concentration with maximum concentration reached usually during a summer period.

Variable/ Locality	I	II	III	IV	V
particulate organic C in sediment < 1 mm [%]	0.9	0.9	0.6	1.3	0.7
interstitial dissolved O^2 saturation [%]	80.5	88.1	82.3	38.5	50.9
ferrous iron [mg L^{-1}]	< 1	< 1	1.8	8.1	4.2
acetate [mmol L^{-1}]	0.21	0.34	0.52	1.87	0.29
interstitial CH4 concentration [μg L^{-1}]	4.9	0.7	8.1	2 480.2	42.8
methanogenic potential [pM CH_4 mL^{-1} WW $hour^{-1}$]	6.6	1.9	2.9	80.7	9.7
methanotrophic activity [nM CH_4 mL^{-1} WW $hour^{-1}$]	0.3	1.3	28.5	30.3	25.1
average daily interstitial water temperature [°C]	8.7	9.4	11.6	11.2	11.4

Table 2. Selected physicochemical parameters (annual means) of the hyporheic interstitial water and sediments of studied localities taken from the depth 25-30 cm.

3.2. Methanogenic potential and methanotrophic activity of sediments

Methanogenic potential (MP) was found to be significantly higher in the upper sediment layer compared to that from deeper sediment layer. Generally, average MP varied between 0.74-158.6 pM CH_4 mL^{-1} WW $hour^{-1}$ with the highest values found at site IV. Average

methanotrophic activity (MA) varied between 0.02– 31.3 nM CH_4 mL^{-1} WW $hour^{-1}$ and the highest values were found to be at the downstream localities while sediment from sites located upstream showed much lower or even negative activity. Similar to MP, values of MA were significantly higher in sediments from upper layers compared to those from deeper layers (e.g. Figs. 3c, 3d).

3.3. Methane concentration along the longitudinal profile, vertical and temporal pattern, stable isotopes

Methane concentrations ranged between 0.18 – 35.47 μg L^{-1} in surface water and showed no expected trend of gradual increase from upstream localities to those laying downstream. However, significant enhancement of CH_4 concentration was found on locality IV and V, respectively. Concentrations of dissolved CH_4 inboth surface and interstitial waters peaked usually during summer and autumn period (Hlaváčová et al. 2005, Mach et al. in review).

Generally, methane concentrations measured in interstitial water were much higher compared to those from surface stream water and on a long-term basis ranged between 0.19 - 11 698.9 μg L^{-1}. Due to low methane concentrations in interstitial water at localities I and II, vertical distribution of its concentrations was studied only at the downstream located sites III-V. Significant increase of the methane with the sediment depth was observed at the localities IV and V, respectively. Namely locality IV proved to be a methane pool, methane concentrations in a depth of 40 cm were found to be one order of magnitude greater than those from the depth of 20 cm (Tab. 3). Recent data from locality IV show much lower methane concentrations in the upper sediment horizons compared to those from deeper layers (Fig. 3a). Considerable lowering of methane concentration in upper sediment horizons is likely caused by oxidizing activity of methanotrophic bacteria (Fig. 3d). while dissolved oxygen concentration sharply decreased with the sediment depth (Fig. 3b).

Locality	Profile (depth)	CH_4 [μg L^{-1}]
III.	Surface water	1.8
	Interstitial water (depth 20cm)	1.44
	Interstitial water (depth 40 cm)	1.52
IV.	Surface water	5.52
	Interstitial water (depth 20 cm)	1 523.9
	Interstitial water (depth 40 cm)	11 390.54
V.	Surface water	4.72
	Interstitial water (depth 20 cm)	6.92
	Interstitial water (depth 40 cm)	24.4

Table 3. Average concentrations of methane in the vertical sediment profile at localities III-V compared to those from surface water at the same sites

Usually, both the surface and interstitial water were found to be supersaturated compared to the atmosphere with locality IV displaying saturation ratio *R* to be almost 195 000. This high supersaturation greatly promote diffusive fluxes of methane to the atmosphere across air-water interface and is also an important mechanisms for loss of water column CH_4.

Stable carbon isotope signature of carbon dioxide ($\delta^{13}C\text{-}CO_2$) measured in the interstitial water ranged from -19.8 ‰ to -0.8 ‰, while carbon isotope signature of methane ($\delta^{13}C\text{-}CH_4$) ranged between -72 ‰ to -19.8 ‰. This relatively high variation in the methane isotopic values could be caused due to consequential fractionation effects preferring light carbon isotopes like methane oxidation or fractionation through diffusion and through flow of an interstitial water. Contrary, the narrow range of the $\delta^{13}C\text{-}CH_4$ was found in the sediment depth of 40-60 cm where a high methane production has occured. Here, the $\delta^{13}C\text{-}CH_4$ values varied only from -67.9 ‰ to -72 ‰. Apparent fractionation factor (α_C) varied also greatly from 1,004 to 1,076. Usually values of $\alpha_C > 1.065$ and $\alpha_C < 1.055$ are characteristic for environments dominated by hydrogenothropic and acetoclastic methanogenesis, respectively. Our measurements indicate predominant occurrence of a hydrogenothropic methanogenesis in the high methanogenic zones where the most amount of methane is produced and $\delta^{13}C$ of CO_2 values were markedly depleted (i.e. ^{13}C enriched). This could be caused by enhanced carbon dioxide consumption by hydrogenothrophic methanogens, strongly preferring light isotopes. Nevertheless, both acetoclastic and hydrogenotrophic pathways take part in the methanogenesis along the longitudinal profile of the Sitka stream.

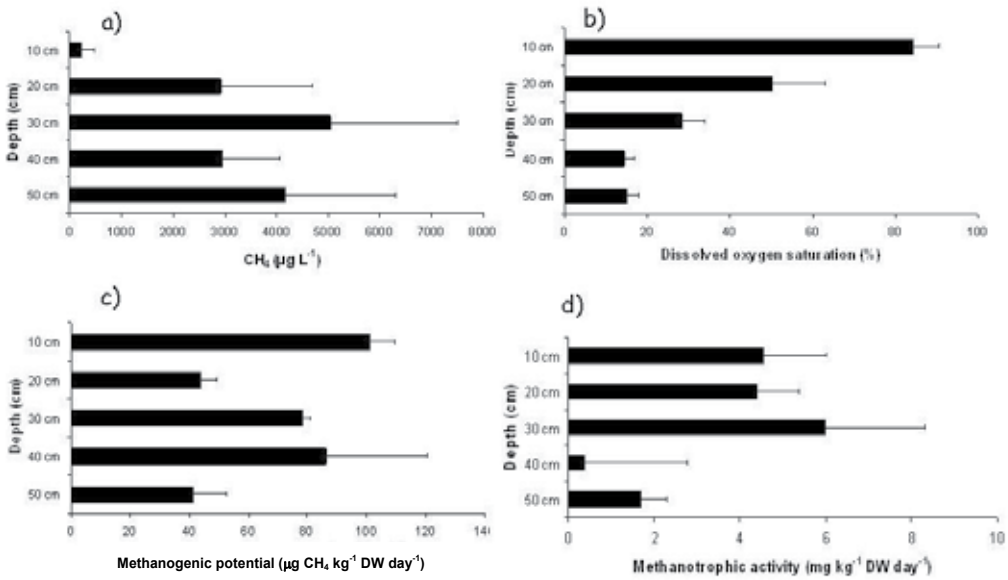

Figure 3. Vertical distribution of methane concentration in the interstitial water at study site IV, horizontal bars indicate 1 SE

3.4. Fluxes of methane across the sediment-water and the air-water interfaces

Methane diffusion rate from deeper sediment layers depends on a methane concentration gradient whilst is affected by oxidation and rate of methanotrophic bacteria consumption. When diffusion fluxes are positive (positive values indicate net CH_4 production), then surface water is enriched by methane which in turn may be a part of downstream transport or is further emitted to the atmosphere (Fig. 4).

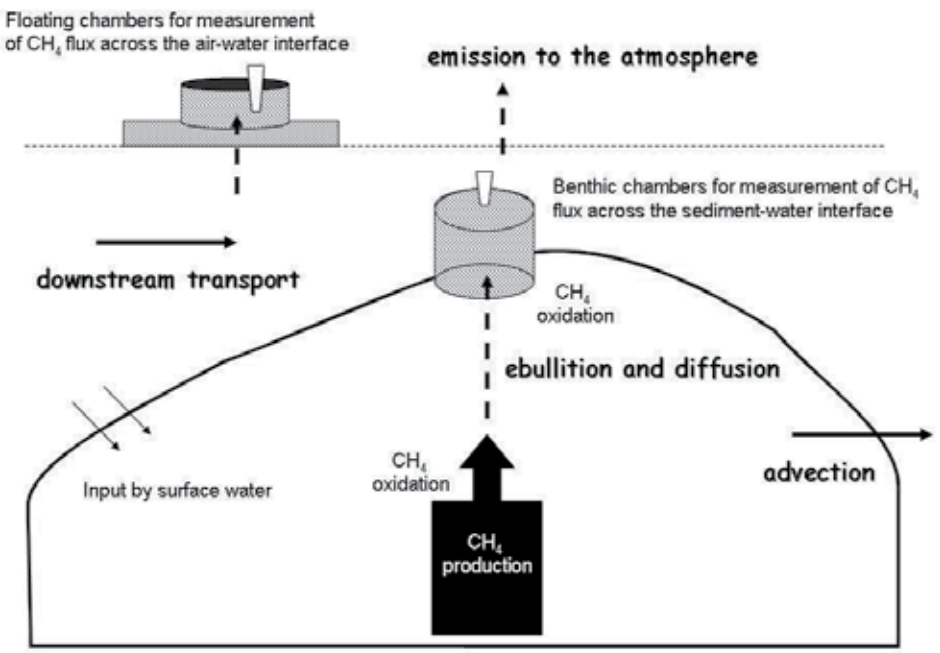

Figure 4. Possible fate of the methane within hyporheic zone and two kinds of chambers for measurement of methane fluxes. Providing that some sites along the longitudinal stream profile should be sources of methane for the stream water, we chose locality IV to be suitable for benthic fluxes measurements.

On the contrary, when the fluxes of methane across the sediment-water interface are negative then all methane produced in the sediments is likely oxidized and consumed by methanotrophic bacteria here or transported via subsurface hyporheic flow.

Calculated diffusive fluxes of CH_4 ranged from 0.03 to 2307.32 $\mu g\ m^{-2}\ day^{-1}$ along the longitudinal profile. The lowest average values of diffusive fluxes were observed at study site II ($0.11 \pm 0.05\ \mu g\ m^{-2}\ day^{-1}$) while the highest average values were those observed at study site IV ($885.81 \pm 697.54\ \mu g\ m^{-2}\ day^{-1}$). Direct benthic fluxes of CH_4 using the benthic chambers were measured at study site IV only and ranged from 0.19 to 82.17 $mg\ m^{-2}\ day^{-1}$. We observed clear negative relationships between benthic methane fluxes and the flow discharge. During higher discharges when the stream water is pushed into sediments, methane diffusing from

deeper sediments upward is either transported by advection through sediments downstream or is probably almost completely oxidized by methanotrophic bacteria due to increasing oxygen supply from the surface stream. As a consequence, very low or no benthic fluxes were recorded during the time of high flow discharge. Compared to calculated diffusive fluxes it is clear that fluxes obtained by direct measurement were approximately 15× higher than the fluxes calculated with using Fick´s first law. Thus, direct benthic fluxes were used for a calculation of water column CH_4 budget.

Gaseous fluxes from surface water to the atmosphere were found at all localities except locality I, where emissions were not mesured directly but were calculated lately using a known relationships between concentrations of gases in surface water and their emissions to the atmosphere found at downstream laying localities II-V. Methane showed an increase in emissions toward downstream where highest surface water concentrations have also occured (Table 4). Methane emissions measured at localities II-V ranged from 0 – 167.35 mg m^{-2} day^{-1} and no gradual increase in downstream end was found in spite of our expectation. However, sharp increase in the amount of methane emitted from the surface water was measured at lowermost localities IV and V (Tab. 4). We found positive, but weak correlation between surface water methane concentrations and measured emissions (r_s = 0.45, $p < 0.05$)(Fig. 5).

Locality/Gas	CH_4 [mg $m^{-2}day^{-1}$]
Locality I.	2.39
Locality II.	0.25 (0 – 0.6) n = 9
locality III.	1.3 (0 – 5.01) n = 10
Locality IV.	32.1 (7.3 – 87.9) n = 8
Locality V.	36.3 (2.8 – 167.4) n = 12

Table 4. Average emissions to the atmosphere and their range in parenthesis and from all localities except locality I. Emissions values for the locality I were calculated using a known relationships between concentrations of methane gas in surface water and its emissions to the atmosphere found at downstream laying localities II-V. *n* means sample size

3.5. Whole-stream emissions E_a

Depending on the time of year we measured the emissions, values of E_a ranged from 430 to 925 kg $year^{-1}$ for methane. Annually, approximately 0.7 tonne of methane was emitted to the atmosphere from the water level of the Sitka stream (total area ca 0.2 km^2). The majority of annual methane emissions (90 %) occured in the lower 7 km of the stream (stretch IV and V) that represents only 1/5 of the total stream area. In addition, contribution of methane emissions to the total annual emissions was found to be the highest during spring-summer period (Mach et al. in review).

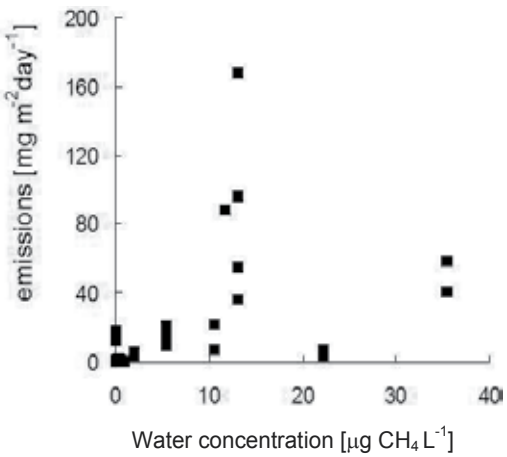

Figure 5. Relationships between atmospheric emissions and surface water concentrations of the methane. Each point represents the mean of five replicate emission measurements and the two replicates of stream water methane concentrations at all

3.6. Sitka stream water column CH_4 budget for the experimental stretch of a stream

The potentially important source and sinks terms for dissolved methane in the water column of the Sitka stream are shown in Figure 6. Previously calculated rates of inputs (benthic fluxes) and loss of dissolved CH_4 through evasion to the atmosphere can be combined together with advection inputs and losses to yield a CH_4 dynamics (budget) for any particular section of the stream.

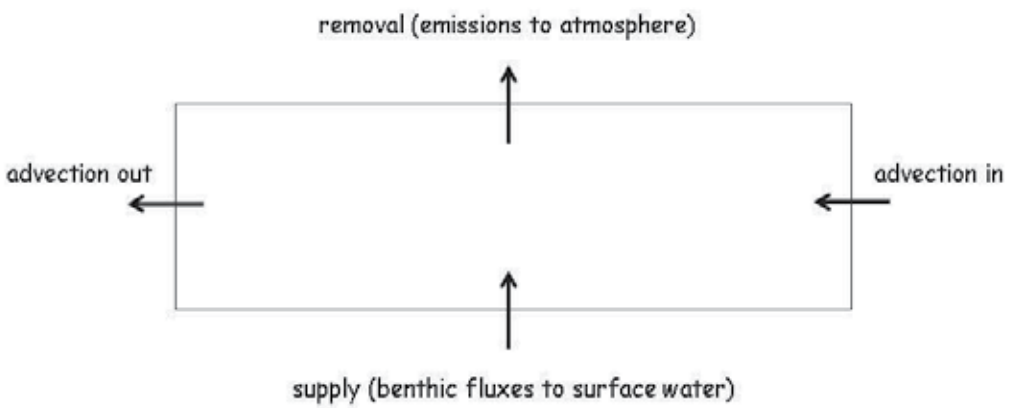

Figure 6. Simple box model used to calculate a CH_4 budget for the Sitke stream experimental section; advection in + supply = advection out + removal (box adjusted after de Angelis & Scranton 1993)

The CH_4 budget determined for the 2011 sampling period in an experimental stream section is summarized in Figure 7. Benthic fluxes were measured along a stream section 45 m long

with an area being ~ 200 m^2. Positive fluxes of CH_4 were found to occur at 30.9 % of the study area. Assuming that average benthic flux of methane across the sediment-water interface was 15.40 mg m^{-2} day^{-1}, the benthic flux of 3081.39 mg CH_4 day^{-1} should occur from the whole area of 200 m^2. Average emission flux of CH_4 across the water-air interface for all study sites was determined to be 14.47 ± 4.73 mg CH_4 m^{-2} day^{-1}. This value is slightly lower than the direct benthic flux of CH_4 and suggests that some portion of methane released from the bottom sediments may contribute to increasing concentration of CH_4 in the surface water. Average flow of the Sitka stream during time of benthic fluxes measurements was 0.351 m^3s^{-1} (i.e. 351 $L^{-1}s^{-1}$). Therefore, we may expect that water column was enriched at least by 187.4 mg (i.e. 0.006 μg L^{-1}) of CH_4 from sediment at 45 m long section near study site IV during one day. Next study site V is located some 4 km downstream from the site IV. Average CH_4 concentration difference in the stream water between these study sites was found to be 3.2 μg L^{-1} of CH_4 indicating that CH_4 supply exceeds slightly CH_4 removal. Methane fluxes from the sediment would contribute to this concentration difference only by 0.6 μg L^{-1}, thus, the immediate difference in the CH_4 budget found between two studied sites IV and V indicates that there must likely be other sources of methane supply to the stream water (Fig. 7). This „missing source" seems to be relatively small (0.9 mg CH_4 0.351 $m^{-3}s^{-1}$), however, net accumulation of CH_4 in the stream water during 4 km section of the Sitka stream below study site IV was almost 78 g CH_4 per one day.

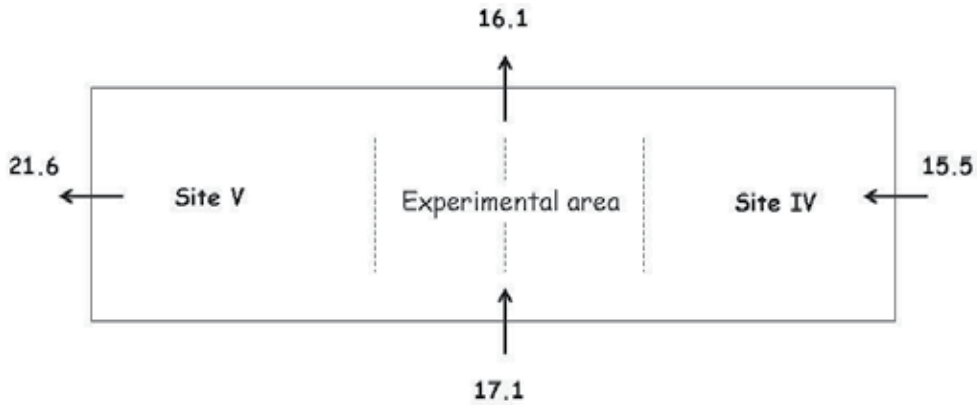

Figure 7. CH_4 budget in mol day^{-1} for a section of the Sitka stream between study sites IV and V (lenght ca 4 km). The arrows correspond to those depicted in Figure 6.

3.7. Fluorescence in situ hybridization (FISH)

Both methanogenic archaea and aerobic methanotrophs were found at all localities along the longitudinal stream profile. The proportion of these groups to the DAPI-stained cells was quite consistent and varied only slightly but a higher proportion to the DAPI-stained cells in deeper sediment layer 25-50 cm was observed. On average 23,4 % of DAPI-stained cells were detected by FISH with a probe for methanogens while type I methanotrophs reached ~ 21,4 % and type II methanotrophs 11,9 %, respectively. All three groups also revealed non-significant higher proportion to the TCN in deeper sediment layer; the abundance of

methanogens and methanotrophs remained almost unchanged with increasing sediment depth. The average abundance of methanogens (0,88 ± 0,28 and 1,07 ± 0,23 x 10^6 cells mL^{-1} in the upper and deeper layer, respectively) and type II methanotrophs (0,44 ± 0,14 x 10^6 cells mL^{-1} and 0,56± 0,1 x 10^6 cells mL^{-1}) increased slightly with the sediment depth , while type I methanotrophs revealed average abundance 0,98 ± 0,23 x 10^6 cells mL^{-1} in the deeper layer being lower compared to abundance 1,07± 0,28 x 10^6 cells mL^{-1} found in upper sediment layer (Buriánková et al. 2012). Very recently, however, using the FISH method we found that abundance of methanogens belonging to three selected families reached their maximum in the sediment depth of 20-30 cm and had closely reflected vertical distribution of acetate concentrations. Species of family *Methanobacteriaceae* grow only with hydrogen, formate and alcohols (except methanol), *Methanosarcinaceae* can grow with all methanogenic substrates except formate, and members of *Methanosaetaceae* grow ecxlusively with acetate as energy source. All three families also showed similar proportion to the DAPI stained cells, ranging in average (depth 10-50 cm) from 9.9% (*Methanosarcinaceae*) to 12.3% (*Methanobacteriaceae*) (Fig. 8).

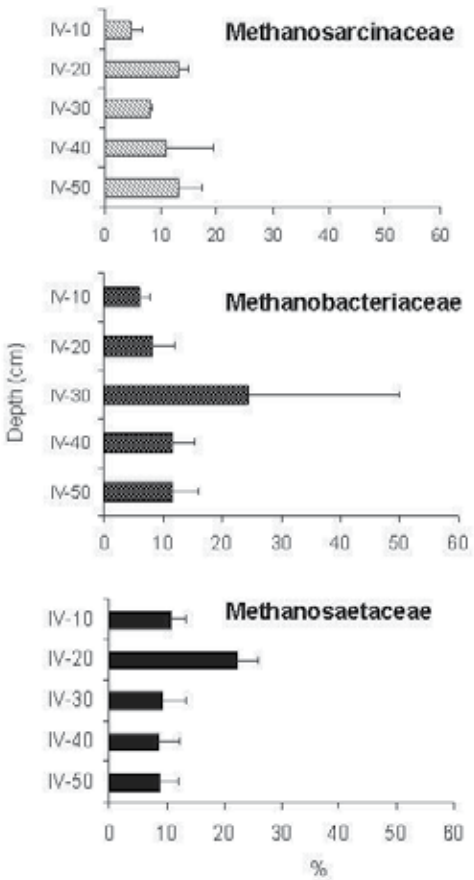

Figure 8. The percentage of chosen methanogenic families as compared to the total bacterial cell numbers found in different sediment layers at locality no. IV, horizontal bars indicate 1 SE

3.8. Denaturing gradient gel electrophoresis and cloning

Methanogenic communities associated with hyporheic sediments at two different depths (0-25 cm and 25-50 cm) along the longitudinal stream profile were compared based on the DGGE patterns. As shown in Fig. 9, the DGGE patterns varied highly among study localities (Fig. 9A), irrespective of the depth (Fig. 9B). However, presence of the bands in all samples indicates that methanogens may occur up to 50 cm of the sediment depth. The number of DGGE bands of the methanogenic archaeal communities was compared either among localites or among different sediment depths. A total of 22 different bands were observed in the DGGE image ranging from 4 (locality II) to 16 (locality IV) in the samples (Fig. 9A).

The number of DGGE bands also ranged from 2 to 10 for the samples from upper layer (0-25 cm) and from 2 to 11 for the samples from deeper layer (25-50 cm), respectively (Fig. 9B). We found no clear trend in the number of DGGE bands with increasing depth (Fig. 9B). Locality IV appears to be the richest in number of DGGE bands. We suppose that this might be due to most favorable conditions prevailing for the methanogens life as indicated by a relatively low grain median size, lower dissolved oxygen concentration or higher concentration of the ferrous iron compared to other localities (cf. Table 2).

The methanogenic community diversity in hyporheic sediment of Sitka stream was also analysed by PCR amplification, cloning and sequencing of methyl coenzyme M reductase (*mcrA*) gene. A total of 60 *mcrA* gene sequences revealed 26 different *mcrA* gene clones.

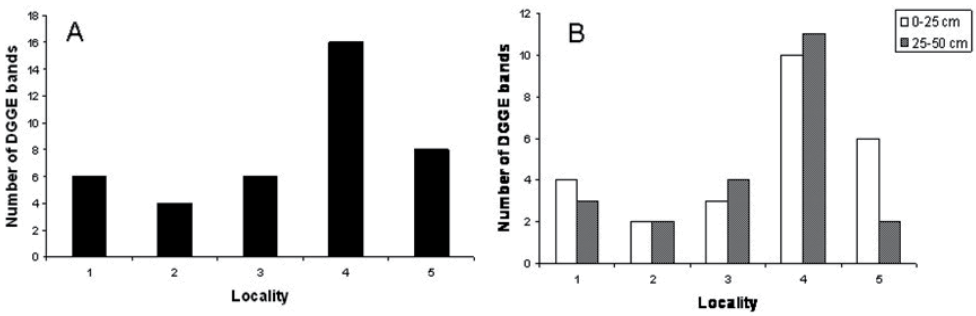

Figure 9. Number of DGGE bands associated with hyporheic sediments at two different depths along the longitudinal stream profile. A – Total number of all bands detected at each locality; B – number of bands found at different sediment depths

Most of the clones showed low affiliation with known species (< 97% nucleotide identity) and probably represented genes of novel methanogenic archeal genera/species, but all of them were closely related to uncultured methanogens from environmental samples (> 97% similarity) retrieved from BLAST. The 25 clones were clustered to four groups and were confirmed to be affiliated to *Methanosarcinales, Methanomicrobiales* and *Methanobacteriales* orders and other unclassified methanogens. The members of all three orders and novel methanogenic cluster were detected to occur in a whole bottom sediment irrespective of a depth, nevertheless, the richness of methanogenic archaea in the sediment was slightly

higher in the upper sediment layer 0-25 cm (15 clones) than in the deeper sediment layer 25-50 cm (11 clones)(Buriánková et al. in review). The clones affiliated with *Methanomicrobiales* predominated in the deeper layer while *Methanosarcinales* clones dominated in the upper sediment layer. This prevalence of *Methanosarcinales* in the upper sediment layer was also confirmed by our FISH analyses as has been mentioned above.

4. Discussion

4.1. Occurence of methane in stream water and sediments

In spite of commonly held view of streams as well-oxygenated habitats, we found both surface and interstitial water to be supersaturated with methane compared to the atmosphere at all five localities (Mach et al. in review). Availability of interstitial habitats for bacteria and archaea carrying out anaerobic processes has been confirmed by our previous (Hlaváčová et al. 2005, 2006; Cupalová & Rulík 2007) and contemporary findings. During this study we found relatively well developed populations of methanogenic archaea at all localities and that all localities also showed positive methanogenic potential. Emissions of methane from water ecosystems results from complex microbial activity in the carbon cycle (production and consumption processes), which depends upon a large number of environmental parameters such as availability of carbon and terminal electron acceptors, flow velocity and turbulence, water depth. In our previous paper (Hlaváčová et al. 2006), we suggested that surface water concentrations, and as a consequence methane gas emissions to the atmosphere would result from downstream transport of gases by stream water (advection in/out), and moreover, from autochthonous microbial metabolism within the hyporheic zone. If so, surface water is continually saturated by gases produced by hyporheic metabolism, leading to supersaturation of surface water and induced diffusion of these gases out of river water (volatizing). Moreover, the run-off and drainage of adjacent soils can also contribute greatly to the degree of greenhouse gas supersaturation (De Angelis & Lilley 1987, Kroeze & Seitzinger 1998, Worral & Lancaster 2005, Wilcock & Sorrell 2008). For example, CH_4 in the estuarine waters may come from microbial production in water, sediment release, riverine input and inputs of methane-rich water from surrounding anoxic environments (Zhang et al. 2008b). For the European estuaries, riverine input contribute much to the estuarine CH_4 due to high CH_4 in the river waters and wetlands also play important roles. However, low CH_4 in the Changjiang Estaury (China) may be resulted from the low CH_4 in the Changjiang water together with the low net microbial production and low input from adjacent salt marshes (Zhang et al. 2008b). Dissolved methane concentrations in a surface water of Sitka stream is consistent with literature data on methane in rivers published by Middelburg et al. (2002) and Zhang et al. (2008b).

4.2. Stable carbon isotopes

A knowledge of the stable carbon isotopic ratio of methane $\delta^{13}C\text{-}CH_4$ in natural systems can be useful in studies of the mechanisms and pathways of CH_4 cycling (Sansone et al. 1997). Values of carbon isotope signature of methane ($\delta^{13}C\text{-}CH_4$) indicate biogenic nature of the

methane, being usually in the range -27 ‰ up to -100 ‰ (Conrad 2004; Michener & Lajtha 2007). Whiticar et al. (1986) demonstrated that methane in freshwater sediments is isotopically distinguished by being relatively enriched in ^{13}C ($\delta^{13}C$ = -65 to -50‰) in contrast to marine sediments (-110 to -60‰). Accordingly, the two precursors of methane, namely acetate and CO_2/H_2, yield methane with markedly different $\delta^{13}C$ values; methane from acetate is relatively enriched in ^{13}C. Average minimum in the carbon isotopic composition of CH_4 (-61.4 ‰) occurred deeper in sediments (60 cm) while average maximum in $\delta^{13}C$-CH_4 occured in the lower sediment depth of 30 cm. Enrichment of ^{13}C in CH_4 probably reflects aerobic CH_4 oxidation because oxidation would result in residual CH_4 with $\delta^{13}C$-CH_4 values less negative than the source CH_4 (Barker & Fritz 1981; Chanton et al. 2004). However, this effect has been observed only at the study site IV.

4.3. Spatial and temporal distributions of emissions

Our working hypotesis suggested that along with the longitudinal profile of a stream, slope and flow conditions also change together with corresponding settling velocity, sediment composition and organic matter content. Thus, according to this prediction, sediment with prevalence of fine-grained particles containg higher amount of organic matter should dominate at the downstream stretches. Moreover, due to prevalence of anoxic environment, production of methane and its emissions was expected to be also higher here compared to that from upstream stretches. Based on our findings, it seems that this presumption is valid for the methane. In addition, we found higher methane concentrations in both the surface and interstitial water at the uppermost locality I compared to lower situated locality II. Similar situation with high methane concentration in the upstream part with subsequent decline further downstream was also reported from USA by Lilley et al. (1996). Dissimilarity of this first stretch is apparent in a comparison with the next, downstream laying stretch (locality II), represented by profile with steep valley and high slope. Generally, there were found very low methane concentrations either in surface or interstitial water and fluxes of emissions to atmosphere were also very low.

Flux rates of gaseous emissions into atmosphere depend on partial pressure of particular gas in the atmosphere and its concentration in a water, water temperature and further on the water depth and flow velocity. Thus, maximum peak of emissions may be expected during summer period and in well torrential stretch of the river. Silvennoinen et al. (2008), for example, found that the most upstream river site, surrounded by forests and drained peatlands, released significant amounts of CO_2 and CH_4. The downstream river sites surrounded by agricultural soils released significant amounts of N_2O whereas the CO_2 and CH_4 concentrations were low compared to the upstream site. When consider seasonal distribution of methane emissions, it is clear, in concordance with above mentioned presumption, that majority of methane emissions was relesed during a warm period of the year (81%). Effect of temperature on methane production was also observed in southeastern USA where the most methane reased to the atmosphere during warm months (Pulliam 1993). In addition, close correlation between methane emissions and temperature was reported also from south part of Baltic Sea; the temperature has been found to be a key factor driving methane emissions (Heyer & Berger 2000).

These findings also indicate that we should be very carefull in making any generalization in total emissions estimation for any given stream or river. Even though some predictions can be made based on gas concentrations measured in the surface or interstitial water, results may be very different. From this point, noteworthy was locality IV; enormous concentrations of a methane found in the deep interstitial water were caused probably by very fine, clayed sediment containing high amount of organic carbon, as well as high DOC concentrations. Supersaturation led also to the enrichment of the surface water with methane - such places may be considered as very important methane sources for surface stream and, consequently source of emissons to the atmosphere.

4.4. Benthic fluxes and potential methane oxidation

CH_4 can be produced and released into overlying near-bottom water through exchange at sediment-water interface. Methane released from the sediments into the overlying water column can be consumed by methanotrophs. Methanotrophs can oxidize as much as 100 % of methane production (Le Mer & Roger 2001). According to the season, 13-70 % of methane was consumed in a Hudson River water column (de Angelis et Scranton 1993). For the Sitka stream, measurement of benthic fluxes into the overlying surface waters indicates that methane consumption by methanotrophic bacteria is likely a dominant way of a methane loss, nevertheless some methane still supports relatively high average methane concentrations in the surface water and, in turn, high emissions to the atmosphere.

The methane production (measured as methanogenic potential) was found to be 3 orders of magnitude lower than the oxidation (methanotrophic activity), thus, almost all methane should be oxidized and consumed by methanotrophic bacteria and no methane would occur within the sediments. However, situation seems to be quite different suggesting that namely methanotrophic activity measured in a laboratory could be overestimated. Since oxidation of methane requires both available methane and oxygen, methanotrophic activity is expected to be high at sites where both methane and dissolved oxygen are available. Therefore, high values of the MA were usually found in the upper layers of the sediments (Segers 1998) or at interface between oxic and anoxic zones, respectively. Relatively high methanotrophic activity found in deeper sediments of the localities III-V indicates that methane oxidation is not restricted only to the surface sediments as is common in lakes but it also takes place at greater depths. It seems likely that oxic zone occurs in a vertical profile of the sediments and that methane diffusing from the deeper layer into the sedimentary aerobic zone is being oxidized by methanotrophs here. Increased methanotrophic activity at this hyporheic oxic-anoxic interface is probably evident also from higher abundance of type II methanotrophs in the same depth layer. Similar pathway of methane cycling has been observed by Kuivila et al. (1988) in well oxygenated sediments of Lake Washington, however, methane oxidation within the sediments would be rather normal in river sediments compared to lakes. All the above mentioned findings support our previous suggestions that coexistence of various metabolic processes in hyporheic sediments is common due to vertical and horizontal mixing of the interstitial water and occurrence of microbial biofilm (Hlaváčová et al. 2005, 2006).

4.5. Methanogens diversity

The presence of relatively rich assemblage of methanogenic archaea in hyporheic river sediments is rather surprising, however it is in accordance with other studies. The number of total different bands (i.e. estimated diversity of the methanoges) observed in the DGGE patterns of the methanogenic archaeal communities was comparable with a number of the DGGE bands found in other studies. For example, Ikenaga et al. (2004) in their study of methanogenic archaeal community in rice roots found 15-19 DGGE bands, while Watanabe et al. (2010) showed 27 bands at different positiosns in the DGGE band pattern obtained from Japanese paddy field soils. Our results from the DGGE analysis are supported by cloning and sequencing of methyl coenzyme M reductase (*mcrA*) gene which also retrieved relatively rich diversity (25 different *mcrA* gene clones) of the methanogenic community in the Sitka stream hyporheic sediments. Similar richness in number of clones was also mentioned in a methanogenic community in Zoige wetland, where 21 different clones were found (Zhang et al. 2008a), while 20 clones were described in the methane cycle of a meromictic lake in France (Biderre-Petit et al. 2011). In addition, soils from Ljubljana marsh (Slovenia) showed 17 clones (Jerman et al. 2009), for example. Both DGGE and *mcrA* gene sequencing results suggest that both hydrogenotrophic and acetoclastic methanogenesis are an integral part of the CH_4 - producing pathway in the hyporheic zone and were represented by appropriate methanogenic populations. Further, these methanogenic archaea form important component of a hyporheic microbial community and may substantially affect CH_4 cycling in the Sitka stream sediments.

5. Conclusion

To our knowledge this study is the first analysis of the composition of active methanogenic/methanotrophic communities in river hyporheic sediments. By use of various molecular methods we have shown that both methanogenic archaea and aerobic methanotrophs can be quantitatively dominant components of hyporheic biofilm community and may affect CH_4 cycling in river sediments. Their distribution within hyporheic sediments, however, only partly reflects potential methane production and consumption rates of the sediments. Rather surprising is the detection of methanotrophs in the deep sediment layer 25-50 cm, indicating that suitable conditions for methane oxidation occur here. In addition, this work constitutes the first estimation of sources, sinks and fluxes of CH_4 in the Sitka stream and in 3rd order stream environment. Fluxes of CH_4 from supersaturated interstitial sediments appear to be a main CH_4 source toward the water column. Compared with CH_4 production rates, the diffusive fluxes are very low due to efficient aerobic oxidation by methanotrophic bacteria, especially during higher flow discharges. Although fluxes to the atmosphere from the Sitka stream seems to be insignificant, they are comparable or higher in comparison with fluxes from other aquatic ecosystems, especially those measured in running waters. Finally, our results suggest that the Sitka Stream is a source of methane into the atmosphere, and loss of carbon via the fluxes of this greenhouse gas out into the ecosystem can participate significantly in river self-purification.

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Chapter 18

How Soil Nutrient Availability Influences Plant Biomass and How Biomass Stimulation Alleviates Heavy Metal Toxicity in Soils: The Cases of Nutrient Use Efficient Genotypes and Phytoremediators, Respectively

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Additional information is available at the end of the chapter

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1. Introduction

There are many factors influencing plant biomass, such as soil humidity, soil and air temperature, photoperiod, solar radiation, precipitations, genotype e.t.c. One of the most important factors influencing biomass is soil nutrient availability. Both nutrient deficiency and toxicity negatively affect total biomass and fruit production [1-10]. So, by controlling the optimum levels of nutrient availability in soil, the production of biomass and, of course, the economic benefit (fruit production) for the farmers can be maximized. In the cases of limited nutrient availability in soils, fertilization seems to be the most usual practice adopted by the farmers in order to ameliorate the low nutrient status. However, since: i) during the last two decades the prices of fertilizers have been dramatically increased, and ii) soil degradation and pollution, as well as underground water pollution, are serious consequences provoked by the exaggerate use of fertilizers, a global concern to reduce the use of fertilizers has been developed. So, the best (most economic and ecological) way in our days to achieve maximum yields is by selecting and growing nutrient efficient genotypes, i.e. genotypes which are able to produce high yields (biomass) in soils with limited nutrient availability. Many researchers studied the influence of genotype on biomass and plant growth (nutrient use efficient genotypes) and found impressive results. According to Chapin and Van Cleve (1991) [11], nutrient use efficiency is defined as the amount of biomass produced per unit of nutrient. So, nutrient use efficient genotypes are those having the ability to produce biomass sufficiently under limited nutrient availability. In our research with different olive cultivars, grown under hydroponics, or in soil substrate, we found significant differences concerning

macro- and micronutrient utilization efficiency among genotypes [12-13]. Possible reasons for differential nutrient utilization efficiency among genotypes may be: i) the genetic material used, i.e. cultivar (differential nutrient uptake, accumulation and distribution among tissues, mechanisms of cultivars/genotypes), ii) differential colonization of their root system mycorrhiza fungi. Chatzistathis et al. (2011) [14] refer that the statistically significant differences in Mn, Fe and Zn utilization efficiency among three Greek olive cultivars ('Chondrolia Chalkidikis', 'Koroneiki' and 'Kothreiki') may be probably ascribed to the differential colonization of their root system by arbuscular mycorrhiza fungus (AMF) (the percentage root colonization by AMF varied from 45% to 73%).

Heavy metal (Cu, Zn, Ni, Pb, Mn, Cr, Cd) toxicity is a very serious problem in soils suffering from: i) industrial and mine activities [15], ii) the exaggerate use of fertilizers, fungicides and insecticides, iii) acidity, iv) waterlogging, v) other urban activities, such as municipal sewage sludges, vi) the use of lead in petrols, paints and other materials [16]. Under these conditions, plant growth and biomass are negatively affected [17-20]. According to Caldelas et al. (2012) [19], not only growth inhibition happened, but also root to shoot dry matter partitioning (R/S) modified (increased 80%) at Cr toxic conditions in Iris pseudacorus L. plants. Some plant species, which may tolerate very high metal concentrations in their tissues, can be used as hyper-accumulators and are very suitable in reducing heavy metal concentrations in contaminated soils [21]. These species are able to accumulate much more metal in their shoots, than in their roots, without suffering from metal toxicity [22]. By successive harvests of the aerial parts of the hyper-accumulator species, the heavy metals concentration can be reduced [23]. Phytoremediation is an emerging technology and is considered for remediation of inorganic- and organic-contaminated sites because of its cost-effectiveness, aesthetic advantages, and long-term applicability. This technique involves the use of the ability of some plant species to absorb and accumulate high concentrations of heavy metal ions [17]. Some of these species may be a few ones from Brassicaceae family, such as raya (Brassica campestris L.) [17] and Thlaspi caerulescens [23], or from other families, such as spinach (Spinacia oleracea L.) [17], Sedum plumbizincicola [24], Amaranthus hypochondriacus [25], Eremochloa ophiuroides [26], Iris pseudacorus L. [19], Ricinus communis L., plant of Euphorbiaceae family [18]. Finally, the tree species Genipa Americana L. may be used as one with great ability as phytostabilizer and rhizofilterer of Cr ions, according to Santana et al. (2012) [20]. Basically, there are two different strategies to phytoextract metals from soils: the first approach is the use of metal hyper-accumulator species. The second one is to use fast-growing, high biomass crops that accumulate moderate to high levels of metals in their shoots for metal phytoremediation, such as Poplar (Populus sp.) [27-28], maize (Zea mays), oat (Avena sativa), sunflower (Helianthus annuus) and rice (Oryza sativa L.) [25]. Generally, the more high biomass producing is one plant species, the more efficient is the phytoremediation effect. So, in order to enhance biomass production under metal toxicity conditions, different strategies, such as the application of chemical amendments, may be adopted [21]. Since Fe deficiency symptoms may be appeared under Cu and Zn toxicity conditions in some species of Brassicaceae family used for phytoremediation, a good practice is to utilize Fe foliar sprays in order to enhance biomass, thus the phytoremediation effect [29].

All the above mentioned topics, concerning the influence of nutrient deficiency and metal toxicity on plant biomass, as well as the importance of using nutrient use efficient genotypes

and cultivars, are within the aim of the present review. Furthermore, the characteristics that should have the plant species used for phytoremediation (fast-growing, high biomass crops) in heavy metal polluted soils are fully analyzed, and the different strategies that should be adopted in order to enhance plant growth and biomass production under so adverse soil conditions are also discussed under the light of the most important and recent research papers.

2. Agronomic, environmental and genotypic factors influencing plant growth

Plant growth (i.e. biomass production) is influenced by many (agronomic environmental and others, such as genetic) factors. Some of the most important factors that influence biomass production are: i) soil humidity, ii) soil and air temperature, iii) air humidity, iv) photoperiod, v) light intensity, vi) soil fertility, i.e. soil nutrient availability, and vii) genotype, and are fully analyzed below.

2.1. Soil humidity

Soil humidity is a very crucial factor influencing root growth, thus nutrient uptake and total biomass. Many plant species are more sensitive in soil humidity shortage during a particular (crucial) period of their growth. In olive trees, if soil humidity shortage happens early spring, shoot elongation, as well as the formation of flowers and fruits, are negatively influenced. If the shortage happens during summer, shoot thickening, rather than shoot elongation, is influenced. Finally, soil humidity shortage reduces olive tree canopy (in order to reduce the transpiration by leaf surface) and favors root system growth (in order to have the ability to exploit greater soil volume and to search for more soil humidity), so that the ratio canopy/root is significantly reduced [30]. On the other hand, under excess soil humidity conditions (waterlogging), when soil oxygen is limited, the root system may suffer from hypoxia, thus, nutrient uptake is negatively influenced. Under extreme anaerobic soil conditions, the presence of pathogen microorganisms, such as *Phytophthora* sp. may lead to root necrosis. According to Therios (2009) [31], for olive trees the mechanism of tolerance to waterlogging is based on the production of adventitious roots near to the soil surface.

2.2. Soil temperature

Soil temperature influences root growth, thus nutrient and water uptake and, of course, biomass production. Most nutrients are absorbed with energy consumption (energetic uptake), so, low and very high soil temperatures negatively influence root growth and nutrient uptake. Furthermore, low soil temperatures induce a water deficit [32].

2.3. Air temperature

Air temperature directly influences photosynthesis, which is the most important physiological function in plants. The optimum temperature for photosynthesis depends on plant species and also on cultivar for the same species. Usually, the optimum temperature

for maximum photosynthetic activity is around 25°C for most vegetative species. When temperature exceeds 35°C photosynthesis is inhibited, thus biomass production may be restrained. High temperatures are associated with a high vapor pressure deficit between leaves and the surrounding air. The same applies to fruit, where high temperatures may cause fruit drop in olive trees [31]. On the other hand, low temperatures act negatively in photosynthesis function and starch is redistributed and is accumulated in organs protected from frost, such as roots. Very low temperatures (<-12°C) damage the leaf canopy, shoot and branches of trees [31].

2.4. Air humidity

Low atmosphere humidity speeds up transpiration by leaf surface. Increase of the rate of transpiration causes reduction of vegetative tissues water content, thus depression in the rate of growth and biomass production.

2.5. Photoperiod

Photoperiod is the duration of light in 24 hours and it is one of the most important factors influencing vegetative growth. Plant species whose vegetative growth is mostly influenced by long day conditions are *Populus robusta*, *Ulmus Americana* and *Aesculus hippocastanus* [30].

2.6. Light intensity

Light, together with CO_2, are the two main factors influencing photosynthetic rate. By increasing light intensity up to an optimum limit the maximum photosynthetic rate, so the greatest biomass production can be achieved.

2.7. Nutrient availability

Limited nutrient availability influences negatively biomass production. Nitrogen deficiency strongly depresses vegetation flush. According to Boussadia et al. (2010) [8], total biomass of two olive cultivars ('Meski' and 'Koroneiki') was strongly reduced (mainly caused by a decrease in leaf dry weight) under severe N deprivation, while in an out-door pot-culture experiment with castor bean plants (*Ricinus communis* L.), conducted by Reddy and Matcha (2010) [9], it was found that among the plant components, leaf dry weight had the greatest decrease; furthermore, root/shoot ratio increased under N deficiency [9]. Phosphorus deficiency caused reduced biomass, photosynthetic activity and nitrogen fixing ability in mungbean (*Vigna aconitifolia*) and mashbean (*Vigna radiata*) [33]. Under P deficiency conditions, genotypic variation in biomass production is evident; according to Pang et al. (2010) [34], who studied in a glasshouse experiment the response of ten perennial herbaceous legume species, found that under low P conditions several legumes produced more biomass than lucerne. Nutrient deficiency may cause physiological and metabolism abnormalities in plants, which may lead to deficiency symptoms. There are two categories of

symptoms: i) General symptoms, such as limited growth and inability of reproduction (flowering and fruit setting), caused by the deficiency of many necessary macro- or micro-nutrients, and ii) typical, characteristic, deficiency symptoms, such as chlorosis, i.e. yellowing (due to Fe deficiency). In both cases biomass production is depressed. In the study of Msilini et al. (2009) [10], bicarbonate treated plants of *Arabidopsis thaliana* suffered from Fe deficiency displayed significantly lower biomass, leaf number and leaf surface, as compared to control plants, and showed slight yellowing of their younger leaves. Under limited nutrient availability, arbuscular mycorrhiza fungi (AMF) may favor nutrient uptake and thus enhance biomass production. Hu et al. (2009) [35] refer that AMF inoculation of maize plants was likely more efficient in extremely P-limited soils. Generally, root colonization by AMF influences positively plant growth under N, P, or micronutrient deficiency conditions [36].

2.8. Genotypic factors (root morphology and architecture, genetic growth capacity e.t.c.)

According to Bayuelo-Jimenez et al. (2011) [3], under P deficiency, P-efficient accessions of maize plants (*Zea mays* L.) had greater root to shoot ratio, nodal rooting, nodal root laterals, nodal root hair density and length of nodal root main axis, and first-order laterals. In our experiments, we also found differential root system morphology among three Greek olive cultivars (the root systems of 'Koroneiki' and 'Chondrolia Chalkidikis' were less branched and more lateral, and with less root hair development and density, than that of 'Kothreiki', which was richly-branched and with much greater root hair development and density), something which was probably the main reason for the great genotypic variations in nutrient uptake and growth among the three cultivars (Chatzistathis, unpublished data). Singh et al. (2010) [37] found that great differences existed among 10 multipurpose tree species, grown in a monoculture tree cropping system on the sodic soils of Gangetic alluvium in north India, concerning plant height, diameter e.t.c.

3. Physiological roles of nutrients

The absolutely necessary nutrients for plant growth are the following: N, P, K, S, Ca, Mg (macronutrients), Zn, Cu, B, Mn, Fe, Mo (micronutrients). Without one of these nutrients, plant organism can not grow normally and survive. The physiological roles of these nutrients are described in detail below.

3.1. Macronutrients

Nitrogen: It is a primary component of nucleic acids, proteins, amino acids, purines, pyrimidines and chlorophyll. Nitrogen exerts a significant effect on plant growth, as it reduces biennial bearing and increases the percentage of perfect flowers. In olive trees, lack of N leads to decreased growth, shorter length of annual shoots (<10cm), fewer leaves, reduced flowering and decreased yield [31].

Phosphorus: P is a component of high-energy substances such as ATP, ADP and AMP; it is also important for nucleic acids and phospholipids. Phosphorus affects root growth and maturation of plant tissues and participates in the metabolism of carbohydrates, lipids and proteins [31].

Potassium: K plays a crucial role in carbohydrate metabolism, in the metabolism of N and protein synthesis, in enzyme activities, in the regulation of the opening and closing of stomata, thus to the operation of photosynthesis, in the improvement of fruit quality and disease tolerance, in the activation of the enzymes peptase, catalase, pyruvic kinase e.t.c. [31,38].

Calcium: It is the element that participates in the formation and integrity of cell membranes, in the integrity and semipermeability of the plasmalemma, it increases the activity of many enzymes, it plays a crucial role in cell elongation and division, in the transfer of carbohydrates e.t.c. [31,38].

Magnesium: It is part of chlorophyll molecule, it activates the enzymes of Crebs' cycle and it also plays a role in oil synthesis [38].

Sulphur: Sulphur plays role in the synthesis of some amino-acids, such as cysteine, cystine, methionine, as well as in proteins synthesis. It also activates some proteolytic enzymes, such as papaine, bromeline e.t.c. Finally, it is part of some vitamins' molecule and that of gloutathione [31,38].

3.2. Micronutrients

Iron: Iron plays an important role in chlorophyll synthesis, without being part of its molecule. Furthermore, it participates in the molecule of Fe-proteins catalase, cytochrome a, b, c, hyperoxidase e.t.c. In addition to that, it is found in the enzymes nitric and nitrate reductase, which are responsible for the transformation of NO_3^- into NH_4^+, as well as in nitrogenase, which is the responsible enzyme for the atmospheric N capturing [38].

Manganese: Manganese is activator of the enzymes of carbohydrates metabolism, those of Crebs' cycle, and of some other enzymes, such as cysteine desulphydrase, glutamyl transferase e.t.c. It also plays a key-role in photosystem II of photosynthesis, and particularly in the reactions liberating O_2. Finally, Mn acts as activator of some enzymes catalyzing oxidation and reduction reactions [38].

Zinc: Zn plays crucial role in tryptophane biosynthesis, which is the previous stage from IAA (auxin) synthesis (direct influence of Zn on plant growth and biomass production). IAA concentration is significantly reduced in vegetative tissues suffering from Zn deficiency. In addition to the above, Zn is part of some metal-enzymes [38].

Copper: Cu is activator of some enzymes, as well as it is part of enzymes catalyzing oxidation and reducing reactions, such as oxidase of ascorbic acid, lactase, nitrate and nitric reductase e.t.c. [38].

Boron: B plays role in the transfer of sugars along cell membranes, as well as in RNA and DNA synthesis. It also participates to cell division process, as well as to the pectine synthesis [38].

Molybdenum: It is part of the enzyme nitrogenase (capturing of atmospheric N) and nitric reductase (transformation of NO_3^- to NO_2^-). Mo also participates to the metabolism of ascorbic acid [38].

As it is clear from all the above physiological roles of nutrients, the deficiency of even one of them in the mineral nutrition of higher plants depresses their growth, thus biomass production. So, in order to achieve the maximum biomass production, apart from the optimum conditions of all the other environmental and agronomic factors influencing plant growth (temperature, soil humidity, photoperiod, light intensity), it should always be taken care of maintaining the optimum levels of all the necessary soil nutrients. This is usually achieved with the correct fertilization program of the different crops. For example, fruit trees have high demands in K, since fruit production is a K sink and reduces its levels in plant level. According to Therios (2009) [31], potassium plays an important role in olive nutrition. Thus, fruit trees should be periodically fertilized (usually K fertilizers applied during autumn, or winter, and are incorporated into the soils) with enhanced doses of potassium fertilizers (usually K_2SO_4). Apart from chemical fertilizers, organic amendments can be also applied under limited nutrient conditions in order to enhance plant growth. According to Hu et al. (2009) [35], stem length, shoot and root biomass, as well as crop yield of maize were all greatly increased by the application of organic amendments on a sandy loam soil. Apart from the application of chemical fertilizers, organic amendments e.t.c., another modern method to improve yields and to increase biomass is the irrigation of crops with FFC H_2O, a commercial product currently utilized by the agriculture, fishery and food industries in Japan. In the study of Konkol et al. (2012) [39], radish and shirona plants irrigated with FFC H_2O developed larger average leaf area by 122% and greater dry weight and stem length by 39% and 31%, respectively, compared to the plants irrigated with deionized H_2O. FFC H_2O offers agriculturalists a simple and effective tool for the fortification of irrigation waters with micronutrients [39].

4. Nutrient utilization efficiency (NUE): The case of nutrient use efficient genotypes

World population is expected to increase from 6.0 billion in 1999 to 8.5 billion by 2025. Such an increase in population will intensify pressure on the world's natural resource base (land, water, and air) to achieve higher food production. Increased food production could be achieved by expanding the land area under crops and by increasing yields per unit area through intensive farming. Chemical fertilizers are one of the expensive inputs used by farmers to achieve desired crop yields [40]. However, during the last years, the prices of fertilizers have been considerably increased. Furthermore, soil degradation and pollution, as well as underground water pollution, are serious consequences provoked by the exaggerate

use of fertilizers during last decades. These two aspects are responsible for the global concern to reduce the use of fertilizers. The best way to do that is by selecting and growing nutrient use efficient genotypes. According to Khoshgoftarmanesh (2009) [41], cultivation and breeding of micronutrient-efficient genotypes in combination with proper agronomic management practices appear as the most sustainable and cost-effective solution for alleviating food-chain micronutrient deficiency.

Nutrient use efficient genotypes are those having the ability to produce high yields under conditions of limited nutrient availability. According to Chapin and Van Cleve (1991) [11] and Gourley et al. (1994) [42], as nutrient utilization efficiency (NUE) is defined the amount of biomass produced per unit of nutrient absorbed. **Nutrient efficiency ratio (NER)** was suggested by Gerloff and Gabelman (1983) [43] to differentiate genotypes into efficient and inefficient nutrient utilizers, i.e. **NER=(Units of Yields, kgs)/(Unit of elements in tissue, kg),** while **Agronomic efficiency (AE)** is expressed as the additional amount of economic yield per unit nutrient applied, i.e. **AE=(Yield F, kg-Yield C, kg)/(quantity of nutrient applied, kg),** where F applies for plants receiving fertilizer and C for plants receiving no fertilizer.

Many researchers found significant differences concerning nutrient utilization efficiency among genotypes (cultivars) of the same plant species [1,12,13,40,44-46] Biomass (shoot and root dry matter production) was used as an indicator in order to assess Zn efficient Chinese maize genotypes, grown for 30 days in a greenhouse pot experiment under Zn limiting conditions [1]. NUE is based on: a) uptake efficiency, b) incorporation efficiency and c) utilization efficiency [40]. The uptake efficiency is the ability of a genotype to absorb nutrients from the soil; however, the great ability to absorb nutrients does not necessarily mean that this genotype is nutrient use efficient. According to Jiang and Ireland (2005) [45], and Jiang (2006) [46], Mn efficient wheat cultivars own this ability to a better internal utilization of Mn, rather than to a higher plant Mn accumulation. We also found in our experiments that, despite the fact that the olive cultivar 'Kothreiki' absorbed and accumulated significantly greater quantity of Mn and Fe in three soil types, compared to 'Koroneiki', the second one was more Mn and Fe-efficient due to its better internal utilization efficiency of Mn and Fe (greater transport of these micronutrients from root to shoots) [12] (Tables 1 and 2). Aziz et al. (2011a) [47] refer that under P deficiency conditions, P content of young leaves in *Brassica* cultivars increased by two folds, indicating remobilization of this nutrient from older leaves and shoot. However, differences in P remobilization among *Brassica* cultivars could not explain the differences in P utilization. Phosphorus efficient wheat genotypes with greater root biomass, higher P uptake potential in shoots and absorption rate of P were generally more tolerant to P deficiency in the growth medium [6]. According to Yang et al. (2011) [48], on average, the K efficient cotton cultivars produced 59% more potential economic yield (dry weight of all reproductive organs) under field conditions even with available soil K at obviously deficient level (60 mg/kg).

The possible causes for the differential nutrient utilization efficiency among genotypes and/or species may be one, or combination of more than one, of the following: a) genetic

reasons (genotypic ability to absorb and utilize efficiently, or inefficiently, soil nutrients), b) mycorrhiza colonization of the root system, c) differential root exudation of organic compounds favorizing nutrient uptake, d) different properties of rhizosphere, e) other reasons. According to Cakmak (2002) [49], integration of plant nutrition research with plant genetics and molecular biology is indispensable in developing plant genotypes with high genetic ability to adapt to nutrient deficient and toxic soil conditions and to allocate more micronutrients into edible plant products. According to Aziz et al. (2011b) [50], *Brassica* cultivars with high biomass and high P contents, such as 'Rainbow' and 'Poorbi Raya', at low available P conditions would be used in further screening experiments to improve P efficiency in *Brassica*. More specifically, a number of genes have been isolated and cloned, which are involved in root exudation of nutrient-mobilizing organic compounds [51,52]. Successful attempts have been made in the past 5 years to develop transgenic plants that produce and release large amounts of organic acids, which are considered to be key compounds involved in the adaptive mechanisms used by plants to tolerate P-deficient soil conditions [53-55]. However, differential root exudation ability in nature exists among different plant species. According to Maruyama et al. (2005) [56], who made a comparison of iron availability in leaves of barley and rice, the difference in the Fe acquisition ability between these two species was affected by the differential mugineic acid secretion. Chatzistathis et al. (2009) [12] refer that, maybe, a similar mechanism was responsible for the differential micronutrient uptake and accumulation between the Greek olive cultivars 'Koroneiki' and 'Kothreiki'. According to the same authors, differential reduction of Fe^{3+} to Fe^{2+}, or acidification capacity of root apoplast (which associates with the increase of Fe^{3+}-chelate reductase and H-ATPase activities) among three Greek olive cultivars should not be excluded from possible causes for the significant differences observed concerning Fe uptake [14]. Mycorrhiza root colonization may be another responsible factor for the differential micronutrient utilization efficiency among genotypes. According to Citernesi et al. (1998) [57], arbuscular mycorrhiza fungi (AMF) influenced root morphology of Italian olive cultivars, thus nutrient uptake and accumulation, as well as plant growth. In our study with olive cultivars 'Koroneiki', 'Kothreiki' and 'Chondrolia Chalkidikis', we found significant differences concerning root colonization by AMF (that varied from 45% to 73%), together with great differences in uptake and utilization efficiency of Mn, Fe and Zn among them (particularly, 1.5 to 10.5 times greater amount of Mn, Fe and Zn accumulated by 'Kothreiki', compared to the other two cultivars, but the differences in plant growth parameters between the three cultivars were not impressive; this is why the micronutrient utilization efficiency by 'Kothreiki' was significantly lower, compared to that of the other two ones). Finally, the different properties of rhizosphere among genotypes may be another important factor influencing nutrient uptake and utilization efficiency, and of course biomass production. According to Rengel (2001) [58], who made a review on genotypic differences in micronutrient use efficiency of many crops, micronutrient-efficient genotypes were capable of increasing soil available micronutrient pools through changing the chemical and microbiological properties of the rhizosphere, as well as by growing thinner and longer roots and by having more efficient uptake and transport mechanisms.

Soil	Cultivar	Micronutrient	Root	Stem	Leaves
Marl		**Mn**			
	Kor		50.2b	38.0a	11.8a
	Koth		74.1a	12.8b	13.1a
Gneiss schist					
	Kor		56.5b	34.2a	9.3a
	Koth		81.3a	10.8b	7.9a
Peridotite					
	Kor		44.0b	44.0a	12.0a
	Koth		76.0a	12.9b	11.1a
Marl		**Fe**			
	Kor		93.7a	3.9a	2.4a
	Koth		98.0a	0.9b	1.1b
Gneiss schist					
	Kor		94.0a	3.7a	2.3a
	Koth		98.8a	0.6b	0.6b
Peridotite					
	Kor		90.8a	7.1a	2.1a
	Koth		98.3a	0.8b	0.9b
Marl		**Zn**			
	Kor		49.3b	29.6a	21.1a
	Koth		64.4a	15.6b	20.0a
Gneiss schist					
	Kor		59.1b	26.7a	14.2a
	Koth		73.7a	14.3b	12.0a
Peridotite					
	Kor		37.3b	33.9a	28.8a
	Koth		65.3a	18.0b	16.7b

The different letters in the same column symbolize statistically significant differences between the two olive cultivars in each of the three soils, for $P \leq 0.05$ (n=6) (SPSS; t-test).

Table 1. Distribution (%) of the total per plant quantity of Mn, Fe and Zn in the three vegetative tissues (root, stem and leaves) of the olive cultivars 'Koroneiki' and 'Kothreiki', when each one was grown in three soils (from parent material Marl, Gneiss schist. and Peridotite) with different physicochemical properties (Chatzistathis et al., 2009).

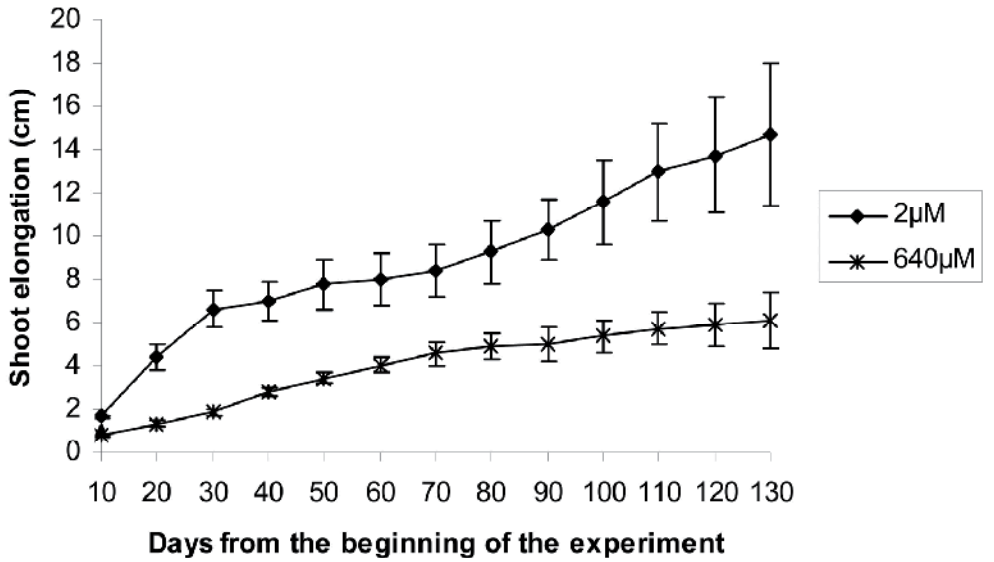
Soil	Cultivar	MnUE	FeUE	ZnUE
Marl		mg of the total plant d.w./μg of the total per plant quantity of micronutrient		
	Kor	31.85a	1.73a	77.53a
	Koth	18.68b	0.65b	68.08a
Gneiss schist				
	Kor	39.87a	1.84a	51.04a
	Koth	17.94b	0.44b	49.15a
Peridotite				
	Kor	23.33a	1.19a	61.75a
	Koth	18.00a	0.58b	72.88a

The different letters in the same column symbolize statistically significant differences between the two cultivars in each of the three soils, for $P \leq 0.05$ (n=6) (SPSS; t-test).

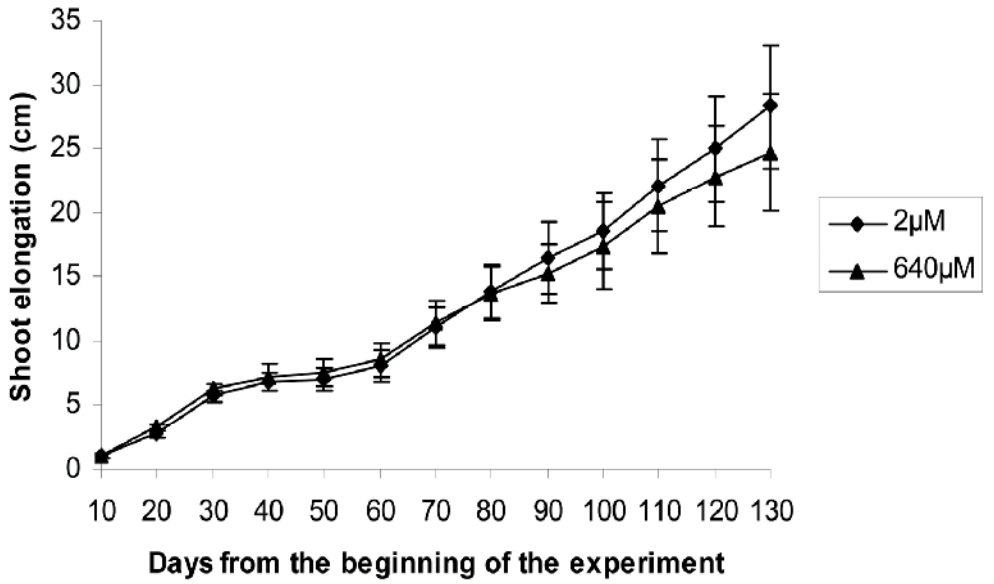
Table 2. Nutrient utilization efficiency (mg of the total plant d.w. /μg of the total per plant quantity of micronutrient or mg of the total per plant quantity of macronutrient) of the olive cultivars 'Koroneiki' and 'Kothreiki', when each of them was grown in three soils (from parent material Marl, Gneiss schist. and Peridotite) with different physicochemical properties (Chatzistathis et al., 2009).

5. The influence of heavy metal toxicity on biomass production

Soil heavy metal contamination has become an increasing problem worldwide. Among the heavy metals, Cu, Zn, Mn, Cd, Pb, Ni and Cr are considered to be the most common toxicity problems causing increasing concern. Growth inhibition and reduced yield are common responses of horticultural crops to nutrient and heavy metal toxicity [2]. Nevertheless, sometimes less common responses happen under metal toxicity conditions. For example, in the case of Pb it has been suggested that inhibition of root growth is one of the primary effects of Pb toxicity through the inhibition of cell division at the root tip [59]. Significant reductions in plant height, as well as in shoot and root dry weight (varying from 3.3% to 54.5%), as compared with that of the controls, were found for *Typha angustifolia* plants in different Cr treatments [60]. Furthermore, according to Caldelas et al. (2012) [19], not only growth inhibition happened (reached 65% dry weight) under Cr toxicity conditions, but also root/shoot partitioning increased by 80%. Under Cr stress conditions, it was found that root and shoot biomass of *Genipa americana* L. were significantly reduced [20]. The biomass reduction of *Genipa americana* trees is ascribed, according to the same authors, to the decreased net photosynthetic rates and to the limitations in stomatal conductance. The disorganization of chloroplast structure and inhibition of electron transport is a possible explanation for the decreased photosynthetic rates of trees exposed to Cr stress [20]. In contrast to the above, Cd and Pb applications induced slight or even significant increase in plant height and biomass. The fact that Cd and Pb addition enhanced Ca and Fe uptake suggests that these two nutrients may play a role in heavy metal detoxification by *Typha angustifolia* plants; furthermore, increased Zn uptake may also contribute to its hyper Pb tolerance, as recorder in the increased biomass over the control plants [60]. According to the



(A)



(B)

Figure 1. Shoot elongation of olive cultivars 'Picual' (A) and 'Koroneiki' (B), when grown under hydroponics at normal (2 μM) and excess Mn conditions (640 μM Mn) (Chatzistathis et al., 2012).

same authors (Bah et al., 2011), plants have mechanisms that allow them to tolerate relatively high concentrations of Pb in their environment without suffering from toxic effects.

Tzerakis et al. (2012) [2] found that excessively high concentrations of Mn and Zn in the leaves of cucumber (reached 900 and 450 mg/kg d.w., respectively), grown hydroponically under toxic Mn and Zn conditions, reduced the fruit biomass due to decreases in the number of fruits per plants, as well as in the net assimilation rate, stomatal conductance and transpiration rate. However, it was found that significant differences concerning biomass production between different species of the same genus exist under metal toxicity conditions; *Melilotus officinalis* seems to be more tolerant to Pb than *Melilotus alba* because no differences in shoot or root length, or number of leaves, were found between control plants and those grown under 200 and 1000 mg/kg Pb [15]. In addition to the above, genotypic differences between cultivars of the same species, concerning biomass production, under metal toxicity conditions may also be observed; Chatzistathis et al. (2012) [13] found that under excess Mn conditions (640 μM), plant growth parameters (shoot elongation, as well as fresh and dry weights of leaves, root and stem) of olive cultivar 'Picual' were significantly decreased, compared to those of the control plants (2 μM), something which did not happen in olive cultivar 'Koroneiki' (no significant differences were recorder between the two Mn treatments) (Figure 1). According to the same authors, some factors related to the better tolerance of 'Koroneiki' not only at whole plant level, but also at tissue and cell level, could take place. Such possible factors could be a better compartmentalization of Mn within cells and/or functionality of Mn detoxification systems [13]. Significant growth reductions of several plant species, grown under Mn toxicity conditions, have been mentioned by several researchers [61-65].

Nickel (Ni) toxicity, which may be a serious problem around industrial areas, can also cause biomass reduction. At high soil Ni levels (>200 mg/kg soil) reduced growth symptoms of *Riccinus communis* plants were observed [18]. According to Baccouch et al. (1998) [66], the higher concentrations of Ni have been reported to retard cell division, elongation, differentiation, as well as to affect plant growth and development. Excess Cd, which causes direct or indirect inhibition of physiological processes, such as transpiration, photosynthesis, oxidative stress, cell elongation, N metabolism and mineral nutrition may lead in growth retardation, leaf chlorosis and low biomass production [67]. According to the same authors, Cd stress could induce serious damage in root cells of grey poplar (*Populus x canescens*). Arsenic (As) toxicity may be another (although less common) problem contributing to soil contamination. Repeated and widespread use of arsenical pesticides has significantly contributed to soil As contamination [4]. According to the same authors, plant growth parameters, such as biomass, shoot height, and root length, decreased with increased As concentrations in all soils.

6. Phytoremediation

Soil pollution represents a risk to human health in various ways including contamination of food, grown in polluted soils, as well as contamination of groundwater surface soils [68].

Classical remediation techniques such as soil washing, excavation, and chelate extraction are all labor-intensive and costly [69].

Phytoremediation of heavy metal contaminated soils is defined as the use of living green plants to transport and concentrate metals from the soil into the aboveground shoots, which are harvested with conventional agricultural methods [70]. The technique is suitable for cultivated land with low to moderate metal contaminated level. According to Jadia and Fulekar (2009) [71], phytoremediation is an environmental friendly technology, which may be useful because it can be carried out *in situ* at relatively low cost, with no secondary pollution and with the topsoil remaining intact. Furthermore, it is a cost-effective method, with aesthetic advantages and long term applicability. It is also a safe alternate to conventional soil clean up [17]. However, a major drawback of phytoremediation is that a given species typically remediates a very limited number of pollutants [24]. For example, a soil may be contaminated with a number of potentially toxic elements, together with persistent organic pollutants [72]. There are two different strategies to phytoextract metals from soils. The first approach is the use of metal hyperaccumulator species, whose shoots or leaves may contain rather high levels of metals [25]. The important traits for valuable hyperaccumulators are the high bioconcentration factor (root-to-soil metal concentration) and the high translocation factor (shoot to root metal concentration) [73]. Another strategy is to use fast-growing, high biomass crops that accumulate moderate levels of metals in their shoots for metal phytoremediation [25]. Phytoextraction ability of some fast growing plant species leads to the idea of connecting biomass production with soil remediation of contaminated industrial zones and regions. This biomass will contain significant amount of heavy metals and its energetic utilization has to be considered carefully to minimize negative environmental impacts [74].

7. Plant species used for phytoremediation

Many species have been used (either as hyperaccumulators, or as fast growing-high biomass crops) to accumulate metals, thus for their phytoremediation ability. Hyperaccumulators are these plant species, which are able to tolerate high metal concentrations in soils and to accumulate much more metal in their shoots than in their roots. By successive harvests of the aerial parts of the hyperaccumulator species, the heavy metals concentration in the soil can be reduced [23]. According to Chaney et al. (1997) [21], in order a plant species to serve the phytoextraction purpose, it should have strong capacities of uptake and accumulation of the heavy metals when it occurs in soil solution. For example, *Sedum plumbizincicola* is an hyperaccumulator that has been shown to have a remarkable capacity to extract Zn and Cd from contaminated soils [75]. In addition, a very good also hyperaccumulator for Zn and Cd phytoextraction is *Thlaspi caerulescens* [23]. *Iris pseudacorus* L. is an ornamental macrophyte of great potential for phytoremediation, to tolerate and accumulate Cr and Zn [19]. Furthermore, many species of *Brassica* are suitable for cultivation under Cu and Zn toxicity conditions and may be used for phytoremediation [29]. *Phragmites australis*, which is a species of *Poaceae* family, may tolerate extremely high concentrations of Zn, Cu, Pb and Cd, thus can be used as heavy metal phytoremediator [76].

Santana et al. (2012) [20] refer that *Genipa americana* L. is a tree species that tolerates high levels of Cr^{3+}, therefore it can be used in recomposition of ciliary forests at Cr-polluted watersheds. According to the same authors, this woody species demonstrates a relevant capacity for phytoremediation of Cr. *Elsholtzia splendens* is regarded as a Cu tolerant and accumulating plant species [77]. Peng et al. (2012) [78] refer that *Eucalyptus urophylla X E.grandis* is a fast growing economic species that contributes to habitat restoration of degraded environments, such as the Pb contaminated ones. On the other hand, concerning Cd phytoextraction ability, only a few plant species have been accepted as Cd hyperaccumulators, including *Brassica juncea, Thlaspi caerulescens* and *Solanum nigrum*. Poplar (*Populus* L.), which is an easy to propagate and establish species and it has also the advantages of rapid growth, high biomass production, as well as the ability to accumulate high heavy metal concentrations, could be used as a Cd-hypaeraccumulator for phytoremediation [27-28,67]. According to Wang et al. (2012) [28], the increase in total Cd uptake by poplar genotypes in Cd contaminated soils is the result of enhanced biomass production under elevated CO_2 conditions. Furthermore, *Amaranthus hypochondriacus* is a high biomass, fast growing and easily cultivated potential Cd hyperaccumulator [25]. Another species was found to be a good phytoremediator concerning its phytoaccumulation and tolerance to Ni stress is *Riccinus communis* L. [18]. Finally, *Justicia gendarussa*, which was proved to be able to tolerate and accumulate high concentration of heavy metals (and especially that of Al), could be used as a potential phytoremediator.

Differences between species, or genotypes of the same species, concerning heavy metal accumulation have been found by many researchers. According to Dheri et al. (2007) [17], the overall mean uptake of Cr in shoot was almost four times and in root was about two times greater in rays, compared to fenugreek. These findings, according to the same authors, indicated that family *Cruciferae* (raya) was most tolerant to Cr toxicity, followed by *Chenopodiaceae* (spinach) and *Leguminosae* (fenugreek). Peng et al. (2012) [78] found that cultivar ST-9 of *Eucalyptus urophylla X E.grandis* was shown to accumulate more Pb than others of the same species, like ST-2, or ST-29.

8. Different strategies adopted in order to enhance biomass production under heavy metal toxicity conditions

Under elevated CO_2 conditions the photosynthetic rate is enhanced, thus biomass production is positively influenced. According to Wang et al. (2012) [28], the increase in total Cd uptake by poplar (*Populus* sp.) and willow (*Salix* sp.) genotypes due to increased biomass production under elevated CO_2 conditions suggests an alternative way of improving the efficiency of phytoremediation in heavy metal contaminated soils.

The use of fertilizers is another useful practice that should be adopted by the researchers in order to enhance biomass production under extreme heavy metal toxicity conditions. Some Brassica species, which are suitable to be used as phytoremediators, may suffer from Fe or Mn deficiency symptoms under Cu, or Zn toxicity conditions. In that case, leaf Fe and Mn fertilizations should be done in order to increase their biomass production [29], thus their

ability to absorb and accumulate great amounts of heavy metals in contaminated soils, i.e. the efficiency of phytoremediation. According to Li et al. (2012) [25], in order to achieve large biomass crops, heavy fertilization has been practiced by farmers. Application of fertilizers not only provides plant nutrients, but may also change the speciation and mobility of heavy metals, thus enhances their uptake. According to Li et al. (2012) [25], NPK fertilization of *Amaranthus hypochondriacus*, a fast growing species grown under Cd toxicity conditions, greatly increased dry biomass by a factor of 2.7-3.8, resulting in a large increment of Cd accumulation. High biomass plants may be beneficed and overcome limitations concerning metal phytoextraction from the application of chemical amendments, including chelators, soil acidifiers, organic acids, ammonium e.t.c. [21]. Mihucz et al. (2012) [79] found that Poplar trees, grown hydroponically under Cd, Ni and Pb stress, increased their heavy metal accumulation by factor 1.6-3.3 when Fe (III) citrate was used.

Mycorrhizal associations may be another factor increasing resistance to heavy metal toxicity, thus reducing the depression of biomass due to toxic conditions. Castillo et al. (2011) [80] found that when *Tagetes erecta* L. colonized by *Glomus intraradices* displayed a higher resistance to Cu toxicity. According to the same authors, *Glomus intraradices* possibly accumulated excess Cu in its vesicles, thereby enhanced Cu tolerance of *Tagetes erecta* L. [80].

Finally, other factors, such as the influence of *Bacillus* sp. on plant growth, in contaminated heavy metal soils, indicate that biomass may be stimulated under so adverse conditions. According to Brunetti et al. (2012) [81], the effect of the amendment with compost and *Bacillus licheniformis* on the growth of three species of *Brassicaceae* family was positive, since it significantly increased their dry matter. Furthermore, the strain of *Bacillus* SLS18 was found to increase the biomass of the species sweet sorghum (*Sorghum bicolor* L.), *Phytolacca acinosa Roxb*., and *Solanum nigrum* L. when grown under Mn and Cd toxicity conditions [82].

9. Conclusion and perspectives

Biomass production is significantly influenced by many environmental, agronomic and other factors. The most important of them are air and soil temperature, soil humidity, photoperiod, light intensity, genotype, and soil nutrient availability. Soil fertility, i.e. the availability of nutrients in the optimum concentration range, greatly influences biomass production. If nutrient concentrations are out of the optimum limits, i.e. in the cases when nutrient deficiency or toxicity occurs, biomass production is depressed. Under nutrient deficient conditions, the farmers use chemical fertilizers in order to enhance yields and fruit production. However, since the prices of fertilizers have been significantly increased during the last two decades, a very good agronomic practice is the utilization of nutrient use efficient genotypes, i.e. the utilization of genotypes which are able to produce high yields under nutrient limited conditions. Although great scientific progress has been taken place during last years concerning nutrient use efficient genotypes, more research is still needed in order to clarify the physiological, genetic, and other mechanisms involved in each plant species.

On the other hand, in heavy metal contaminated soils, many plant species could be used (either as hyperaccumulators, or as fast growing-high biomass crops) in order to accumulate metals, thus to clean-up soils (phytoremediation). Particularly, the use of fast growing-high biomass species, such as Poplar, having also the ability to accumulate high amounts of heavy metals in their tissues, is highly recommended, as the efficiency of phytoremediation reaches its maximum. Particularly, since a given species typically remediates a very limited number of pollutants (i.e. in the cases when soil pollution caused by different heavy metals, or organic pollutants), it is absolutely necessary to investigate the choice of the best species for phytoremediation for each heavy metal. In addition to that, more research is needed in order to find out more strategies (apart from fertilization, the use of different *Bacillus* sp. strains, CO_2 enrichment under controlled atmospheric conditions e.t.c.) to enhance biomass production under heavy metal toxicity conditions, thus to ameliorate the phytoremediation efficiency.

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This two-volume book on biomass is a reflection of the increase in biomass related research and applications, driven by overall higher interest in sustainable energy and food sources, by increased awareness of potentials and pitfalls of using biomass for energy, by the concerns for food supply and by multitude of potential biomass uses as a source material in organic chemistry, bringing in the concept of bio-refinery. It reflects the trend in broadening of biomass related research and an increased focus on second-generation bio-fuels. Its total of 40 chapters spans over diverse areas of biomass research, grouped into 9 themes.

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