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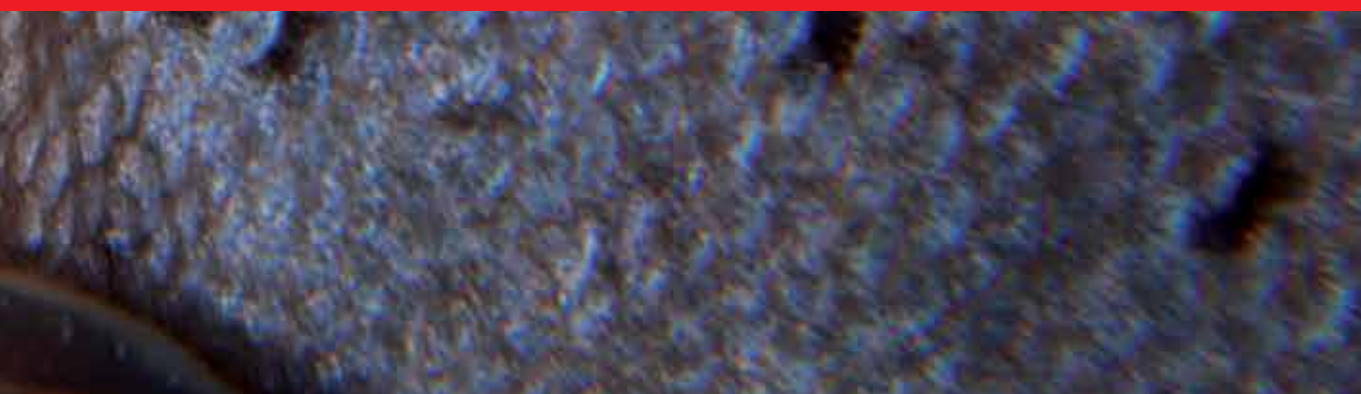
Biological Resources of Water

Edited by Sajal Ray



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Contributors

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Notice

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Meet the editor

Sajal Ray received his MSc and MPhil degrees in Zoology and Environmental Science, respectively, from Calcutta University and his PhD degree from Jadavpur University. His thesis reports immunotoxicity of pesticide in an economically important snail in India. As an awardee of Fogarty Visiting Fellowship, Dr. Ray carried out his postdoctoral research in cardiac pathology at the National Institutes of Health, USA. His research interest is studying the immunological responses of mollusc, sponge, crab and earthworm exposed to pollutants. His team is engaged in understanding the evolutionary mechanism of immunity in phylogeny. He presented his research in various conferences including the World Congress of Malacology at Washington, DC. Sajal Ray, currently a professor of Zoology at Calcutta University, has been teaching Zoology for nearly 30 years at the postgraduate level.

Contents

Preface

Growth of human civilisation, from time immemorial, depended largely on biological resources. Ancient humans understood the significance of biological resources needed for their survival and perpetuation. Hunter-gatherer mode of life of many primitive societies relied on forest and river resources for alleviation of hunger. They derived nutrition from such sources that are thought to influence their general biological traits like physical and mental development, reproduction, migration and immunity. Problem related to its steady and uniform availability pushed these societies towards farming, agriculture and fishery. The earliest form of fishery dealt with hunting and trapping of aquatic species with or without tools and fishing devices. This primitive form of capture fishery was later developed into a systematic culture of aquatic species ensuring larger and definitive harvestation. The practice of primitive fishing technologies and gears among some of the primitive tribes indicates their traditional dependence over aquatic bioresource. Archaeologists unearthed an oyster shell mound among debris near the banks of a river at modern-day Maryland, which is thought to be an early human settlement in the USA. Shellfish like oysters were important dietary resource in the early riverside settlement. Biological resources of water, which include fish, oyster, mussel, crab, prawn, octopus, sponge and plant species among others, bear dietary, nutritional, pharmaceutical, ecological and economical significance and prospect. Many of the biological resources of water are either edible or act as a source of ethnomedicine and contribute greatly on global economics related to aquaculture. However, the basics of biology, ecology and taxonomy of many aquatic species are yet to be investigated thoroughly. Deterioration of air and water quality associated with habitat destruction has been identified as a major ecological threat for the aquatic flora and fauna with resource potential. This situation has been worsened in recent years under the backdrop of climate change.

Issues related to rational utilisation, conservation and access to the aquatic bioresources are linked with sustainable resource management and honouring the common natural property right of the masses of many underdeveloped and developing countries. In this book, with two sections, issues concerning biology, ecology, physiology, genetic characterisation, conservation and management of the aquatic bioresources have been discussed with supportive scientific data. It will provide a holistic view of the past and present status of the important biological resources of high ecological and economic values. In Section 1, entitled "Biology, Ecology and Physiological Chemistry", the authors discuss the pivotal issues related to biology, diversity and molecular physiology of the established biological resources of water. Molecular physiology of egg-laying in cuttlefish, an important biological resource of marine ecosystem, is discussed. The authors highlight the significance of chemical communication in the process of egg aggregation. Molecular orchestration of neuropeptides involved in environmental cue integration, ovarian regulatory peptides and sex pheromones is explained.

Composition and developmental characteristics of egg case formation are elucidated. Molecular events ensuring the successful reproduction of cuttlefish would provide a better understanding of the reproductive physiology and management of this resource in its natural habitat. Endocrine-disrupting chemical compounds are posing serious ecophysiological threats to many aquatic organisms with high resource potential in recent years. Freshwater pond snails are established as unique indicators of aquatic contamination. Various reproductive parameters like fecundity, oocyte and egg mass production, egg mass quality, time window of cell division in the offspring and metabolite content of zygote are experimentally evaluated for reproductive markers of aquatic contamination of steroids in snail. The prospect of pond snail as a model of environmental risk assessment is discussed from a novel viewpoint. A polymarker approach involving the physiological and behavioural parameters related to snail reproduction is considered for risk assessment of steroid contamination of water. Genetic characteristics of brook trout populations are discussed at the zone of contact from the viewpoint of evolution, distribution and management of this bioresource. Ecological implication of invasion of alien species of fish is mentioned. In another chapter, the authors explain the mosaic-like complexity of Ice Age environment, which influenced the evolution of mollusc at the regional and local scale.

Conclusion is drawn on the basis of paleontological evidence of Ice Age gastropod refugia of the Carpathian basin of Central Europe. Biodiversity status of many aquatic bioresources has been exhibiting structural and functional alteration primarily in recent years due to climate change and human activities. Thus, the need for routine examination of the resource diversity is stressed upon. The diversity, distribution and abundance of malacological resource are estimated in the southeastern Gulf of California, Mexico, an important biodiversity zone. The authors report the current biodiversity status of molluscs collected from the nonsimilar ecological zones of Mexico.

Fishery, pollution and climate change are reported to affect the distribution, habitat, behaviour and life history traits of marine snail (*Phorcus* sp.) of Portugal. Anatomy, taxonomy, distribution, ecology, feeding habit, growth rate and reproductive status of these marine molluscs are discussed with reference to their resource potential. Monitoring potential of water toxicity of these keystone species is discussed too. Guatemala, a megadiverse country, supports a wide range of natural food resource in the form of crustacean organisms to its human inhabitants. Freshwater prawns, crabs and crayfish constitute a traditional source of diet for many of its local populations. The species richness of major decapod crustaceans with resource potential is reported. The authors mention the roles of constructing dam and agricultural activities as detrimental factors for declining the population density of these native species in rivers and aquatic reservoirs. This section also includes chapters contributed on the physiological and biochemical significance of biological resources of water. Black sea mussel of Bulgarian Black Sea Coast is an edible variety of marine mollusc with high commercial and nutritional values. The seasonal dynamics of fatty acids, cholesterol, fat-soluble vitamins and carotenoids of this species are discussed. The commercial and nutritional prospects of black sea mussel of this region are reported with reference to the relative availability of important bioactive molecules traced in them. These mussels, with high prospect in aquaculture, are reported as a source of many therapeutic compounds. Sponges are the earliest evolved Metazoa distributed both in marine and freshwater ecosystems. They are the potential sources of drugs against cancer, bacteria and fungus infections. The authors in this chapter review the sources of these drugs and other pharmacoactive compounds in sponges.

Sponge as a source of antifouling and antibiofilm compounds is reported. The chemical ecology of aquatic molluscs is discussed with reference to the dynamics of multiple water quality parameters like temperature, salinity, pH and related food sources. The authors indicate these parameters as influencing agents of various natural compounds as well as the biological traits like male-female ratio, reproduction and breeding cycle of molluscs.

In Section 2, entitled "Conservation and Sustainable Management", the authors discuss the status of conservation and eco-friendly management of different live resources of water, both nonchordate and chordate. It describes the importance of genetic application in conservation of neotropical freshwater fish. Important regulatory aspects of fishery management are also highlighted. Conservation status of various organisms including the dolphin and gharial of Girwa river of India is presented by the scientists of the field. The authors also mention the avifaunal diversity of Girwa river. Construction of dam, unavailability of food species and unrestricted netting and killing are also reported as the major concerns for the conservation of river dolphin, a unique bioresource of India. The prospect of shrimp and prawn farming in Indonesia is reported with reference to "zero water discharge" technology. This novel method of crustacean culture is prescribed as an efficient methodology alternative to the conventional one. Detailed methodologies including the designing of construction system for microbial culture, conditioning of system and microbial manipulation are discussed for culture of this bioresource. An increase of 10–20% of survivability of culturable species indicates the technical and economic prospects of the technology. This edited volume comprises informative chapters dealing with the basic and applied aspects of bioresource research. Important issues related to biology, ecology, diversity status, pharmaceutics, threat factors and innovative technologies for sustainable management of different phyletic forms of aquatic bioresource are addressed. This book will also provide the future direction of research in this field.

I thankfully acknowledge Ms. Dajana Pemac of IntechOpen for her sincere cooperation that I received during every step of editing of this book. I accepted the technical assistance from my doctoral research students Santanu, Abhisek and Arunodaya of Aquatic Toxicology Laboratory of the Department of Zoology of the Calcutta University. My special thanks go to my wife Dr. Mitali Ray, daughters Shubhalakshmi and Bishnupriya and my son-in-law Arka for their cheerful encouragement.

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Section 1

Biology, Ecology and Physiological Chemistry

Chapter 1

Egg-Laying in the Cuttlefish *Sepia officinalis*

Céline Zatylny-Gaudin and Joël Henry

Additional information is available at the end of the chapter

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Abstract

This chapter reviews studies about egg-laying in the cuttlefish Sepia officinalis. Egg masses are spawned in specific mating and spawning coastal areas where mates aggregate between April and June in the English Channel and all year long in the Mediterranean Sea. Environmental cues are clearly involved in the aggregation process, but chemical communication also plays a determining role in these complex mechanisms. The successive steps of egg-laying are orchestrated by three classes of regulatory peptides: (1) neuropeptides that integrate environmental cues, (2) ovarian regulatory peptides that modulate the activity of the genital tract, and (3) sex pheromones expressed and released by the oviduct gland. After egg-laying, embryo protection is ensured for 8-10 weeks by a multilayer capsule secreted by the accessory sex glands. The oviduct gland secretes the inner layer of the egg case. The main nidamental gland secretes the main polysaccharides and glycoproteins, such as Sepia Egg Case Proteins, involved in capsule formation and in embryo protection. The accessory nidamental gland expresses specific proteins inherent in the structural organization of the gland, and hosts symbiotic bacteria. Similarly to salivary glands, this gland secretes immune factors possibly associated with gamete and/or embryo protection.

Keywords: reproduction, egg-laying, neuropeptides, ovarian peptides, egg-case, common cuttlefish

1. Introduction

Phylogenetically, anatomically, and physiologically speaking, cephalopods are indeed mollusks. Yet, they possess special characteristics that distinguish them from other molluscan classes, especially the other two major classes, gastropods and bivalves.

First of all, they are the most mobile mollusks. They come in the form of pelagic species capable of performing large-amplitude horizontal and vertical migrations, like squid. Octopoda are in turn largely territorial and therefore sedentary, while the common cuttlefish *Sepia officinalis* exhibits a nectobenthic behavior associated with low or medium migration amplitude depending on the latitude.

Unlike other classes of molluscs, cephalopods possess a cephalopodium whose eight arms play an important role in predation but also in mating and egg-laying during the formation of the egg mass (**Figure 1**). These arms are also used for handling prey during catches by capping [1].

This ability to handle prey is quite unique in the marine environment and only found in primates and in some mammals. It is probably related to the exceptional development of the central nervous system (CNS). This CNS is protected by a cartilaginous skull and is located between the eyes, which are capable of forming an image.

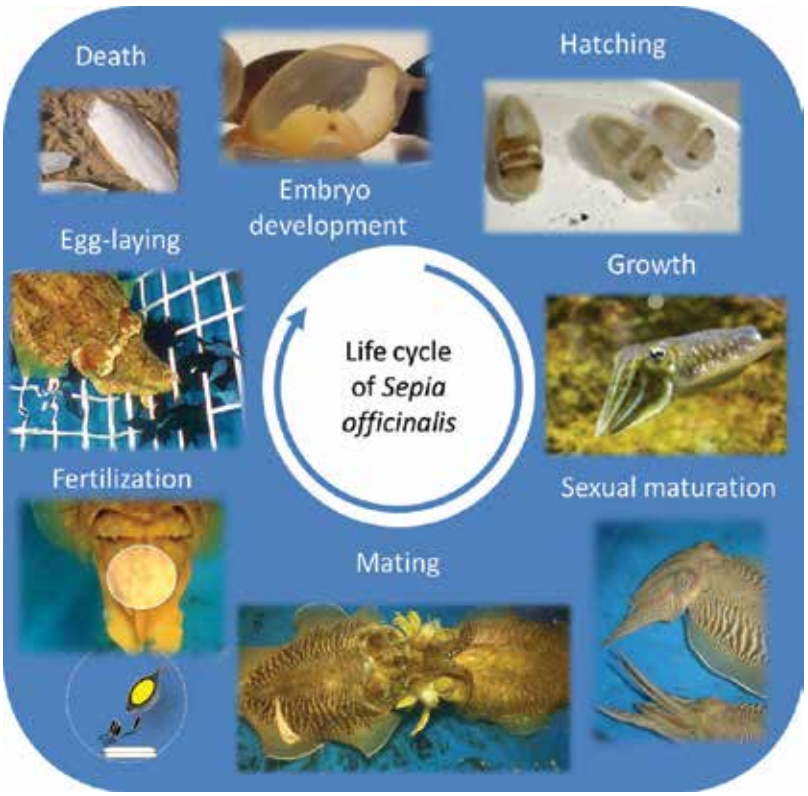

Figure 1. Main successive steps of the life cycle of the cuttlefish *Sepia officinalis*. (Photo credits: V. Cornet, J. Henry, C. Zatylny-Gaudin).

2. Egg-laying: description

The cuttlefish *Sepia officinalis* is a semelparous species with a life cycle that varies depending on the geographical location of the population: 15–16 months in the Mediterranean Sea versus 20–22 months in the English Channel. Spawning is associated with a stereotyped behavior. In aquaria, sexually mature females that have not spawned yet manipulate eggs laid earlier by conspecifics, while increasing their ventilation rate.

The different behavioral sequences leading to the laying of eggs are gradually repeated and eventually lead to the laying of the first egg (personal observation). A female can lay dozens of eggs at once, probably 150–200 eggs, which roughly corresponds to the storage capacity of the genital coelom, before a pause that allows it to replenish its stock of mature oocytes (stage V) through asynchronous gametogenesis. It also restores the capsular products secreted by the oviduct gland and the nidamental glands. Some females are then able to lay a second batch of eggs and probably several successive spawns. We do not know exactly how many eggs are laid before the programmed death of the animal. Fertility is probably quite variable among females and is very difficult to estimate.

3. Egg-laying: regulation

The first work on the contractile structures of the female reproductive system led to the identification of numerous myotropic or myosuppressor regulatory peptides. The first of them, a neuropeptide belonging to APGWamide family, was identified from a sample of optic lobes purified by rpHPLC on the basis of its myosuppressive effect on the contraction of the distal oviduct [2].

Then, new myotropic bioassays on the contractile organs of the female genital apparatus were performed, and the bases of the functional control of spawning and the related activities were laid, for example, blocking or inducing oocyte transport in the oviduct and the secretion of internal and external oocyte capsules before fertilization.

From the papers published on this topic between 1997 and 2006, it appears that the successive steps of egg-laying are mainly governed by two classes of regulatory peptides: neuropeptides involved in the integration of environmental cues and ovarian regulatory peptides that modulate the activity of the genital tract [2–8]. The recent development of "-omics" approaches based on *de novo* RNAseq and mass spectrometry led to the identification of transcripts and mature cleavage products.

Using a transcriptomic approach, Enault and collaborators [9] discovered a third category of regulatory peptides, namely the sex pheromones expressed and released by the oviduct gland, and cleaved from three protein precursors into bioactive peptides ranging between 1.3 and 8 kDa.

Thanks to the sequencing of the neurotranscriptome, several neuropeptide families involved in the regulation of egg-laying were identified on the basis of expression pattern and tissue localization out of the 38 families composing the cuttlefish neuropeptidome [10]. Finally, the RNA sequencing of ovary tissue revealed that most of the ovarian regulatory peptides involved in oocyte release were cleaved from a single yolk protein (unpublished results).

4. Neuropeptides

As mentioned above, the first neuropeptide identified in cuttlefish on the basis of its ability to modulate the contractile activity of the oviduct was characterized by Henry and collaborators [2] from the optic lobes of egg-laying females (**Figure 2**).

It is the GWamide, a dipeptide that belongs to the APGWamide family and is derived from enzymatic cleavage of the APGWamide by a CNS dipeptidyl aminopeptidase (DPAP).

DPAP activity appears to be an alternative mechanism for the maturation of precursors into bioactive proteins or peptides such as peptides produced by amphibian skin, precursors of lytic peptides in honeybee venom, bactericidal peptides secreted in the insect hemolymph, or extracellular proteases in yeasts [11].

In gastropods, another molluscan class, the APGWamide is involved in the control of male behavior. In the pond snail *Lymnaea stagnalis*, this tetrapeptide detected in the penile nerve regulates penis erection [12–14].

Besides, FMRFamide-related peptides (FaRPs) also occur in the nervous fibers of the female accessory sex glands. Their occurrence was demonstrated by immunohistochemistry. Perfused FaRPs induce strong modifications of the contractile activity of the distal oviduct [3]. The involvement of two neuropeptide families—APGWa-RPs and FaRPs—in the control of egg-laying suggests a complex regulation of the successive steps leading to the formation of the egg mass.

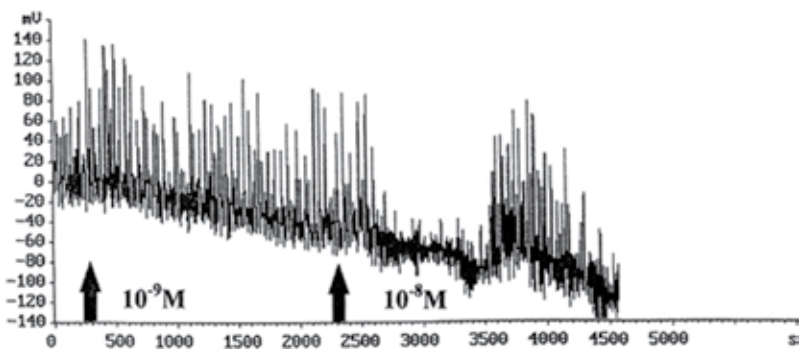

Figure 2. Decrease of the tonus, frequency, and amplitude of oviduct contractions following treatment with 10^{-8} M synthetic GWamide. A dose of 10^{-9} M GWamide did not induce contractions; therefore, the threshold for GWamide activity appears to be between 10^{-8} and 10^{-9} M [2].

The identification of the cuttlefish neuropeptidome by *de novo* RNAseq and mass spectrometry screening was the next step that provided an overview of the neuropeptidome *via* a deep structural and functional investigation [10].

Based on the filtering criteria applied to the 38 identified neuropeptide families—expression level, neuropeptide tissue mapping, and mRNA localization—seven neuropeptide families were finally selected: allatostatins, APGWamide, crustacean cardioactive peptides (CCAPs), FaRPs, FLGamide, myomodulins, and small cardioactive peptide (SCP).

Several neuropeptides cleaved from the protein precursor of **allatostatin A1** and **A2** issued from alternative splicing were detected by nanoliquid chromatography tandem mass spectrometry (nLC-MS/MS) in the oviduct gland and the main nidamental glands, suggesting a role in egg capsule secretion. In insects, **FGLamide allatostatins** (also called **allatostatins A** or **buccalins**) are involved in reproduction [15] and feeding decisions interacting with the adipokinetic hormone (AKH) and insulin-like peptides [16]. In cuttlefish, **allatostatins A1** and **A2** were also detected in the hemolymph, in accordance with the status of neurohormones that could regulate the biosynthesis of egg capsule products during vitellogenesis.

The **APGWamide** was detected by nLC-MS/MS in the CNS, and the **GWamide** was characterized from the CNS [2]. Moreover, large amounts of mRNAs were found in the OGs, MNGs, and ANGs of egg-laying females. Similar observations were reported in the pond snail *Lymnaea stagnalis* by van Minnen and Bergman [17]. High amounts of mRNAs encoding the egg-laying hormone were detected in the nerve terminals after a stimulus, as well as polyribosomes, supporting that the translation of egg-laying hormone transcripts could occur in the axonal compartment. These data are supported by recent papers revealing the occurrence of rough endoplasmic reticulum, smooth reticulum, and Golgi apparatus in the axonal compartment [18]. Otherwise, Martin and Kim [19] used Aplysia as a model to show that netrin-1, already known to promote translation in axonal growth cones [20], increased translation of subcellular mRNAs localized at the level of dendrites or axons by binding the cytoplasmic domain of the netrin-1 receptor called DCC (for deleted colorectal cancer). The rapid reaction of female cuttlefish can be related to the state of readiness of the axons that innervate the ASGs.

The three neuropeptides predicted from the protein precursor of **CCAPs** were detected by nLC-MS/MS in the CNS, the oviduct gland and the main nidamental glands [10]. **CCAPs** are also overexpressed in the sub-esophageal mass of egg-laying females (as opposed to mature males), which is the only part of the CNS that innervates the genital apparatus. This neuropeptide family is strongly suspected to regulate egg capsule secretion.

CCAPs were initially described in crustaceans. They are usually C-terminally amidated neuropeptides in arthropods [21–24], as well as in mollusks [14, 25–27], whereas the cuttlefish preprohormone predicted three nonamidated peptides confirmed by nLC-MS/MS analysis.

The four neuropeptides predicted from the protein precursor of **FaRPs** were detected by nLC-MS/MS in the CNS, and the decapeptide ALSGDAFLRFamide was the only one detected in the neurohemal area connected to the sub-esophageal mass and in the oviduct gland and main nidamental glands, confirming the immunostaining results obtained by Henry and collaborators [3]

in the ASGs of mature females. These neuropeptides are widely distributed in the animal kingdom and involved in many physiological regulation processes in mollusks such as heart activity [28], amylase secretion [29], feeding [30], and reproduction [31]. In cuttlefish, they are believed to regulate egg capsule secretion and oocyte transport in the oviduct [3] and also chromatophore control pathways [32].

FLGamide is a novel neuropeptide family never described so far in the animal kingdom although blastn revealed a similar precursor annotated "uncharacterized protein" (ELU03112) in the polychaete worm *C. teleta* [33]. Most of the eight neuropeptides predicted from the two protein precursors were detected by nLC-MS/MS in cuttlefish CNS, oviduct gland, main nidamental glands, and ovarian stroma [10]. It is the only neuropeptide family detected in the ovarian stroma, suggesting a putative involvement in the release of smooth oocytes into the genital coelom. But recent unpublished experiments show that these neuropeptides do not induce any modification on contractile activity when perfused into the ovarian stroma. As they are also detected in the hemolymph, they are thought to be involved in vitellogenesis regulation.

In the oviduct gland, recent unpublished experiments show that they regulate oocyte transport in the oviduct. They could also induce egg capsule biosynthesis and secretion.

Myomodulins were detected by nLC-MS/MS in the CNS, the oviduct gland and the main nidamental glands [10], and so were mRNAs, as described for **APGWamide** and **CCAPs**. They appear to be closely associated to accessory sex glands involved in egg capsule secretion.

In the tropical abalone *Haliotis asinine*, egg-laying is characterized by a dramatic increase in the expression of **APGWamide**, **myomodulins,** and **insulin** within 12 h of the spawning event. Expression strongly decreases 24 h after spawning, demonstrating that these neuropeptides have a regulatory role in the release of gametes [34]. In cuttlefish, the mRNAs of these three neuropeptides are recovered at the level of the oviduct gland and main nidamental glands, suggesting that they are involved in the rapid response of the genital apparatus after mating.

The detection of the **small cardioactive peptide (SCP)** by mass spectrometry proved very difficult and was restricted to the oviduct gland. As already described for **CCAPs**, **SCP** is also overexpressed in the sub-esophageal mass of egg-laying females (as opposed to mature males). This neuropeptide could be related to the secretion of the internal layer of the egg capsule and also in the release of oocytes into the mantle cavity. The preprohormones of all these neuropeptides are presented in **Figure 3**. The OG and MNGs are closely associated in egg capsule elaboration. Just before fertilization, oocytes are embedded into two layers of egg capsule proteins: the inner layer is secreted by the oviduct gland and the outer layer by the main nidamental glands. These glands synthesize and secrete most of the egg capsule constituents. The similar function of OG and MNGs could explain why they share so many common regulatory neuropeptides. Three categories can be distinguished among them (**Table 1**): (1) **APGWamide** neuropeptides and **myomodulins** whose mRNAs are recovered in OG and MNGs, probably located in the axon ends; they can be associated to a rapid responsiveness following mating; (2) **allatostatins A**, **CCAPs**, and **SCP** neuropeptides, overexpressed in the sub-esophageal mass

of egg-laying females and detected in OG and MNGs; they stimulate the mechanical contraction of glands for egg capsule secretion when the oocyte reaches the distal oviduct embedded by the oviduct gland, just before being released into the mantle cavity; (3) **FLGamide** and **FMRFamide** neuropeptides are detected in OG and MNGs and also in the hemolymph; they modulate oviduct contractions and are probably involved in the mechanical secretion of the egg capsule. Moreover, as they are circulating peptides, they could also be involved in the

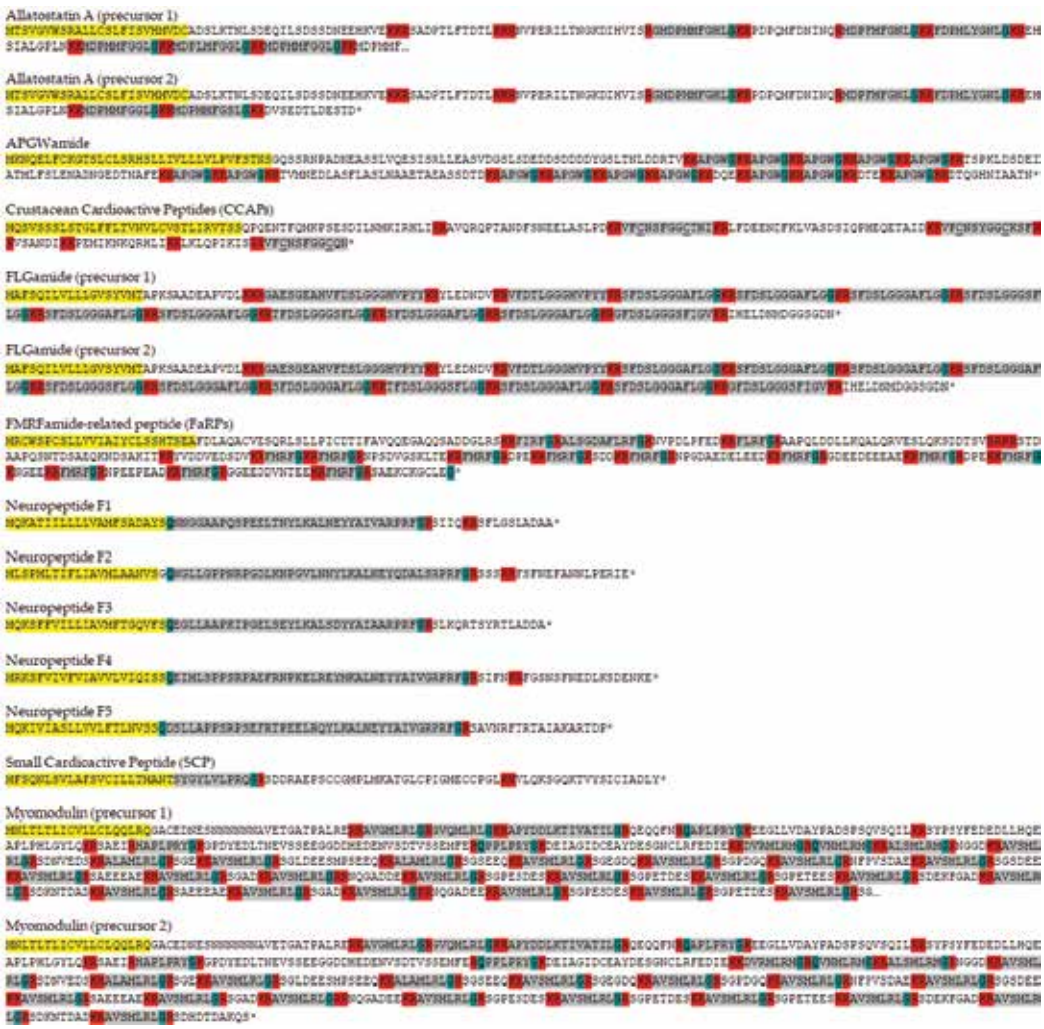

Figure 3. Preprohormones of allatostatins A1 and A2, APGWamide, crustacean cardioactive peptide, FLGamide, FMRFamide-related peptides, myomodulin, neuropeptides F1, F2, F3, F4, F5, and small cardioactive peptide, that all encode neuropeptides detected in the nerve endings and/or in the CNS of *S. officinalis* egg-laying females or by *in silico* data mining. Predicted signal peptides are highlighted in yellow, convertase cleavage sites in red, cysteines are underlined, glycine residues predicted to be converted into C-terminal amides in blue-gray, as well as glutamine residues predicted to be converted into pyroglutamate. Neuropeptides are highlighted in gray, and the stop codons at the end of the coding sequences are indicated by an asterisk [10].

regulation of the synthesis of yolk proteins and/or egg capsule proteins. In addition to the neuropeptides directly involved in the regulation of egg-laying, RNAseq revealed a substantial overexpression of neuropeptides Y (NPY), also called neuropeptides F (NPF) in protostomes because of a C-terminal tyrosine amide substituted by a phenylalanine amide (**Figure 3**). The five transcripts of cuttlefish NPF are unequally overexpressed.

The expression level of NPF 1, the most overexpressed of them, reaches 45-fold the expression level found in mature males in the same part of the CNS. NPFs are probably overexpressed in females to stimulate feeding in order to support gametogenesis and egg capsule synthesis between each spawning step. In this species, asynchronous gametogenesis allows females to resume egg-laying by replenishing their batch of mature oocytes and biosynthesizing egg capsule products until they die.

More generally, the overexpression of many neuropeptides found in the sub-esophageal mass of egg-laying females (as compared to males) could be due to the regulation associated to the production of several batches of oocytes that contain a large quantity of vitellus for embryonic development and to the mobilization of the energy needed to carry out gametogenesis. By contrast, in English Channel males, gametogenesis ends 6 months before reproduction and produces a much smaller volume of gametes than in females. The energy required for male gametogenesis is probably very low compared with the energy required for female gametogenesis.

Finally, a 36-amino-acid neuropeptide called Egg-Laying Hormone (ELH) can induce egg-laying following a single intramuscular injection in the foot of Gastropods. In cuttlefish, ELH

	Occurrence of neuropeptides			Overexpression of transcript	Localization of transcripts			Functional status
	OG	MNG	OS	subEM	OG	MNG	ANG	
APGWamide	NO	NO	NO	NO	YES	YES	YES	neuromodulator
Myomodulins	YES	YES	NO	NO	YES	YES	YES	neuromodulator
Allatostatins A	YES	YES	NO	NO	NO	NO	NO	neuromodulator
CCAPs	YES	YES	NO	YES	NO	NO	NO	neuromodulator
SCP	YES	NO	NO	YES	NO	NO	NO	neuromodulator
FaRPs	YES	YES	NO	NO	NO	NO	NO	neurohormone neuromodulator
FLGamide	YES	YES	YES	NO	NO	NO	NO	neurohormone neuromodulator

ANG: accessory nidamental gland; MNG: main nidamental gland; OG: oviduct gland; OS: ovarian stroma; SubEM: sub-esophageal mass.

Table 1. Tissue mapping of neuropeptides and mRNAs in the female cuttlefish.

remains unknown despite *in silico* data mining from transcriptomic data associated to nLC-MS/MS screenings of the CNS of egg-laying females. Considering that cephalopods are the only class among the three main molluscan classes in which no ELH was ever identified, we can hypothesize that the reason is the loss of the ELH gene or more probably a low level of structural conservation leading to a failure of the data mining strategy or an insufficient depth of RNAseq. Further deeper RNA sequencing will probably allow for the identification of this neuropeptide in cephalopods.

5. Sex pheromones

During a short life cycle of about 22 months, English Channel cuttlefish can perform four horizontal migrations from the Normandy coasts to the western part of the English Channel [35]. After a last migration to reach specific mating and spawning coastal areas, cuttlefish aggregate for mating and egg-laying between April and June on the Normandy coasts. This behavior suggests the occurrence of some kind of chemical communication *via* waterborne molecules that induce the aggregation of mates. Chemical communication in cuttlefish was first demonstrated by Boal and collaborators [36, 37] using y-mazes. They showed that recently laid eggs, ovary extracts and nidamental glands, induced an increase in ventilation rate and the attraction of sexually mature cuttlefish in the arm of the y-maze containing purified extract *versus* artificial sea water. In the same way, Cummins and collaborators [38] identified a 10 kDa protein in Loligo termed Loligo β-microsemino-protein (Loligo β-MSP) that immediately changes the behavior of male squid from calm swimming and schooling to extreme fighting. Loligo β-MSP is synthesized in the accessory sex gland of females—the oviduct gland, the main and accessory nidamental glands—and is secreted with the proteins of the outer tunic of egg capsules. When a male is attracted to the eggs visually, upon touching them and contacting Loligo β-MSP, it immediately escalates into intense physical fighting with any nearby males.

Loligo β-MSP was originally discovered in human seminal plasma and prostatic fluids [39]. It is only described in other vertebrates [40–44] and in the basal chordate amphioxus [45]. It is a highly variable 91-amino-acid protein, with 10 spatially conserved cysteine residues than can potentially form five intramolecular disulfide bonds, giving resistance to proteolytic cleavage to prolong its activity on the egg surface.

In *Sepia officinalis*, Enault and collaborators [9] identified three major related transcripts encoding secreted peptides and expressed in the oviduct gland. RT-PCR and mass spectrometry analyses revealed that transcripts and expression products were co-localized in the oviduct gland. The two very similar protein precursors termed SPα and SPα' (**Figure 4**) diverge by only four amino acids in the α3 and α3′ peptides. They yield seven putative expression products ranging from 1.3 kDa (α5) to 7 kDa (α3 and α3′).

All peptides except α1 contained at least one cysteine, and two of them, α3 and α3′, are C-terminally amidated like many bioactive peptides (**Figure 5A**).

The third protein precursor, termed SPβ, shares 56.7% similarity with SPα and SPα' (**Figure 4**) and yields five putative expression products ranging from 1.1 kDa (peptide β1) to 8.3 kDa

Figure 4. Amino acid alignments of the three protein precursors SPα-α′ and SP β. Red asterisks indicate conserved cysteines. Predicted signal sequences are highlighted in yellow, the conserved sequence between SPs in green, differences between Spα and SPα′ in blue, and potential basic residue cleavage sites in red [9].

(peptide β3), with C-terminal amidation (peptide β2), disulfide bonds (peptides β3, β4, β5), or N-glycosylation (peptide β3) (**Figure 5B**).

For most of the expression products derived from SPα-α′ and SPβ, predicted post-translational modifications such as C-terminal amidation and disulfide bonds have been confirmed by nLC-MS/MS analysis. These modifications can provide a strong protection against protease and peptidase activity and can be expected to confer the peptides a long life in marine environments. As most of the predicted peptides were recovered by nLC-MS/MS analysis, the processing of SPα-α′ and SPβ should lead to the release of a cocktail of waterborne pheromones. Peptides α3 and α2 strongly stimulate the contraction of the penis and the gills when they are applied on these parts (**Figure 6**).

Therefore, peptides expressed and secreted by a female's accessory sex gland can modulate the activity of a male's genital apparatus. Recent unpublished data show that the protein precursors SPα and SPβ are also able to release a second batch of high-molecular-weight (22–26 kDa) pheromones secreted with the egg capsule proteins and integrated to the inner layer of the egg capsule. Finally, they are detected in the sea water around egg masses once they have crossed the outer layer of the egg capsule. The presence of these high-molecular-weight pheromones identified by proteomic analysis of the oviduct gland and egg capsule also implies the presence of high-molecular-weight polypeptides/proteins derived from SP precursors. These analytical results demonstrate that two modes

of cleavage of SP precursors coexist and generate low-molecular-weight peptides (prohormone convertase cleavages) and also 22–26 kDa polypeptides/proteins released by the eggs into the surrounding medium.

The mechanism that leads to the release of both low- and high-molecular-weight pheromones processed from a same protein precursor has to be elucidated. The occurrence of C-terminal amidation for peptides β2 and α3 demonstrates that two distinct processings are performed in the Golgi apparatus, which means that low-molecular-weight pheromones (LMWPs) are not degradation products of high-molecular-weight pheromones (HMWPs).

The present functional hypothesis could be that LMWPs induce mating and the release of oocytes into the mantle cavity, and that HMWPs, as described in *Aplysia*, facilitate the

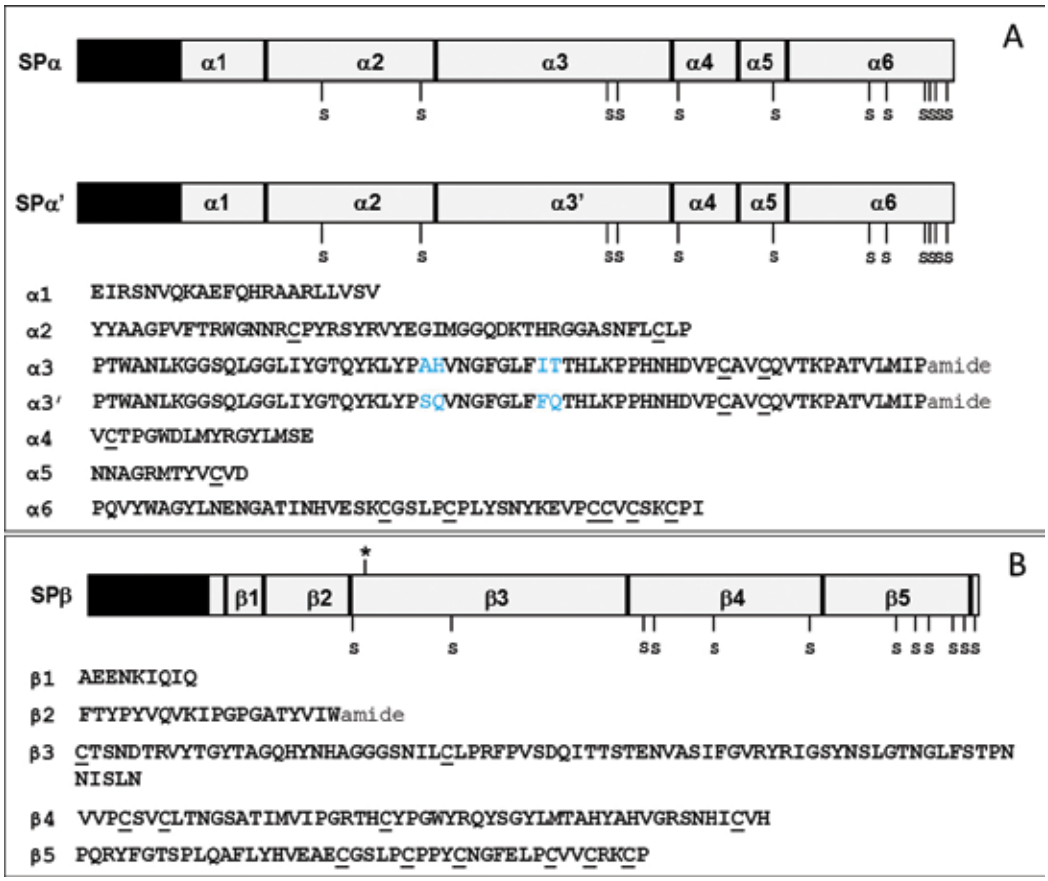

Figure 5. Schematic diagrams showing the organization of *Sepia officinalis* pheromone precursors (A) SP α and α′; (B) SPβ. Precursors encode a complex cocktail of peptides and polypeptides resulting from dibasic cleavages. Black box, signal peptide; vertical black line, potential dibasic residue cleavage site; asterisk, predicted N-linked glycosylation site; S, Cys residue [9].

aggregation of mature cuttlefish in the coastal egg-laying areas. All these data confirm that cuttlefish eggs are a source of pheromones, as described in other mollusks such as marine gastropods of the genus *Aplysia* [46–48]. Behavioral tests now have to be performed to clarify the mechanism of action of LMWPs and HMWPs in sexually mature cuttlefish.

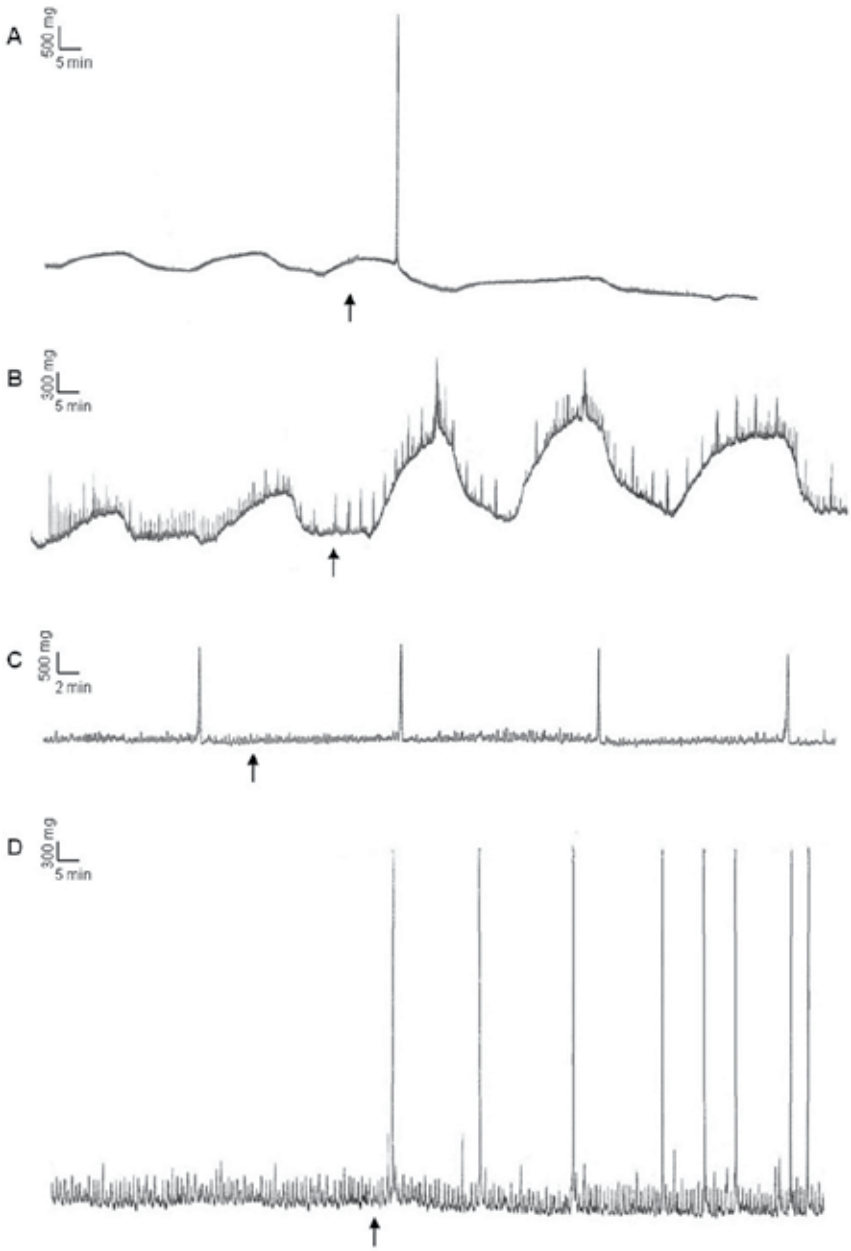

Figure 6. Bio-activity of synthetic β2 and α3 peptides. β2-induced contractions on (A) female gill and (B) penis from a threshold of 10^{-8} M. No activity on (C) rectum. α3-induced contractions on (D) penis from a threshold of 10^{-9} M [25].

6. Ovarian regulatory peptides

The role of the ovary in the regulation of the synthesis of capsular products secreted by MNGs was highlighted for the first time by Henry and Boucaud-Camou [49]. Ovary extract stimulated the incorporation of ^{3}HLeucine and ^{14}CGlucose into the proteins and polysaccharides of primocultures of glandular cells from main nidamental glands.

Seawater used for incubating oocytes also modified the contractile activity when applied on perfused oviduct (**Figure 7A**). The first ovarian regulatory peptide ever characterized was the tetrapeptide ILME [4], followed by SepOvotropin [5], SepCRPs (Sepia Capsule Releasing Peptides) [6, 8], and OJPs (Ovarian Jelly Peptides) [7]. All these peptides modulate the contraction of the distal oviduct, and some of them also regulate the contraction of the main nidamental glands (**Figures 7B**, **C**, and **8A–E**). They are expressed in vitellogenic follicles and smooth oocytes and secreted into the lumen of the oviduct during egg-laying to regulate the contractions that permit oocyte transport to the mantle cavity. They are suspected to be key-players in the synchronization of the accessory sex glands and oviduct. This regulation takes into account the number of oocytes stored in the genital coelom, which substantially fluctuates according to the successive spawning events.

A recent transcriptomic approach showed that SepOvotropin, SepCRPs, and OJPs are cleaved from a single large protein precursor of 1634 amino acids expressed in the ovarian follicle and smooth oocytes and as yet never described in the animal kingdom (**Figure 9A**).

The occurrence of a signal peptide reveals that the expression products released by this protein precursor are secreted. The spatial and temporal expression patterns of the transcripts show that it is probably a yolk protein (unpublished results: **Figure 9B**) involved in embryo development. This implies that yolk proteins could be submitted to successive processes, leading to the release of regulatory peptides. A comparison of the protein precursors with the primary sequences obtained from MS/MS analysis, and Edman degradation revealed some mistakes probably due to the tool used to determine molecular weights (ionic trap) and to analyze MS/MS spectra by a *de novo* strategy. In SepCRPs, there was a mistake about the

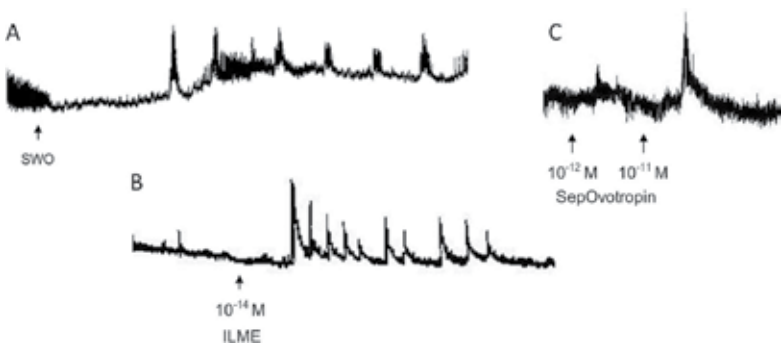

Figure 7. Perfusion of distal oviduct with (A) seawater used for incubating mature oocytes (SWO), (B) the synthetic peptide ILME and (C) synthetic SepOvotropin [4, 5].

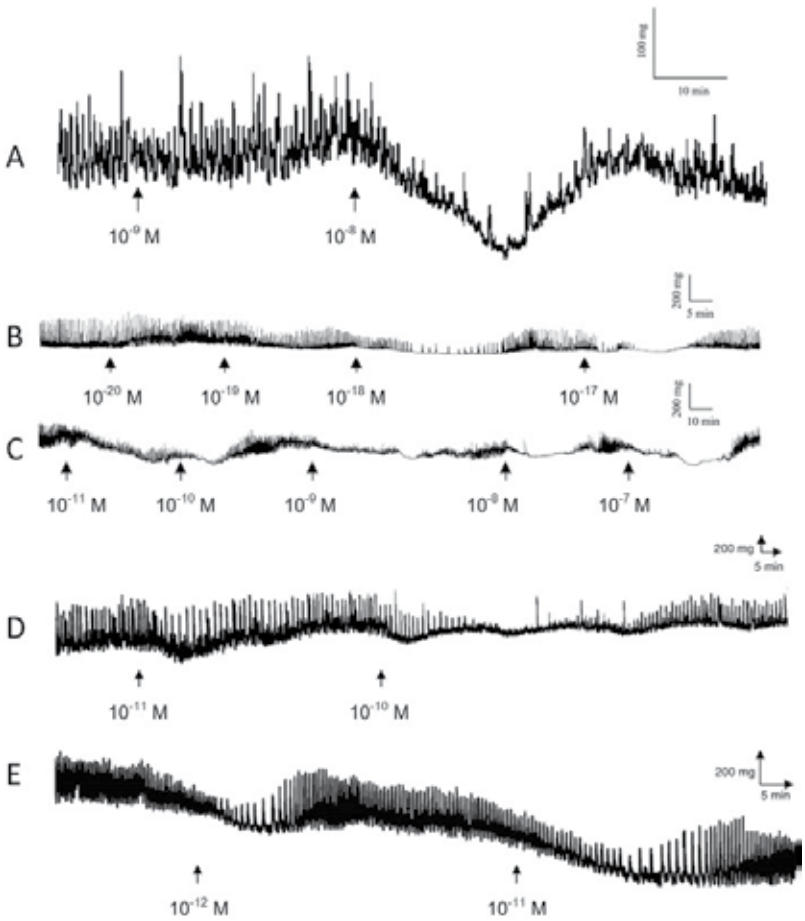

Figure 8. Effects of increasing concentrations of SepCRP on (A) the main nidamental gland and the whole female genital tract, (B) before the laying of a first batch of eggs, and (C) after the laying of a first batch of eggs. Effects of increasing concentrations of DQVKIVL on the whole female genital tract (D) and on the main nidamental gland (E) [6–8].

amino acid in position 5: aspartate (D) should be replaced by asparagine (N) (EISLNKDEVK instead of EISLNKD). In OJPs, the same amino acids should be swapped in the C-terminal moiety (EISLNKDEVK instead of EISLNKD), and in position 2, glutamate (E) should be replaced by glutamine (Q). In each case, the amino acids involved have very similar molecular masses that only diverge by 1 Da.

Finally, SepCRPs and OJPs are smaller families initially described in [6–8].

A similar mistake was made at the level of SepOvotropin because the sequence PKDSML LLQVPVYamide has the same molecular weight as PKDSMoxLLLQVPVMox. The primary sequence of SepOvotropin released by the protein precursor is PKDSMLLLQVPVM. The corrected sequences of SepOvotropin, SepCRPs and OJPs are summarized in **Table 2**. They reveal the occurrence of a conserved domain suggesting that they could bind the same receptor.

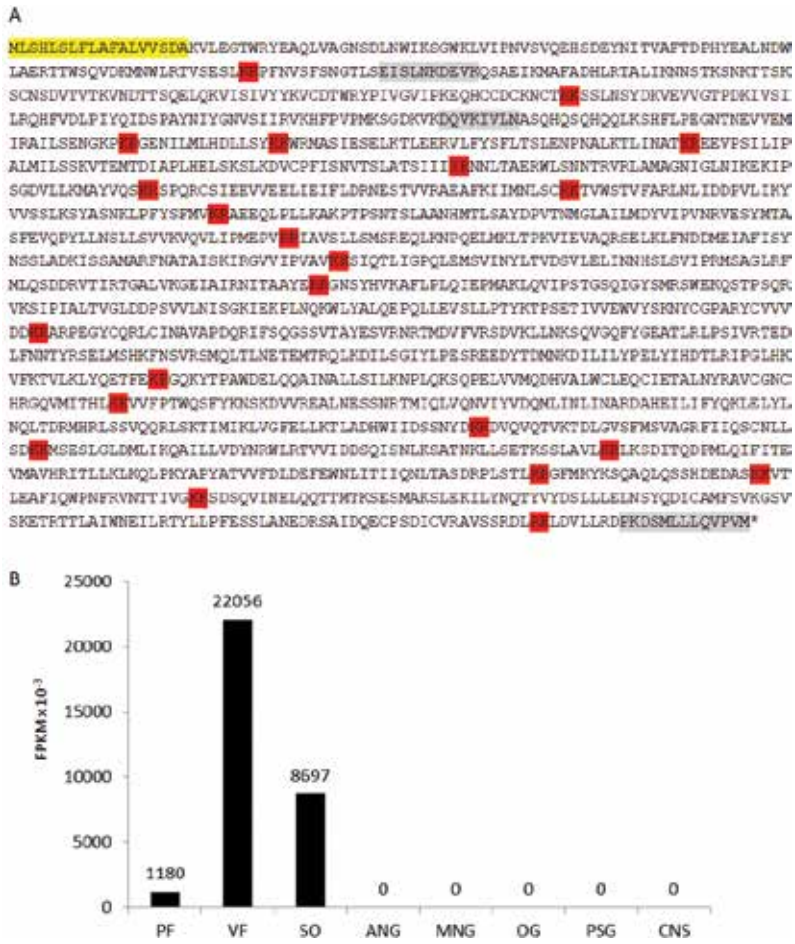

Figure 9. (A) Protein precursor of yolk-protein-releasing SepOvotropin, SepCRPs, and OJPs. The predicted signal peptide is highlighted in yellow and the convertase cleavage sites in red. Ovarian regulatory peptides are highlighted in gray, and the stop codon at the end of the coding sequence is indicated by an asterisk. (B) Expression pattern of the yolk protein. PF: previtellogenic follicles; VF: vitellogenic follicles; SO: smooth oocytes; ANG: accessory nidamental gland; MNG: main nidamental gland; OG: oviduct gland; PSG: posterior salivary gland; CNS: central nervous system, FPKM: fragments per kilobase of exon per million fragments mapped x 10^{-3}.

As for tetrapeptide ILME, it could be cleaved from the protein precursor of a retinol-binding protein (**Figure 10**) expressed in the ovarian follicles and the oocytes. *In silico*, data mining showed that it was the only protein precursor expressed in the ovary and containing the sequence ILME. The specificity of ovarian regulatory peptides lies in the fact that they come from the secondary cleavage of functional proteins. As they are cleaved at atypical cleavage sites, this makes it difficult to predict their primary sequence on the basis of protein precursor structure.

Similar regulatory peptides have been described in insects, such as TMOFs for "Trypsin-Modulating-Oostatic Factors." Bioactive peptides cleaved from vitellin membrane proteins [50] control egg development [51] and inhibit ecdysone biosynthesis [52].

Peptides	Primary sequences
SepOvotropin	PKDSMLLLQVPVM
SepCRPs	SLNKD
	ISLNKD
	EISLNKD
	EISLNKDEVK
	ESLNKDEV
	ISLNKDEV
	EISLNKDEV
OJPs	DQVKIVL
	DQVKIVLN

Table 2. Primary sequences of ovarian regulatory peptides.

MKWVSNFAVFCLVFSLAVSFSKFGTQATNSKRATTTIDPPSEKKCRVNNFVVQKNFNASLYQGHWFVI
SWNKHSMAVEHPFLSKFVSIRNAEAYYTLRRDGNFRFLTGGMISRMFCQQDEIVAYVMNRTAPQKLTV
QISPKDRYPQWVMQTDYTGYAVIYSCLKVASNGMCEPGNAVVQSMNRKPTGHTPTQQAKVESVARRLC
VDPSELKIVGYDGRCPELDPKNFPQRKILMEGVCFIFFLIVSIIGIIYFTCCQSPAKEKKKEHAK*

Figure 10. Protein precursor of a retinol-binding protein able to release the tetra-peptide ILME. The predicted signal peptide is highlighted in yellow and the convertase cleavage sites in red. ILME is highlighted in gray, and the stop codon at the end of the coding sequence is indicated by an asterisk.

Egg-laying regulation in cuttlefish is a complex mechanism that involves peptide and protein regulatory factors of different nature produced by the central nervous system, the ovary, and the ASGs.

The neuropeptides trigger egg-laying by integrating environmental stimuli across a neurosensory network. The ovarian regulatory peptides synchronize oocyte transport and egg capsule secretion, and their concentration is correlated to the number of smooth oocytes stored in the genital coelom. As they are short and unprotected peptides, they have a short life time after secretion, hence a very dynamic regulation.

The waterborne sex pheromones cleaved from three protein precursors overexpressed in the oviduct gland stimulate and facilitate mating and reproduction behaviors by aggregating mates in egg-laying areas. Short pheromones participate to the release of oocytes in the mantle cavity, and large pheromones are suspected to modulate reproduction behaviors by aggregating mates in egg-laying areas.

These multiple regulatory layers can be correlated with the complexity of the successive steps of the egg-laying mechanism that involves the ovary and ASGs and is performed thanks to a

stereotyped behavior: (1) ovulation, with the release of mature oocytes in the genital coelom, (2) oocyte transport by the oviduct, (3) secretion of the inner egg capsule by the OG, (4) secretion of the outer egg capsule by the MNGs, (5) black pigmentation of the egg capsule by the ink bag, (6) fertilization of oocytes by the sperm stored in the female's copulatory pouch, and (7) attachment of eggs to the sea bottom to form an egg mass.

7. The egg case: structure and function during embryo development

After the spawning period, the genitors die and leave their eggs in the marine environment without any parental protection. Thus, the sustainability of the species depends on the reproduction success and more precisely on the ability of the eggs to complete their development.

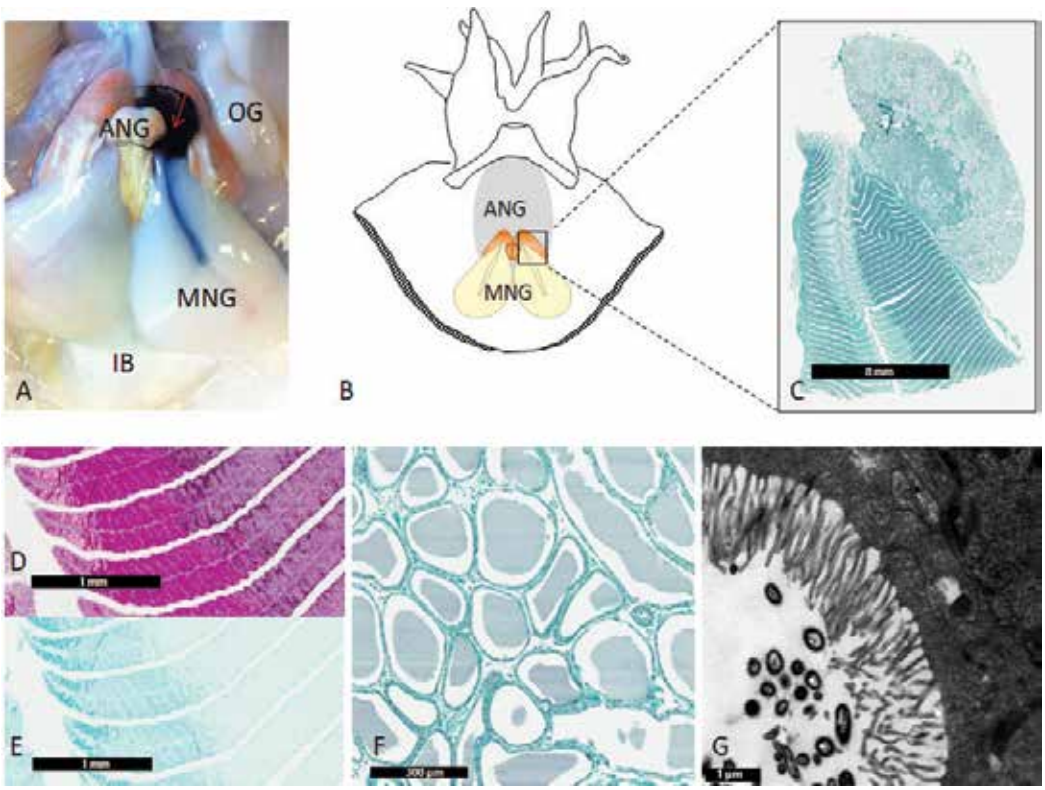

Figure 11. (A) Photograph of female reproductive glands during secretion of the egg case (red arrow). ANG, accessory nidamental gland; IB, ink bag; MNG, main nidamental gland; OG, oviduct gland. (B) Schematic representation of a mature female cuttlefish in ventral view showing the localization of the MNG and ANG. (C) Longitudinal section of the MNG and ANG stained in Prenant-Gabe triple staining. Longitudinal section of the MNG lamellae stained in alcian blue and periodic acid of Schiff highlighting the secretion of acid mucopolysaccharides (D), and neutral mucopolysaccharides and glycoproteins (E). (F) Longitudinal section of the ANG stained in Prenant-Gabe triple staining showing that tubules are composed of a single layer of ciliated epithelium and filled with bacteria in the lumen. (G) Thin section of the lumenal surface of accessory nidamental gland tubules showing a single layer of ciliated epithelium with microvilli and a few lumenal bacteria in TEM (x 12,000). (Photo credits: V. Cornet. D. Goux).

Cuttlefish eggs are large oocytes containing all the nutrient reserves required for embryo development. To withstand physical and microbial threats, mature oocytes are enclosed within a protective egg case produced by secretions of the female genital apparatus [53, 54]. This egg case is composed of two distinct envelopes. The inner layer is in direct contact with the chorion surrounding the oocyte; it is formed by secretions added, while the egg passes through the oviduct gland [53]. The oviduct gland secretes proteins and polypeptides. The main proteins secreted by this gland correspond to sex pheromones. Afterward, the oocyte is released inside the mantle cavity and embedded with an outer layer secreted by the two nidamental glands and stained with ink (**Figure 11A**).

8. Nidamental glands: a specificity in decabrachia cephalopods

The genital apparatus of *Sepia officinalis* contains two pairs of accessory reproductive glands partly involved in egg case formation (**Figure 11**). The main nidamental glands (MNGs) are related to the accessory nidamental glands (ANGs). The two paired glands are located on the ventral side of the visceral mass. The histological structure of these glands in cuttlefish is similar to the structure of squid (*Loligo forbesi*) nidamental glands [55].

The main nidamental gland and the oviduct gland both present a lamellar structure (**Figure 11C**). Each lamella consists of a central lamina of connective tissue covered with a glandular epithelium at the origin of the polysaccharides labeled by periodic acid-Schiff (PAS)-positive deposits (**Figure 11D**). The cells located at the free end of the lamellae produce particularly acid mucopolysaccharides and glycoprotein secretions revealed by alcian blue (**Figure 11E**), while the other cells secrete neutral mucopolysaccharides. During egg case formation, the secretions are released into the lumen and are led out through a duct opening onto the mantle cavity at the anterior end of the gland (**Figure 11A**). MNG and ANG structures substantially differ.

The ANG is divided into four lobes attached to the anterior end of the MNG by conjunctive tissue. Histological observations of ANG reveal a tubular gland harboring symbiotic bacteria. These symbionts are enclosed in the lumen of tubular structures that nearly completely fill the gland (**Figure 11F**). The wall of each tubule appears to be composed of a single layer of ciliated epithelium with microvilli (**Figure 11G**). The role of this gland in reproduction is unclear. Some clues suggest its involvement in egg case formation at the spawning period. During sexual maturation, the ANG indeed increases in size and changes in color from white to bright orange at the time of spawning (**Figure 11A**). It also harbors a dense consortium of bacteria that secrete carotenoids at the origin of the intense orange color of ANGs in mature females [56].

Using 16S RNA gene sequencing, many bacterial taxa were identified in ANGs, including *Agrobacterium, Roseobacter, Sporichthya, Rhodobium, Xanthobacter*, and *Clostridium* [57]. The origin of the bacterial symbionts in cuttlefish remains undetermined. Although the presence of bacteria in the egg capsule suggests vertical transmission, we cannot exclude horizontal transmission as in *Loligo opalescens* [58]. In squid, ANGs develop only a few months after hatching from a single layer of cells containing many cilia and microvilli [58].

The conserved innate immune Toll/NF-κB pathway was described for the first time in *Sepia officinalis* ANG [59]. The transcriptomic analysis of ANG led to the identification of different constitutive elements of the Toll/NF-κB pathway. Five related Toll receptors (TLRs) have been characterized. Among them, TLRα shares 89% sequence identity with the unique TLR found in the light organ of *E. scolopes*. In addition, eight phosphorylation cascade elements have been demonstrated such as IRAK, TRAF6, and Rel/NF-κB. These immune pathway proteins (α2-macroglobulin-like protein, CD-63 antigen, transferrin) are probably involved in the establishment and maintenance of the bacterial symbionts like those in the light organ of *E. scolopes* [60]. Although several studies have been carried out about the subject, the real function of this ANG and its symbionts remains unknown. Several studies in squid suggest protection of the egg via the secretion of antimicrobial or antifouling compounds by ANG or its symbionts [61, 62], but no molecule has been characterized yet.

The function of the main nidamental gland (MNG) in egg case formation is clearer (**Figure 11A**). This white gland secretes the mucopolysaccharides and glycoproteins that form the egg case.

A recent unpublished analysis of the MNG proteome reveals the occurrence of proteins involved in glycolysis/gluconeogenesis (6-phosphofructo-2-kinase, type-2 Hexokinase, Pyruvate kinase, Glyceraldehyde 3-phosphate dehydrogenase, Fructose-1,6-bisphosphatase, fructose-bisphosphate aldolase) and in glycogenolysis/glycogenesis (Glycogen phosphorylase, Glycogen synthase). These results indicate a large amount of energy production and consumption by the MNG due to an intense production and secretion of egg case components. Some of the identified proteins are also involved in the metabolism of polysaccharides or glycoproteins, like glycosyltransferases, which catalyze the transfer of oligosaccharide moieties from activated nucleotide sugars to nucleophilic glycosyl acceptor molecules or GDP-mannose pyrophosphorylase, involved in the production of N-linked oligosaccharides. Finally, the MNG secretes the main capsular components, the Egg Case Proteins, involved in the formation of a narrow mesh that provides elasticity and resistance properties to the egg case [63].

9. The oral cavity: completion of the eggs

At the time of fertilization in the oral cavity of the female cuttlefish, the oocytes are already wrapped in the thick and complex egg case. The female's arms form a chamber to keep the freshly embedded oocytes near the oral copulatory pouch where spermatophores have been deposited by the male during mating (**Figure 1**). Fertilization of the oocytes by spermatozoa is facilitated by a diffusible chemoattractant factor: SepSAP (Sepia Attracting Sperm Peptide). This hexapeptide is expressed in the vitellogenic follicles and released by embedded oocytes through the various capsular envelopes to facilitate fertilization by increasing chances of gamete collision. SepSAP has an attractant effect on sperm from low concentrations around 10^{-17} M [64].

During fertilization, the eggs are also in contact with saliva. As early as 1934, Jecklin suggested that salivary secretions could protect the eggs during spawning [54]. A recent study of the transcriptome and proteome of *Sepia officinalis* posterior salivary gland seems to confirm this

hypothesis. In addition to enzymes and toxins such as cephalotoxins and CRISPs (Cysteine Rich Secreted Proteins), cuttlefish saliva contains many immune effectors like α-macroglobulin, lysozyme, Bactericidal/Permeability-Increasing proteins (BPIs), and Lipopolysaccharide-Binding Proteins (LBPs) [65]. These salivary proteins very likely play a role in gamete protection or/and in improving fertilization.

After 2 or 3 minutes in the oral cavity, the eggs are deposited by the female's arms on a natural structure like marine eelgrass (*Zostera marina*) or an artificial one like a rope.

10. The cuttlefish egg case

During its development, the embryo is only secured by its egg case. The morphological evolution of the egg and its capsule from laying to hatching occurs in three phases during which the capsule undergoes major changes (**Figure 12**). The different steps described below correspond to embryonic development [66] first defined the different embryonic stages by performing a morphological study of the cuttlefish embryo during its development. The telolecithal egg presents a meroblastic discoidal cleavage (stages 1–9) associating blastomeres in central position and blastocones on its fringe. During epibolic gastrulation (stages 10–15), blastocones disappear under the ectoderm plate following the peripheral ring of blastula cells that will form the ring-shaped endo-mesoderm. At the end of gastrulation, the vitelline syncytium and extra-embryonic ectoderm completely surround the yolk and internalize the vegetal pole to form the yolk sac. The cleavage period corresponds to the first phase (P1) of egg evolution. A few hours after laying, the egg cell is covered with a lamina propria and surrounded by a thick gelatinous capsule (1.4 mm, ±0.6 mm). In contact with seawater, the gelatinous and fluid capsule polymerize. This reduces the volume of the egg by about 30% (**Figure 12**) and its thickness by 50%.

After 15 days of incubation and following polymerization (**Figure 13A**), capsule thickness is down to 614 microns (±150 microns) (**Figure 13B**), and the outer and inner layers can be distinguished. Polymerization of the capsule proteins helps tighten the layers of coiled outer and inner envelopes, highlighting an increasing melanin gradient from the inner layers to the outer layers. The egg is then tightly wrapped by a hardened, strong yet elastic capsule. These morphological characteristics of the capsule define the second phase of egg evolution (P2), which lasts from the 7th day to the end of the first month and corresponds to gastrulation and the beginning of organogenesis. The embryo develops within the limits of a disk located at the animal pole, at the surface, or above the yolk mass (**Figure 12**), while the capsule size and thickness remain unchanged. The initiation of organogenesis marks the beginning of the last phase (P3) that ends with hatching. The embryo in early organogenesis does not yet fill the perivitelline space. However, the capsule has become permeable to let in water and solutes. Thus, the accumulation of fluids in the perivitelline space causes the capsule to stretch, and its thickness continues to decrease (437.9 (±104) μm). Organogenesis corresponds to 2/3 of the development period, and it follows after the closure of the yolk sac and ends with hatching and can be divided into three phases (**Figure 12**). (1) During discoid or early organogenesis

(stages 15–20), the embryo forms a disk at the animal pole. The different embryonic territories build up above the yolk mass. (2) The second phase corresponds to an extension phase (stages 20–23). The brachial crown tightens on the yolk mass; the embryo straightens into the anteroposterior axis. Its rear end corresponding to the mantle gradually moves apart, leaving the brachial crown, mouth, and eyes toward the yolk. (3) The final growth phase (stage 23 to hatching) begins once the organs are found in their final topology.

After 72 days of incubation, a few days before hatching, the embryo completes its growth and has assimilated much of the yolk reserves. It now fills most of the available space in the egg and is surrounded by a large amount of perivitelline fluid (about 1 ml), stretching the capsule to its maximum (**Figure 13D**).

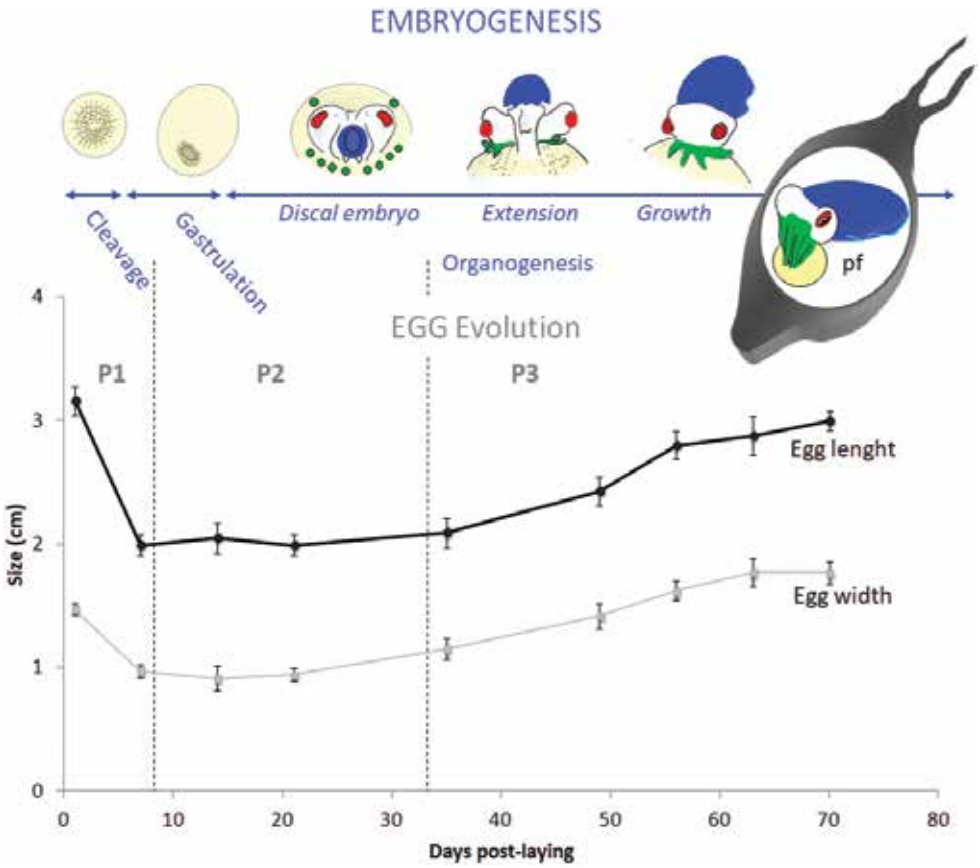

Figure 12. Evolution of *Sepia officinalis* egg size during embryogenesis at 16°C. Evolution phases of the egg case: P1, polymerization of the egg case; P2, stabilization of the egg case; P3, thinning and delamination of the egg case. Illustration of different stages of embryogenesis during cleavage, gastrulation and organogenesis. Yellow: vitellus, red: future eyes, blue: future mantle and shell, green: future arms; pf, perivitelline fluid.

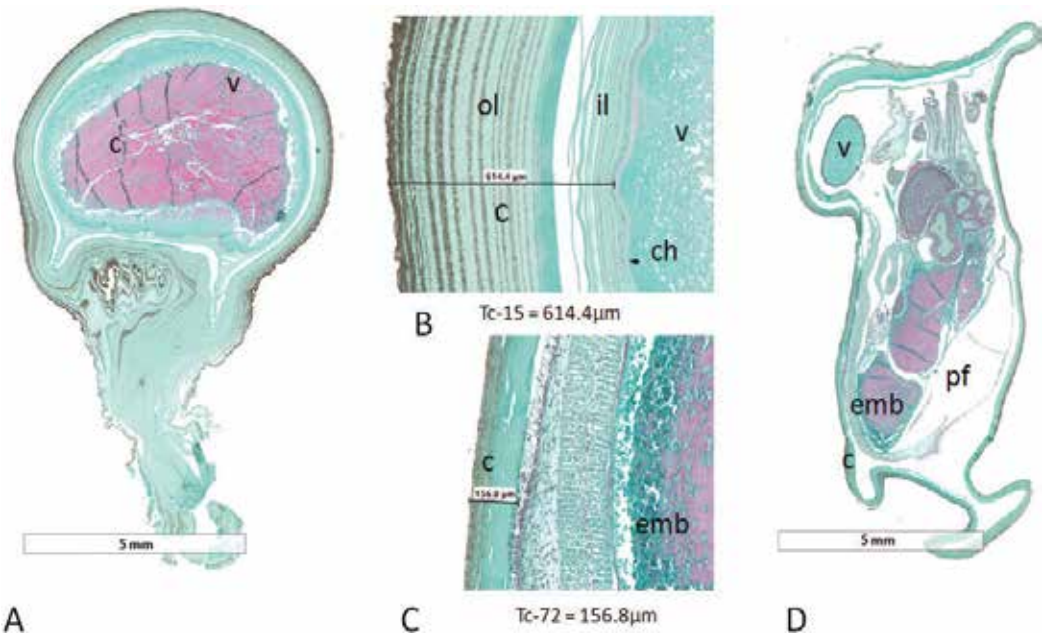

Figure 13. Longitudinal sections of the egg after 15 days (A) and 72 days (D). ANG stained in Prenant-Gabe triple staining. Magnification of the egg case including capsule thickness after 15 days (B) and 72 days (C). C, capsule or egg case; ch, chorion; emb, embryo; il, inner layer; pf, perivitelline fluid; ol, outer layer; Ct, capsule thickness; v, vitellus. (photo credits: V. Cornet).

At this stage, the embryo's features are similar to the adult's; the embryo enters a linear growth phase. All essential elements of the brachial device, the nervous system, the palleal, and visceral parts are now in place Organogenesis ends with the transfer of the outer yolk sac to the inner yolk sac, enabling faster assimilation of energy resources.

The outer and inner capsule envelopes have now completely merged, and the outermost layers of the capsule including melanin appear to be delaminated (**Figure 13C**). Thus, at the time of hatching, the capsule has undergone significant changes: it has become extremely thin (156.8 (±110) microns) and friable, so that it will break easily and release the juvenile.

At the time of hatching (Stage 30), 75–80 days after egg-laying and at 16°C, the release of enzymes by the Hoyle organ located on the end of the dorsal mantle facilitate the emergence of the juvenile [67]. Hatching is also facilitated by the thinning of the capsule.

11. Egg case composition

The capsule of *Sepia officinalis* eggs has a specific black color (**Figure 14A**). Only females belonging to the Sepiidae family include melanin granules into the egg capsule. Melanin is secreted by the ink bag and is integrated into egg case via secretions from the main nidamental gland (**Figure 11A**). Other compounds of the ink such as proteins may well also integrate the capsule.

Structural analysis of the egg capsule by photonic microscopy reveals a lamellar structure of the inner and outer envelopes (**Figure 13B**), with successive spirally wound layers. The outer envelope contains melanin deposits gathered in layers that become increasingly intense. Observations of the outer envelope by Transmission Electron Microscopy showed the presence of melanin deposits and revealed the occurrence of isolated or grouped structures whose size ranged between 0.4 and 1 μm, corresponding to bacterial structures (**Figure 13B** and **D**). These bacteria probably come from the accessory nidamental gland. The egg case ultrastructure shows a narrow mesh composed of glycoproteins and polysaccharides.

SepECP 1 and SepECP2 are cationic, cysteine-rich protein of 71 and 74 kDa, respectively (**Figure 15**). These two proteins were characterized as the main constituents of the cuttlefish egg case [16]. SepECPs are only secreted by females, mainly by the MNG and also by the oviduct gland. These two proteins are highly cationic, with 73 positively charged residues for ECP1 and 43 for ECP2. They exhibit bacteriostatic activity against a few pathogenic GRAM- bacteria from the Vibrio genus. Their bacteriostatic activity could explain the occurrence of

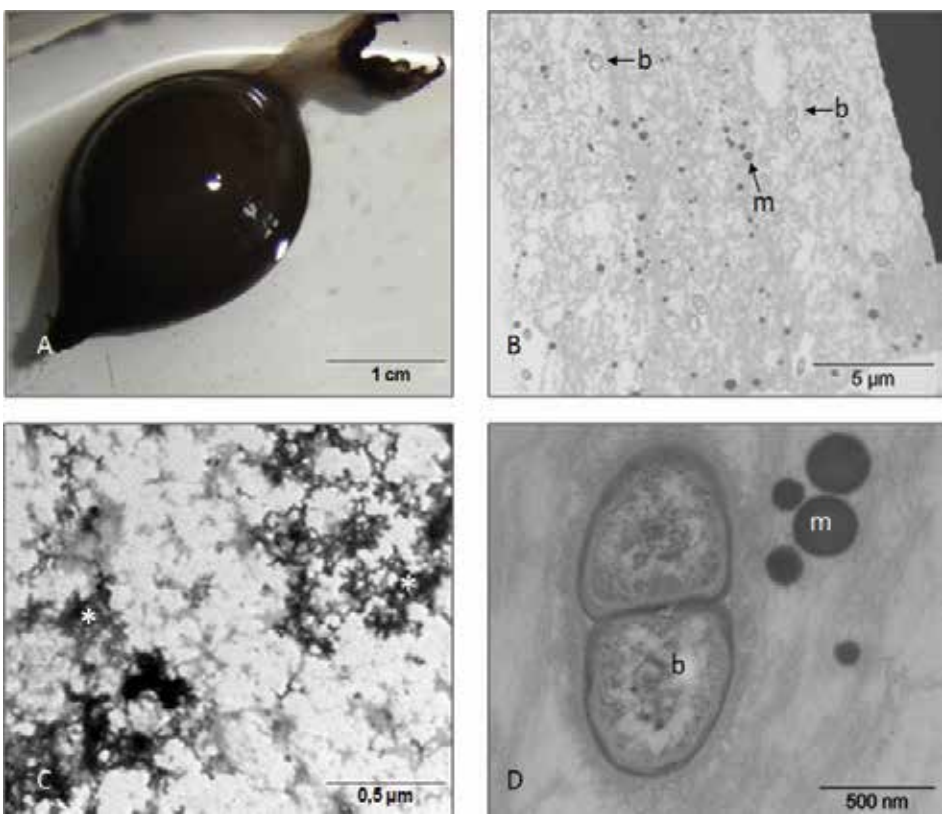

Figure 14. Photographs of the *Sepia officinalis* egg case and its components. (A) Freshly laid egg. (B and C) thin sections of the outer layer of the egg case in TEM. (D) Dividing bacteria and melanin granules. (C) Observation in TEM of SepECPs extracted from the egg case. White asterisks correspond to the protein network; b, bacteria; m, melanin. (Photo credits: C. Zatylny-Gaudin, V. Cornet, D. Goux).

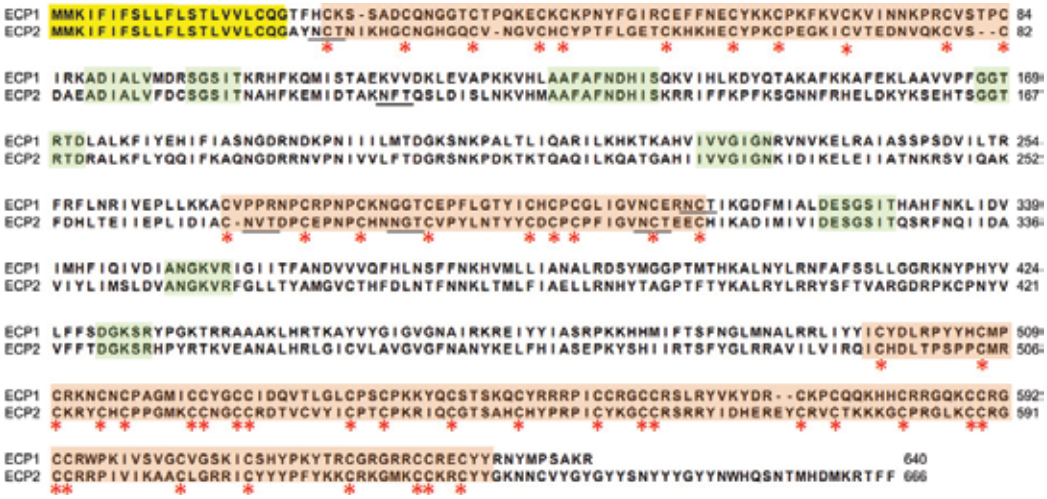

Figure 15. Amino acid alignments of the SepECP precursors. Yellow: signal peptide; orange: conserved cysteine domains; green: conserved motifs up to five amino acids. Red asterisks indicate conserved cysteines, and underlined sequences correspond to potential glycosylation sites.

bacteria in the egg case, corresponding to potential bacterial symbionts. The two SepECPs display 48 conserved cysteines grouped in three cysteine domains (**Figure 15**). These cysteines could be implied in intramolecular and intermolecular disulfide bonds involved in the formation of heterodimers. SepECPS are indeed involved in the formation of a network (**Figure 14C**) or dense matrix protecting the embryo against mechanical shocks and microbial infection during its development. No infection or biofilm is observed on cuttlefish eggs under controlled conditions or in natural environments. The capsule seems very effective: both antifouling and antibacterial coatings prevent pathogenic bacteria from proliferating [63].

During embryo development, the egg case becomes increasingly thin, but it retains elasticity to allow for embryonic growth. SepECPs are probably cleaved during the last phase (P3) to allow for hatching. During this phase, when the capsule seems more fragile, the embryo keeps developing without being affected by pathogens. When they are degraded, highly cationic SepECPs probably generate antibacterial cationic peptides. Last of all, a role of the perivitelline fluid in embryo protection should not be ruled out.

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Chapter 2

Pond Snail Reproduction as Model in the Environmental Risk Assessment: Reality and Doubts

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Éva Molnár and Tibor Kiss

Additional information is available at the end of the chapter

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Abstract

In European limnetic systems, the most relevant endocrine-disrupting chemicals (EDCs) of steroid type are the natural and synthetic hormones, phytosterols, pesticides, biocides and other chemicals produced by the plastic industry. Their presence in aquatic ecosystems represents a potentially adverse environmental and public health impact. Furthermore, this is a warning signal that the current handling of pharmaceuticals needs to be further improved. Nowadays, it has become clear that EDCs have specific disturbing effects on the neuroendocrine system of invertebrate and vertebrate aquatic animals, particularly gastropods. Among a latter, pond snail (*Lymnaea stagnalis*) has been used as the first aquatic non-arthropod test organism in studying the effect of EDCs because they are sensitive to various anthropogenic steroids, like progestogens. Investigating a variety of reproductive endpoints of *Lymnaea*, such as fecundity, oocyte production, egg mass production, the quality of egg masses, the shell size in development and after egg-laying, the time window of cell division in the offspring, the metabolite content of single-cell zygotes and egg albumen has concluded that progestogen contaminations in water are detrimental for reproduction and early stage development of *Lymnaea*. This chapter is an attempt to show whether *Lymnaea reproduction*, despite many altering reproductive endpoints, is a suitable model for environmental risk assessment or not.

Keywords: endocrine-disrupting chemicals, progestogens, molluscs, *Lymnaea stagnalis*, reproduction model

1. Introduction

In the last few years, it has become clear that a wide variety of environmental contaminants have specific effects on neuroendocrine system of aquatic species. The frequent detection of

endocrine-disrupting chemicals (EDCs) in the aquatic environment and a high consumption of contraceptives all over the world reflect a rapidly growing concern on their environmental impact. EDCs interfere with the body's endocrine system mimicking or partly mimicking naturally occurring hormones in the body and induce adverse developmental, reproductive, neurological (cognitive and behavior) and immune effects in both humans and wildlife [1]. In addition, the high frequencies of detection of these contaminants in aquatic environments and the incomplete removal of them during passage through sewage treatment plants may pose the greatest risk during prenatal and early postnatal development when organ and neural systems are developing. The increasing and continuous occurrence of steroidal estrogen and progestogen compounds in the environment can lead to toxicological effects on non-target organisms, therefore, it is important on the whole to assess the environmental risk posed by these contaminants.

Molluscs like gastropods and bivalves have been used as non-target model organisms in studying environmental contamination for a long. They proved to be effective model animals because they are ubiquitous, have highly conserved control and regulatory biochemical pathways that are often homologous to vertebrate systems and they are extremely sensitive to anthropogenic inputs [2–4]. For example, the bivalves, by virtue their ability to accumulate toxic substances (due to their sessile and filtering life style) in their body are considered as excellent indicators of ecosystem health [5]. Furthermore, molluscs are ecologically crucial organisms, which are essential to the biosphere and to the human economy. They are the second most diverse animal group (10 taxonomic classes) encompassing more than 400,000 species, they are ecologically and commercially important as food and non-food resources. Among them terrestrial gastropods are destructive agricultural pests causing economic damage to a wide variety of plants including horticulture, field crops and forestry. In addition they are of importance in medical and veterinary practice, since they serve as intermediate hosts for several human and animal diseases, such as schistosomiasis and helminth diseases [6]. Both terrestrial (e.g. *Helix pomatia*), marine (e.g. *Aplysia californica*) and freshwater (e.g. *Lymnaea stagnalis*) snails have proved to be excellent models, due to their "simple" nervous system, in neurophysiology and behavioral ecology [7–12]. Gastropod model organisms play an important role for immunology [13], reproductive and developmental biology (which is facilitated by several genome and transcriptome projects that are currently underway) [14–16], neurobiology, especially on learning and memory formation [17–22]. Some species, in particular simple pond snail (*Lymnaea stagnalis*) have been widely applied in pollution biomonitoring programs, and widely used in a variety of ecotoxicological studies [23–28]. Based on earlier investigations the reproduction test of *L. stagnalis* was officially approved by the national coordinators of the Organization for Economic Cooperation and Development (OECD) member countries as test guidelines. *L. stagnalis* and the New Zealand mudsnail (*Potamopyrgus antipodarum*) have been the first aquatic non-arthropod-tests, which were successfully validated within the Conceptual Framework for Endocrine Disrupters [3, 29, 30]. Therefore, in this chapter one of the most relevant mollusc of European limnetic systems, the hermaphroditic *L. stagnalis* is particularly presented to model the various physiological effects on its reproductive and developmental parameters eliciting by acute or chronic exposures of endocrine-disrupting substances. A variety of endpoints are assessed and collected, including fecundity, oocyte production, egg mass

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production, the quality of egg masses, the shell size in successive development and following egg-laying, the time window of cell division in the offspring, the metabolite content of single-cell zygotes and egg albumen before and after the treatment of parents.

It has been shown that recent research aims to combine molecular level investigation with cellular, organismal, behavior and environmental research. In this chapter, an attempt is made to summarize data particularly obtained on *L. stagnalis* so far, and to discuss the molecular mechanisms, the functional and ecological consequence of EDCs and the advantages of snail preparations as tools for ecotoxicological research. Comparison of the data obtained on molluscs with those obtained on the lower vertebrates, will definitively contribute to the better understanding of the impact caused by EDCs, like steroid hormones, present in our environment.

2. Steroid type EDCs in the aquatic environment

The release of human pharmaceuticals (as xenobiotics) into aquatic ecosystems is a serious environmental risk which results in an acute and chronic contamination of non-target invertebrate (e.g. molluscs) and vertebrate (e.g. fish) freshwater organisms [31]. Among the most critical environment contaminants are EDCs, which are defined as an exogenous substance that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism. It is concluded that endocrine disruption is not considered a toxicological end point per se but a functional change that may lead to adverse effects in both non-target and target organisms, as well. EDCs act as agonist or antagonists at multiple sites via complex mechanisms of action including: receptor-mediated mechanisms, synthesis and/or metabolism of hormones, neuropeptides and neurotransmitters, as well as transport pathways [32].

In European limnetic system, the most relevant steroid type EDCs are follows: natural (e.g. progesterone, estradiol, testosterone [33–35] and synthetic (e.g. drospirenone, levonorgestrel, ethinylestradiol, cyproterone acetate (CPA), t-methyltestosterone [23, 33–35]) hormones, phytosterols (e.g. β-sitosterol [23]), pesticides (e.g. octylphenol, chlordecone [35, 36]), fungicides (e.g. vinclozolin (VZ), pyraclostrobin [25, 28]), biocides (e.g. tributyltin [23, 36]) and other chemicals produced in the plastic industry (e.g. bisphenol A [36]). One of the most cited examples to steroidal EDCs is the tributyltin (TBT) in molluscs. It caused imposex and intersex development as two masculinization phenomena in more than 260 species of gastropod worldwide, and severe losses of invertebrate biodiversity in waters [5, 37]. Several studies on perturbations of mollusc reproduction following exposure to low concentrations (ng/L range) of steroid type EDCs have already been reported. These more recent studies collectively provide evidence for possible detrimental effects of steroidal EDCs on *L. stagnalis* reproduction and embryonic development. However, the underlying mechanisms between exposure to EDCs and a variety of biologic outcomes, their potential long-term side effects of these molecules on molluscs remain largely unknown. This book chapter is mainly focused on synthetic steroids because they have become one of the most harmful pharmaceutical pollutants in molluscs.

Synthetic steroids, like estrogens and progestogens, are potent endocrine disrupters, which can modify diverse physiological, hormonal and behavioral processes in freshwater species,

and subsequently affect their capacity to reproduce, develop and grow [38, 39]. Estrogens and progestogens in combination are widely used as synthetic oral contraceptives (SOCs) [40]. SOC residues or their metabolites are eliminated from the human body unchanged or in the form of active metabolites in a remarkable amount [41, 42]. These biologically active agents enter into the waste water treatment plants (WWTP) where the generally applied treatment process is not suitable to eliminate them perfectly [42–45]. Consequently, synthetic steroid hormone residues enter the aquatic environment (e.g. surface waters) manly through cleaned effluents. The first review, which describes the presence of estrogen and progestogen hormones in natural surface waters was published by Richardson and Bowron [46]. In fact, very few pharmaceutical chemicals were identified due to the limitations of the early gas chromatography and HPLC techniques. The development of analytical techniques (e.g. liquid chromatographic-mass spectrometric method with solid-phase extraction, see later) decreased the limit of detection, resulting in an increasing number of detectable SOCs in surface and ground water, as well [47, 48]. Nowadays, their reported presence are in a concentration range from a few ng/L to often tens or hundreds of ng/L (estrogens: 0.20–480.00 ng/L, progestogens: 0.07–22.20 ng/L) in surface waters [47, 49–51]. The catchment area of the largest shallow lake of Central Europe is a habitat of several molluscs (e.g. *L. stagnalis, Anodonta cygnaea, Dreissena polymorpha*) and fish species (e.g. *Rutilus rutilus, Alburnus alburnus, Abramis brama, Carassius carassius, Cyprinus carpio, Perca fluviatilis*), where the estrogen and progestogen concentrations were found between 0.07–0.68 ng/L and 0.23–13.67 ng/L, respectively [33, 34]. The presence of steroid hormones has also been found in the drinking water, which is a warning sign that the current handling of pharmaceuticals may lead to future global human health problems [52–56]. It has already been described that exogenous steroid contaminations have wide range genotoxicity, neurotoxicity and germ cell-damaging effects in humans. For example, ethinylestradiol may modify brain structure, function, and consequently, behaviors pattern during the female brain development [57]. Furthermore, accumulating evidence suggest that human exposure to steroids is related to the impairment of male reproductive function (e.g. decreased sperm number) and can interrupt other hormonally regulated metabolic processes, particularly if exposure occurs during early development [58].

2.1. Methods in detection of steroidal EDCs

Measurements of multi-residue analysis require a rapid, sensitive, robust and reliable method with fast response time (high-throughput). These analytical measurements are essentially determined by two crucial things, one is the limit of detection, and the other is the sample (matrix) complexity. The subject of detection limits in analytical chemistry has improved since the 1970s and these resulted that the amount of detectable analytes, such as EDCs, are decreased [59–62]. Nowadays, the mass spectrometry based methods are extended and their detection limits are almost low ppm or ppb, which are below the environmentally relevant concentrations at the time. Other problem with the detection and quantification of an analyte can result from different matrix effects, sample concentration or other conditions, such as instrument sensitivity and reagent purity. In general, these matrices have different type of waters (e.g. wastewater influents or effluents, ground-, surface- and tap waters) and various solid samples (sediment, sludge, biological matrices). Sample preparation techniques can enhance the performance results for better recovery, increased sensitivity and lower detection limits [63].

Multi-residue analysis, as a field of study encompassing steroid EDCs residue analysis, has made considerable advances regarding selectivity and detection limits. Before analytical procedures, in order to keep track levels of EDCs, it is recommended that (e.g. deuterated) internal standards are added to the water or solid samples [64]. In general, there are several extraction methods, such as liquid-liquid extraction (LLE), solid-phase extraction (SPE), solid-phase micro-extraction (SPME), stir-bar sorptive extraction (SBSE), selective pressurized liquid extraction (SPLE), Soxhlet extraction (SE), ultrasonic extraction (USE), microwave-assisted extraction (MAE) and accelerated solvent extraction (ASE) [65–68]. The majority of current analytical methods for separation and detection of various steroidal EDCs, for example, use liquid chromatography-tandem mass spectrometry (LC-MS/MS) because its versatility, specificity and selectivity are very well [69]. Other possibility to detection and quantitative measurement of steroidal EDCs is also offered by gas chromatography (GC) with electron capture detection and confirmation by MS [70].

In case of water samples, the main steps of analytical methods are the filtration (e.g. glass microfiber filters), extraction and purification (e.g. SPE), finally quantitative measured by using LC-MS/MS. Generally, around 0.1 ng/L limit of quantification (LOQ) value are achieved [33, 34, 71, 72]. The detection of steroid EDCs from various solid samples are complicated because more sample preparation steps are required (drying, homogenization, destruction, extraction and purification). The most commonly applied extraction methods are USE, MAE and SPLE for solid environmental matrices, such as sediment or biological tissues [64–68]. After extraction procedure, off-line SPE and LC-MS/MS are utilized for EDCs analysis [64, 73, 74].

2.2. Progestogens as neuroendocrine disruptors: an outlook on the world of fish

Together with synthetic estrogenic steroids, progestogens are among the most important group of environmental pharmaceuticals of concern. A large number of studies investigating the occurrence and effects of natural and synthetic estrogen hormones (e.g. ethinylestradiol, estradiol, estrone and estriol), and the risk is now well documented [47, 49, 75, 76]. Several studies have also been conducted on the risk related to anti-androgens [77], but contrast to these, surprisingly, relatively few data are published about the occurrence of progestogens in different waters [34, 41, 49] and mainly their neuroendocrine effects on non-target freshwater organisms, including particularly invertebrates [49].

Progesterone (PRG) is an endogenous steroid hormone involved in the female menstrual cycle, pregnancy and the embryogenesis of humans and other vertebrate species. In turn progestins are a group of natural and synthetic molecules that have effects similar to those exerted by PRG. The endogenous PRG and its analogue progestins together are generally referred to as progestogens (or gestagens). The most important and frequent synthetic progestogens are the follows drospirenone (DRO), levonorgestrel (LNG), gestodene (GES), norethindrone (NET) and ciproterone acetate (CPA). The progestogens that are used in hormonal contraceptives are LNG (e.g. Alesse, Trivora-28, Plan B, Mirena), DRO (e.g. Yasmin, Yasminelle), GES (e.g. Femodene) and CPA (e.g. Diane-35, Dianette). There are approximately 20 different progestogens used in human and veterinary medicine. Despite significant use, their ecotoxicological implications are poorly understood in environment. According to Fent, only about 50% of the progestogens in use have been analyzed for their environmental occurrence and effects in aquatic organisms [49].

For example, in fish, the main natural progestin is 17α,20β-dihydroxy-4-pregnen-3-one (DHP). In females, DHP is responsible for maturation of oocytes [78] and ovulation [79], while in males it is involved in spermiation and sperm motility [80]. Synthetic progestogen contaminations altered hormone levels [81], induced transcriptional effects in adults [82] and embryos [83], altered sex development and induced development of male secondary sexual characteristics in female fish [81, 84]. Therefore, there are evidences that progestogen contamination interferes with endogen steroids and adversely affect fish reproduction. According to literature data, LNG and GES significantly reduce egg production in fathead minnow (*Pimephales promelas*) [81, 84]. At environmental ng/L concentrations, progestogens could interfere with natural pheromones, therefore also impair the physiological responses and spawning behavior in fish [85, 86]. In addition, based on earlier work it has been shown that chronic exposure to a mixture of PRG, LNG, DRO induce complex molecular changes both in brain, liver and serum of roach (*Rutilus rutilus*) [87]. Collectively in vertebrates, progestogens activate nuclear PRG receptors [88], but also may activate other steroid receptors, such as the androgen, estrogen, glucocorticoid and mineralocorticoid receptors, exerting combinations of progestogenic, (anti)androgenic, (anti) estrogenic, glucocorticoidogenic and anti-mineralocorticoidogenic effects [89].

3. Molluscs as "possible and valuable" model animals in environmental tests

3.1. Sex steroid-like receptors in molluscs

PRG receptor immunoreactive elements were identified in the reproductive system of the female *Octopus vulgaris* [90]. According to Tosti, the PRG receptors are physiologically active because the external application of PRG stimulates the activation of spermatozoa in *Octopus* [91]. In contrast to cephalopods, no progestogen-like receptors have been identified in snails so far. The androgen-like receptor immunopositive cells has already been described in ovotestis of *Biomphalaria alexandrina*, and there is some (inferred) evidence of a role for androgen-like molecules in the reproductive cycle of molluscs [92–94]. But the fact is that homolog or orthologue sequences were not identified in molluscs despite investigations searching for the androgen receptor gene [95]. In contrast, estrogen receptor orthologues have previously been reported in number of freshwater and marine molluscs, such as *Aplysia californica, Biomphalaria glabrata*, *Bithynia tentaculata, Marisa cornuarietis*, *Potamopyrgus antipodarum*, *Nucella lapillus*, *Chlamys farreri, Crassostrea gigas*, *Lottia gigantean, Mytilus edulis*, *Octopus vulgaris* and *Sepiella japonica* [93, 96, 97]. The existence of an estrogen or estrogen-related receptor has been confirmed in *Lymnaeidae* sp. (e.g. *L. ollula*) [98], however in *L. stagnalis* is not investigated so far. The amino acid sequence of endocrine receptor of oyster (*Saccostrea glomerata*) contains a DNA-binding domain and a ligand-binding domain which are conserved among vertebrate endocrine receptors [99]. However, it is worth to mention that real function of identified estrogen receptors are questionable at present, because ligand studies show that the receptor homolog is non-sensitive to estrogen in the oyster, for example [100]. Even so, many researchers speculate that the most steroid pollutants act through the estrogen or androgen-like receptors in molluscs [93]. It is also known that the endocrine effect of TBT (steroid biocide) appear

through binding to a nuclear receptor (the retinoid × receptor) in *Nucella lapillus*. The natural ligand (9-cis-retinoic acid) of the retinoid × receptor induces similar imposex in females of *N. lapillus* than TBT at similar concentration [101, 102]. Despite the contradictory observations and opinions about the presence of steroid-like receptors in molluscs, as well as the limited genetic evidence for steroid receptors, binding proteins for classical vertebrate-type steroids have been described. However, it has not yet been demonstrated that this binding is coupled to an endocrine biological response. Some researchers speculate that vertebrate-like steroids, such as estorgen, can also/just act through non-genomic mechanisms in mollusc. Non-genomic action of steroids are expressed through cell surface membrane receptors (not nuclear receptors) and in this case they also can results direct local "ionotropic" effects (e.g. modification ion fluxes) and/or they can activate second messenger kinase cascade system during "metabotropic" pathway (e.g. cAMP-MAPK-PKC) [103, 104].

3.2. Endocrine steroid system of molluscs: evidences and questions

Despite many published studies reporting presence of vertebrate-like sex steroids, steroidogenic enzymes and steroid receptors in molluscs, the endocrine system is the most uncleared and contradictory topic of molluscan research. It is generally accepted that vertebrate-type steroids, as PRG, estradiol or testosterone, are presented in various molluscan tissues (e.g. gonads, haemolymph) and they are physiologically potent molecules performing hormonal functions. Regarding their endogenous biosynthesis, evidences are contradictory. At present is unknown whether vertebrate-type sex steroids are formed endogenously during steroidogenesis or they are taken up from their environment through the feeding because it is known that many plant species contain vertebrate-like sex steroids [105]. Since PRG, estradiol and testosterone as functional hormones in mollusc are the same as those of vertebrates, and vertebrates continuously excrete them not just via urine and faces, but via their body surface or gills (in fish), the other possibility is that observed "molluscan" steroids just come from contamination [95, 106]. At the same time, several papers have been published presenting evidence of steroidogenic activity and steroid metabolism in molluscs [107, 108]. For example, beside other metabolic enzymes (e.g. 5α-reductase, sulfotransferase, and acyl-CoA acyltransferases) the occurrence and activity of two key steroidogenic enzymes 3α/β-hydroxysteroid dehydrogenase (HSD) and 17β-HSD are presented in several molluscan species. The 3α/β-HSD is the key enzyme in conversion of prognenolone (P5) to PRG. This enzyme has been described in *Ariolimax californicus*, *Aplysia depilans*, *Helix pomatia*, *Mytilus edulis* and *Octopus vulgaris*. The 17β-HSD is crucial molecule in the last step of steroid syntheses and the primary metabolism. The 17β-HSD catalyzes the interconversion of androstenedione to testosterone, estrone to 17ß-estradiol and androstenedione to dihydrotestosterone. The 17β-HSD enzyme has been detected in many snails (e.g. *Marisa cornuarietis, Ilyanassa obsolete, Hexaplex trunculus, Bolinus brandaris* and *Helix aspersa*), bivalves (e.g. *Crassostreas gigas, Crassostreas virginica, M. edulis, M. galloprovincialis, Ruditapes decussate* and *Patinopecten yessoensis*) and cephalopods (e.g. *Sepia officinalis and O. vulgaris*) so far. These observations comprise a series of indications about the existence of steroidogenesis in different molluscs [107, 108]. At present, no data are available about the expression of key enzymes in *L. stagnalis*, however the cholesterol which is the direct precursor of P5 has been described in its neurons [109]. According to literature

data, *L. stagnalis* was able to transform PRG from injected labeled P5 [110]. The P5 is a key molecule in the biosynthetic pathway of main vertebrate steroids, such as PRG, 17ß-estradiol and testosterone which have also been proposed as functional hormones in molluscs [95].

Steroidogenesis and steroid metabolism play and important role in the regulation of endogenous steroid level in molluscs. As a result of their endogenous biosynthesis, active P5 (e.g. in *M. edulis, Astacus leptodactylus* and *Nephrops norvegicus*), PRG (e.g. in *M. edulis, Mya arenaria* and *O. vulgaris*), androstenedione (e.g. in *M. edulis, H. aspersa, A. leptodactylus* and *Neomysis integer*), estron (e.g. in *M. edulis, Arion ater rufus, H. aspersa* and *Asterias rubens*) and testosterone (e.g. in *M. edulis, Arion ater rufus, A. leptodactylus* and *N. integer*) have been described in several molluscan species [108]. In addition, it has been published that the physiological concentration of these sex steroids are related to changes in the reproductive cycle and their level are higher in one sex than other, or their level are changed during EDC contamination. Furthermore, another vertebrate-type hormone, the gonadotropin-releasing hormone (GnRH) stimulates the synthesis and release of "molluscan"sex steroids from the gonads, and elicited contractions of the oviduct, for example, in cephalopods. This result suggests that octopus-GnRH induces the gonadal maturation and oviposition by regulating sex steroidogenesis [111]. GnRH-like hormone has also been identified in two freshwater snails, *Helisoma trivolvis* and *L. stagnalis*, presumably with a similar control function than in cephalopods [112]. From a phylogenetic point of view, observations of steroidogenesis and vertebrate-type steroids are very interesting because they indicate a common origin of a sex hormonal system between molluscs and vertebrates. However, much more information is needed to fully understand the physiological function of sex steroid hormones in molluscs. But also noticeable that according to valuable Scott's reviews, despite many studies starting over 55 years ago, these data are questionable from several reasons. For example, the mollusc genome (so far known) does not contain genes for key enzymes that are necessary to transform cholesterol (precursor molecule in steroid biosynthesis) [95, 106].

3.3. Reproductive system and behavior of *L. stagnalis*

The reproductive biology of *L. stagnalis* has been well-studied [14, 15, 113, 114]. It is a hermaphrodite species, but during mating behavior one individual acts as male and the other as female. The snail playing the male role climbs on the shell of the prospective female, moves over the shell in a counter-clockwise direction until he reaches the area of the female gonophore. The preputium (muscular structure that surrounds the penis) is then partially everted through the male pore. This is followed by probing for the female pore by the preputium, insertion of this organ into this pore followed by penis eversion and intromission. Each of the four stages prior to intromission is variable in duration but the intromission is more constant. The whole mating behavior may last for several hours. During oviposition (egg-laying), masses containing 50–100 eggs embedded in a gelatinous mass are deposited on the substrate, from which juvenile snails of adult form emerge following about 10 days of intracapsular embryogenic development, without any free-living larval stages [113, 114]. Egg-laying consists of a sequence of behavioral events beginning with a rest period when the animal ceases to locomote, then a turning phase characterized by counter-clockwise shell movements and high frequency rasping to clean the substrate, followed by oviposition and a final phase

called inspection when the snail moves along the length of the egg mass brushing it with lips and tentacles. Resting and turning last for about an hour each, oviposition 10 minutes and inspection about 2 minutes. Egg-laying in *L. stagnalis* is an example of a complex behavior that is triggered by the release of multiple neuropeptide transmitters from neuroendocrine centers within the central nervous system that act on other neural circuits controlling egg-laying behavior [12]. Weather the action of described sex steroids is receptor-mediated or not is uncleared in *L. stagnalis* at present. But it is a fact that vertebrate-like sex steroids might have a key role in reproduction in snails. Temporal variation in some steroid titers that coincide with reproductive stages have been observed.

3.4. Progestogen effects in *L. stagnalis*

Cyproterone acetate (CPA) is a commonly used synthetic progestogen compound in oral contraceptives, which also has anti-androgen effects in vertebrates. The vinclozolin (VZ) is mainly known as anti-androgen, which can also bind to estrogen and progestogen receptors in vertebrates. Using these progestogens, Ducrot and Giusti published no significant difference in shell length of adult *L. stagnalis* between control and treated groups after 21 days of exposure to any of the tested chemicals [25, 27]. However, Ducrot observed slower growing among juveniles, sub-adults and young adults exposed to the highest concentration of VZ (2500 μg/L) during the first week, but partly recovered during the second and third weeks, so that growth pattern did not lead to a significant decrease in the mean shell size compared to control group. In Giusti's experiments, neither CPA (2–50 μg/L), nor VZ (10–240 ng/L) induced more than 10% mortality. According to Ducrot, significant mortality occurred in treated adult animals exposed to the highest concentration of VZ, whereas the feeding activity was stopped in this group. CPA and VZ had no significant effect on cumulated oviposition and fertility in adult snails in single chemical treatment approach, however 2 and 10 μg/L CPA as well as 10–240 ng/L VZ treatments induced a significant decrease in egg number per egg mass compared to control. However, in this experiment no clear concentration-response relationship was described [27, 106]. CPA, VZ and tributyltin (TBT) were also tested on other three gastropod species, *Marisa cornuarietis*, *Nucella lapillus* and *Nassarius reticulatus* in a chronic experiment. In this investigation, the snails were treated by nominal CPA concentration of 1.25 mg/L for 12 month or by nominal VZ concentration of 0.03–1.0 μg/L for up to 5 month. It was reported that no mortality at used concentrations, but a significant decrease in the length of the penis and accessory male sex organs was observed in both species [106, 115]. Since progestogen contaminations are expected be found in the environment as mixtures, Zrinyi and her co-workers applied them together and environmentally relevant concentration range in *L. stagnalis* reproduction tests [116]. In a 10 ng/L eqi-concentration mixture of PRG, LNG, GES and DRO treatment approach resulted that the oocyte production of individual animals significantly changed in the treated group at the end of the 21-day long experiment. The number of laid oocyte per egg mass of individual animals shows continuous growth in the treated group week by week. Their number at the end of the third week was in average of twofold higher compared to the control. The control animals produced the same amount of oocytes weekly during experiment.

Beside egg number assessment, egg abnormalities can also be observe (e.g. polyembryonic egg, atrophied albumen, unfertilized oocyte or dead zygote in eggs), which refer to reproductive

status and can determine the egg quality. Egg quality showed no significant difference in CPA and VZ treatments, however polyembryony was the most frequent phenomena. A 3-week long CPA treatment resulted in significant increase of the frequency of polyembryony in concentration dependent manner from 2 μg/L concentration [27]. In addition, the whole egg mass quality was also assessed in progestogen mixture treatment of adult snails with a three-graded scheme, which integrates the number of polyembryonic eggs with eggs containing dead zygotes. This endpoint resulted in a significant difference by the first week (**Figure 1**), but did not show difference at the end of a 3-week long experiment [116]. Based on published data, following the time window of early embryonic development in *L. stagnalis* could be able to another well endpoint for investigation of external progestogen contaminations. Progestogen mixture applied in environmental relevant concentration (10 ng/L) had significant effect on cell proliferation during early embryonic development. At the end of a 3-week long treatment period of the adult snails, the freshly laid zygotes were observed from the single-cell to the eight-cell stage. In zygotes obtained from hormone-treated adults, a significantly different accelerated cell proliferation could be noticed compared to controls, however the hatching time was unchanged [116]. In single-cell zygotes as well as egg albumen, a partial metabolomic analysis was also carried out using capillary microsampling combined ion mobility separation mass spectrometric technique. It was observed that the molecular composition of zygotes or egg albumen does not differ after steroid treatment of adults, but some semi-quantitative metabolic ratio (e.g. adenylate energy charge (AEC), redox ratio or hexose utilization) can express difference between the groups. These ratios could be used as marked endpoint in assessment of progestogen exposition in snails. The hexose utilization defined by UDP-hexNAc/UDP-hex ratio significantly decreased in single-cell zygote cytoplasm after a 3-week long progestogen mixture treatment of adults. This result partly could explain the observed accelerated cell division in zygotes obtained from treated parents. At the same time, AEC indicating the energy state of the cell was unchanged. This endpoint was significantly increased only in the albumen (obtained from treated adults) during the metamorphosis, which is the half-time of the average hatching time [116].

Figure 1. Evaluation of the egg mass quality in *Lymnaea*. Figure demonstrates the categories of the egg mass quality in the control and with the 10 ng/L progestogen-treated groups. The egg mass was presented in good (white), fair (gray) and poor (black) quality in the 1st, 2nd and 3rd weeks in both groups. On the 1st week, the egg mass quality was significantly different between the groups (Kruskal-Wallis $Chi^2 = 6.31$; $P < 0.05$; $n = 41$), while this was statistically not different in next 2 weeks (Kruskal-Wallis $Chi^2 = 3.65$; $P = 0.56$; $n = 50$ and $Chi^2 = 0.15$; $P = 0.70$; $n = 43$). $P < 0.05$ is signed by asterisk (*).

Based on several published data, we conclude that progestogen contaminations in water ecosystem are harmful for reproduction and early stage development of *L. stagnalis*. But, taken separately, progestogens might not present a risk for the snails, however since they are expected be found in the environment as mixtures (similar then in earlier experiment), there is a risk of additive or even synergistic effects [41]. Together with synthetic estrogenic steroids, progestogens are among the most important group of environmental pharmaceuticals of concern. However, in contrast to estrogens, progestogens have received only little attention so far and their environmental risks are not sufficiently known. Further investigations are needed to fully understand the synergistic effects of mixed progestogens or combined effects of progestogens and estrogens in freshwater organisms, such as molluscs.

3.5. *L. stagnalis* became a "real" test animal in EDC experiment

The OECD Conceptual Framework for Testing and Assessment of Endocrine Disruptors is available from 2004 (revised and completed with mollusc's tests in 2012) [117]. This recommendation provides a guide with a five-level assessment but not indented to be a testing strategy of various EDCs. Another OECD reproductive toxicity test guideline with the pond snail *L. stagnalis* is also available from 2010 [25] and this optimized in 2016 using the steroidal TBT [30]. Several studies published data with different endpoints (number of egg mass, eggs, survival and shell size) recommended in OECD guidelines in progestogen exposure of *L. stagnalis* [23–28, 116]. Based on endpoint results coming from steroid, progestogen effects in snail reproduction, the pond snail, *L. stagnalis*, beside a mudsnail, *Potamopyrgus antipodarum*, has been the first aquatic non-arthropod-tests, which were successfully validated within the Conceptual Framework for Endocrine Disrupters [3].

4. General considerations: ecotoxicologist versus physiologist

Nowadays, we realized that a wide variety of environmental contaminants have specific effects on neuroendocrine system of aquatic species, including snails. For among them, *L. stagnalis* has been used as non-target model organisms in studying environmental contamination long time because they are sensitive to anthropogenic steroids, such as progestogens. Investigating a variety of reproductive endpoints, such as fecundity, oocyte production, egg mass production, the quality of egg masses, the shell size in development and after egg-laying, the time window of cell division in the offspring, the metabolite content of single-cell zygotes and egg albumen, it is concluded that progestogen contaminations in water are detrimental for reproduction and early stage development of *L. stagnalis*. Based on its endpoint results, the *L. stagnalis* has become the first aquatic non-arthropod-tests, validated successfully within the Conceptual Framework for Endocrine Disrupters. In this context, the proposed model is ecotoxicologically correct because it has well detectable effects. But if we are interested in physiological mechanisms of steroids (progestogen), many uncleared questions and contradictory observations are detected. For example, how progestogen contamination influences the *Lymnaea* reproduction is difficult to explain because progestogen and androgen

receptors until recently were not observed. At the same time, the identified estrogen receptor was found to be insensitive to estrogen. Whether estrogen binds to its receptor and the hormone-receptor complex remains inactive or it does not binds at all is unknown. Furthermore, according to some assumptions, the key enzymes for steroidogenesis are also missing in gastropods, therefore, the biosynthesis of vertebrate-type steroid hormones are questionable. If it is true, how can endogen "gastropod" steroids control the reproduction pathways?

We guess that some researchers did not consider that active state of many (terrestrial and freshwater) gastropod species depend on the season. For example, hibernation, aestivation or inactive state is an evolutionary mode of adaptation of animal species to unfavorable environmental conditions, such as low temperature or lack of food in autumn or winter. During the inactive state, normally no reproduction is observed in nature. This observation can be explained either by the low metabolism in unfavorable conditions (no steroid hormone synthesis) of the snails or vertebrate-type steroids cannot be taken up from environment through the feeding. Most of recent experiments on *Lymnaea* are performed on bred animals. It is possible that the evolutionary conserved seasonal (normal) rhythm of steroidogenesis and endogen level of steroids controlling reproduction will be damaged in artificial laboratory conditions. In this case, the steroids will be present at steady level instead of the normal wavering and the egg laying is continuous during all year. This is not a normal physiological rhythm for the laboratory-bred stock animals. If this artificial condition persists for a long time, it may occur that animals try to defense their metabolic status by inactivating receptors or reducing their steroid hormone levels. In this condition, the detection of receptors or hormones is sticky by IHC, ELISA or other analytical methods. The conclusion will be an artifact about the presence and distribution of steroid receptor or hormone in different tissue of snail.

The fact is that vertebrate-like steroid hormones undoubtedly can be detected in molluscs. Whether they are synthetized and performed physiologically, relevant functions taken up from the environment is firmly not established yet. The solution to mention problems are for scientists to apply more robust experimental designs and animals in sufficient conditions [95].

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Chapter 3

Genetic Characteristics of Southern and Northern Brook Trout (*Salvelinus fontinalis*) Populations at the Zone of Contact

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Additional information is available at the end of the chapter

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Abstract

Population genetic evidence suggests differentiation among evolutionarily significant units of southern and northern Appalachian brook trout, with the zone of contact in southwestern Virginia. Before this differentiation was recognized, brook trout of northern origin were stocked throughout the southeastern United States. In order to determine this differentiation, established allozyme markers were used to classify 56 southwest Virginia populations as southern, northern, or introgressed. Variation at 4 polymorphic loci, including the diagnostic creatine kinase (*CK-A2**) locus, indicated that 19 populations were of southern origin, 5 of northern origin, and 32 of mixed genetic origin. Data compiled among genetic studies of brook trout in the southern Appalachians showed that the southern/northern break is sharp, occurring at the New/Roanoke-James watershed divide. New River drainage populations exhibited the southern allele at high frequency, suggesting their historic native character as southern, with presence of northern alleles due to stocking or stream capture events. In conclusion, the present study suggests that management of southern Appalachian brook trout should include: (1) genetically cognizant planning of stocking events, (2) management of populations on a stream-by-stream basis, (3) prioritized conservation of pure southern brook trout populations, and (4) use of southern Appalachian hatchery stocks in restoration efforts.

Keywords: southern Appalachian brook trout, conservation, population genetics, trout management, restoration

1. Introduction

Brook trout, *Salvelinus fontinalis*, is the only salmonid native to the southern Appalachian Mountains, and it is distributed across eastern North America from Canada to Georgia [1].

This species was once abundant in coldwater lakes and streams throughout its range, but environmental disturbances such as deforestation, development, and pollution: and the introduction of non-native rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*) have drastically reduced the number and sizes of wild populations [2].

Beginning in the mid-1800s, fishery managers began stocking hatchery-reared brook trout extensively. However, hatchery-reared brook trout often exhibit lower growth, yield, survival, and natural reproduction than locally adapted wild populations [3, 4]. Further, the hybridization of hatchery-derived fish with wild populations can compromise the genetic integrity and fitness of receiving populations by introducing foreign alleles and breaking up locally adapted gene complexes [5, 6]. The stocking of northern-derived hatchery brook trout is of particular concern in the southern part of its range due to significant population genetic differentiation between southern and northern lineages of brook trout. Genetic differences between the two lineages may be large enough to justify distinction at the subspecies level [7, 8]. In addition, screening of allozyme [7–16], mitochondrial DNA [17–19], and microsatellite nuclear DNA [20, 21] markers has uncovered smaller scale genetic variation throughout the geographic range of brook trout. Differentiation at smaller geographic scales may reflect different colonization histories, as well as differential effects of selective and non-selective population genetic processes.

Native southern Appalachian brook trout (SABT) populations share several biological characteristics [22]. Food availability being a limiting factor in these systems, adult fish are typically small (<229 mm total length) and life span seldom exceeds 3 years [23, 24]. Native SABT and introduced northern-lineage brook trout differed in terms of survival in the laboratory and diet in a natural stream [25]. Comparison of external microbial assemblages suggested that SABT exhibit greater ability to inhibit microbial growth in their epidermal mucus than do northern brook trout of hatchery ancestry [26]. Demonstration that SABT are genetically distinct from northern-origin hatchery stocks led management agencies to assess the heritage of populations within their jurisdiction, for example, in the Great Smoky Mountains National Park [8, 13], Tennessee [11], North Carolina [12, 16, 27], and Georgia [10]. Molecular and adaptive differentiation may warrant management of brook trout populations or groups of populations as evolutionary significant units [28], although some of their population genetic differentiation may reflect stocking history.

The zone of contact between the southern and northern lineages of Appalachian brook trout is roughly at the New River watershed [14, 15, 29]. Against the background of decline of the southern form and history of stocking with non-native strains, genetic characterization of brook trout populations at the zone of contact is needed to support informed management decisions and conserve the native form of the species. The objective of this study was to use established allozyme markers to wild Appalachian brook trout populations at the zone of contact in southwest Virginia as southern or northern lineages or introgressed.

2. Methods

2.1. Sampling

Seventy-eight historic wild brook trout streams from the New, James, Holston, and Yadkin river drainages [30] were sampled by backpack electrofishing. Brook trout tissue samples were collected from 916 individuals from 56 streams (**Table 1**). Sample sizes ranged from 8 to 26 individuals per stream. Fish were anesthetized, and two samples of dorsal muscle tissue (from fish greater than 120 mm TL) were collected non-lethally using an 18-gauge Monopty Biopsy Instrument (C.R. Bard, Inc., Covington, GA) and immediately placed on dry ice. Anesthetized fish were fully revived in fresh water prior to release. A limited number of fish of <120 mm total length were sacrificed to sample streams from which few adults were collected. Samples were stored at −80°C.

2.2. Protein analysis

Genetic analysis was performed using cellulose acetate gel electrophoresis to observe variability at nine loci encoding five polymorphic enzymes: creatine kinase (*CK-A2**), aspartate aminotransferase (*sAAT-1,2**), glycerol-3-phosphate dehydrogenase (*G3PDH**), glucose-6-phosphate isomerase (*GPI-A**, *GPI-B1,2**), and malate dehydrogenase (*sMDH-B1,2**). Muscle tissue was homogenized in 200 μl of 0.09 M tris-HCl (pH 8.0), and subjected to electrophoresis in tris-glycine buffer (pH 7.5 or 8.0) for 45 min, followed by staining for enzyme activity. Electrophoretic conditions and histochemical staining procedures were modified from those described by Hebert and Beaton [31] and Galbreath et al. [16]. Individuals from the Paint Bank Hatchery in Virginia were included in the analysis as a northern reference population because the hatchery is known to culture the northern lineage. The North Carolina Wildlife Resource Commission provided tissue samples from individuals from Charles Creek of the North Toe River drainage, a known SABT population, for use as a reference population.

2.3. Data analysis

Allele frequencies for *CK-A2**, *G3PDH**, *GPI-A**, and *MDH-B1,2** were calculated for all populations using the Excel Microsatellite Toolkit [32]. Allele frequencies could not be calculated for *sAAT-1,2** and *GPI-B1,2** using that program because both enzymes are encoded by isoloci (i.e., duplicated loci with alleles of overlapping mobility). Since genotypes among heterozygous individuals could not be determined with certainty for *sAAT-1,2**, phenotype frequencies were calculated using the program FDASH [33]. The *GPI-B1,2** isoloci contain multiple alleles that could not be assigned to either locus with confidence; hence, they were treated as a single tetraploid locus and allele frequencies were estimated using the program AUTOTET [34]. Initially, allele frequency data from all nine marker loci were used to calculate genetic distance, population differentiation, contingency-table analysis of heterogeneity among populations, and hierarchical cluster analysis using the program BIOSYS-1 [35]. The same statistics then were calculated using only the five marker loci with unambiguous interpretation of allelic expression (i.e., omitting data from *sAAT-1,2** and *GPI-B1,2**), to determine any effect of

		*CK-A2**		*G3PDH**		*GPI-A**			*MDH-B1,2**					
	N	***78***	***100***	***45***	***100***	***87***	***100***	***115***	***100***	***145***	***P***	***A***	***HO***	***HE***
Controls														
Charles Creek, NC	5		1.00		1.00		1.00		1.00					
Paint Bank Hatchery	16	1.00		0.44	0.56		1.00		1.00					
Holston River drainage														
Grassy Branch	12		1.00		1.00		1.00		1.00		0.00	1.0	0.000	0.000
Henshew Branch	20	1.00		0.45	0.55		1.00		1.00		0.25	1.3	0.125	0.127
Parks Creek	10	0.05	0.95		1.00		1.00		1.00		0.25	1.3	0.025	0.025
Pennington Branch	12	0.08	0.92		1.00		1.00		1.00		0.25	1.3	0.042	0.040
Roaring Fork	8	0.56	0.44		1.00	0.69	0.31		1.00		0.50	1.5	0.188	0.246
Sturgill Branch	16	0.19	0.81		1.00		1.00		0.75	0.25	0.50	1.5	0.219	0.175
James River drainage														
Barbours Creek	20	1.00		0.08	0.93		1.00		1.00		0.25	1.3	0.021	0.036
Ewins Run	20	1.00			1.00		1.00		1.00		0.00	1.0	0.000	0.000
Pickles Branch	20	1.00			1.00		1.00		1.00		0.00	1.0	0.000	0.000
New River drainage														
Bear Creek	23	0.02	0.98		1.00		1.00		1.00		0.25	1.3	0.016	0.016
Big Horse Creek	18		1.00		1.00		1.00		1.00		0.25	1.3	0.011	0.011
Big Laurel Creek	11	0.05	0.95	0.09	0.91		1.00		1.00		0.00	1.0	0.000	0.000
Big Reed Island Creek	20	0.08	0.93		1.00		1.00		0.95	0.05	0.50	1.5	0.068	0.066
Bournes Branch	16	0.03	0.97		1.00		1.00		1.00		0.50	1.5	0.063	0.061
Buffalo Branch	16	0.06	0.94		1.00		0.97	0.03	1.00		0.75	1.8	0.125	0.111
Cabin Creek	20	0.05	0.95		1.00		1.00		1.00		0.50	1.5	0.047	0.046
Chestnut Creek	17	0.12	0.88		1.00		1.00		1.00		0.25	1.3	0.000	0.024
Chisholm Creek	12		1.00		1.00		1.00		0.96	0.04	0.25	1.3	0.021	0.021
Crooked Creek	15		1.00		1.00		1.00		1.00		0.25	1.3	0.059	0.053
Ding Branch	26	0.25	0.75	0.02	0.98		1.00		0.94	0.06	0.00	1.0	0.000	0.000

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		CK-A2*		***G3PDH****		***GPI-A****			***MDH-B1,2****					
	N	****78***	****100***	****45***	****100***	****87***	****100***	****115***	****100***	****145***	***P***	***A***	***HO***	***HE***
East Fork Cove Creek	14	0.11	0.89		1.00		0.93	0.07	1.00		0.75	1.8	0.145	0.133
East Fork Crooked Creek	20	0.03	0.98		1.00		0.98	0.02	1.00		0.50	1.5	0.089	0.084
East Fork Dry Run	20		1.00		1.00		1.00		1.00		0.50	1.5	0.025	0.025
East Fork Little Reed Island	10		1.00		1.00		1.00		1.00		0.00	1.0	0.000	0.000
Elkhorn Creek	10		1.00		1.00		1.00		0.95	0.05	0.25	1.3	0.125	0.097
Fox Creek	20	0.18	0.83		1.00		0.95	0.05	0.88	0.12	0.25	1.3	0.025	0.025
Grassy Creek	9		1.00		1.00		1.00		1.00		0.75	1.8	0.150	0.154
Howell Creek	20	0.05	0.95		1.00		1.00		0.98	0.02	0.00	1.0	0.000	0.000
Laurel Branch	22	0.23	0.77		1.00		1.00		0.98	0.02	0.50	1.5	0.038	0.037
Laurel Creek	10		1.00		1.00		1.00		1.00		0.00	1.0	0.000	0.000
Laurel Creek	20	0.10	0.90		1.00		0.98	0.02	1.00		0.50	1.5	0.063	0.059
Little Indian Creek	19	0.79	0.21		1.00		1.00		0.95	0.05	0.50	1.5	0.125	0.101
Little Snake Creek	8		1.00		1.00		1.00		1.00		0.50	1.5	0.132	0.111
Little Stony Creek	14	0.11	0.89		1.00		0.96	0.04	1.00		0.00	1.0	0.000	0.000
Little Wilson Creek	19	0.21	0.79	0.03	0.97		1.00		0.82	0.18	0.50	1.5	0.071	0.067
Middle Fox Creek	12	0.04	0.96		1.00	0.04	0.96		0.58	0.42	0.00	1.0	0.000	0.000
Mill Creek	17	0.12	0.88		1.00		1.00		0.82	0.18	0.75	1.8	0.184	0.176
NB Elk Creek	14	0.25	0.75		1.00		1.00		1.00		0.75	1.8	0.250	0.168
NF Stony Creek	21	0.02	0.98		1.00		0.98	0.02	1.00		0.50	1.5	0.147	0.128
No Business Creek	20	0.20	0.80	0.03	0.98		1.00		0.90	0.10	0.50	1.5	0.024	0.024
Oldfield Creek	12		1.00		1.00		1.00		1.00		0.75	1.8	0.163	0.141
Opossum Creek	17	0.03	0.97		1.00		1.00		0.72	0.28	0.00	1.0	0.000	0.000
Pearis Thompson Branch	17	1.00		0.15	0.85		1.00		0.91	0.09	0.50	1.5	0.155	0.119

		*CK-A2**		*G3PDH**		*GPI-A**			*MDH-B1,2**					
	N	****78***	****100***	****45***	****100***	****87***	****100***	****115***	****100***	****145***	***P***	***A***	***HO***	***HE***
Ripshin Creek	10	0.15	0.85		1.00		1.00		0.75	0.25	0.50	1.5	0.200	0.166
Roads Creek	11		1.00		1.00		0.95	0.05	1.00		0.25	1.3	0.023	0.023
Snake Creek	20		1.00		1.00		1.00		0.98	0.02	0.50	1.5	0.200	0.166
Standrock Branch	20		1.00		1.00		1.00		1.00		0.25	1.3	0.013	0.013
Stony Creek	20	0.18	0.83	0.03	0.98		1.00		0.95	0.05	0.25	1.3	0.100	0.111
Sulfur Springs Branch	10	0.30	0.70		1.00		1.00		1.00		0.00	1.0	0.000	0.000
Tory Creek	19		1.00		1.00		1.00		1.00		0.00	1.0	0.000	0.000
Upper West Fork Dry Run	10		1.00		1.00		1.00		1.00		0.00	1.0	0.000	0.000
West Fork Dry Run	19		1.00		1.00		1.00		1.00		0.25	1.3	0.063	0.057
Whitetop Creek	12	0.13	0.88		1.00		1.00		1.00		0.00	1.0	0.000	0.000
West Fork Furnace Creek	17	0.12	0.88		1.00		1.00		0.97	0.03	0.50	1.5	0.044	0.068
Yadkin River drainage														
Pauls Creek	20		1.00		1.00		1.00		1.00		0.00	1.0	0.000	0.000
South Fork Stewarts Creek	24		1.00		1.00		1.00		1.00		0.00	1.0	0.000	0.000

Charles Creek, a known southern-strain population, was included as a southern-strain reference group. Individuals from Paint Bank Hatchery, which cultures the northern strain, were included as a northern-strain reference group. Abbreviations: number of individuals analyzed (N), proportion of polymorphic loci (*P*), mean number of alleles per locus (*A*), expected heterozygosity (H_o), and observed heterozygosity (H_e).

Table 1. Allele frequencies and genetic diversity at four polymorphic loci (*CK-A2**, *G3PDH**, *GPI-A**, *sMDH-B1,2**) in wild brook trout populations in 56 southwest Virginia streams, grouped by drainage.

omitting these data from analysis. Similar conclusions were drawn from analysis of both data sets. Here, we report results based on analysis of the reduced dataset only.

Initial characterization of the genetic origin of each population was based on allele frequencies at the diagnostic *CK-A2** locus. Allele frequencies at the other markers were compared to those observed in northern and SABT populations characterized in previous studies [7–16]. Individual heterozygosity and polymorphism were calculated across five loci to assess levels of genetic diversity within each population [32]. Arlequin [36] was used to test for departures from Hardy-Weinberg equilibrium and to perform analysis of molecular variance (AMOVA) to characterize the distribution of the genetic diversity within and among populations and river basins. Cluster analysis using the unweighted pair-group with arithmetic averaging algorithm (UPGMA, [37]) was performed using BIOSYS-1 [35], and a dendrogram was built based on Nei's unbiased genetic distance [38].

Allele frequency data from previous studies of brook trout population genetics were compiled and combined with the results from this study to gain a better understanding of the geographic distribution of SABT in Virginia, as well as the genetic composition of brook trout populations throughout the Appalachian portions of the native range.

3. Results

Of 56 wild brook trout populations from 4 major river drainages analyzed in this study, 19 were fixed for the diagnostic *CK-A2**100 allele, and were designated as pure SABT populations (**Table 1**). Five populations fixed for the *CK-A2**78 allele were designated as northern, and 32 populations exhibiting variation at the *CK-A2** locus were designated as introgressed. The three James watershed populations exhibited alleles characteristic of northern-form brook trout. Populations in other watersheds were characterized as southern ($n = 19$), northern ($n = 2$), or introgressed ($n = 32$).

Only the Cabin Creek population (New River drainage, Grayson County) deviated significantly ($p < 0.05$) from Hardy-Weinberg equilibrium at the *CK-A2** locus. No other deviations from Hardy-Weinberg equilibrium were detected, indicating that the respective populations were in reasonable conformance with assumptions underlying the model. The proportions of polymorphic loci (*P*), the mean number of alleles per locus (*A*), and mean heterozygosities (*H*) for each population are listed in **Table 1**. Observed mean *P* and H_0 values were lowest in the putative southern populations ($P = 0.05$, $H_0 = 0.004$; **Table 2**). The introgressed populations exhibited the highest means for metrics of genetic variability ($P = 0.48$, $H_0 = 0.099$), and the northern populations exhibited intermediate means ($P = 0.20$, $H_0 = 0.053$). Grouped by drainage, Yadkin River populations had the lowest means ($P = 0$, $H_0 = 0$), followed by James River

Group	*N*	*P*	*A*	*Ho*	*He*
Holston River drainage	6	0.29	1.3	0.100	0.102
James River drainage	3	0.08	1.1	0.007	0.012
New River drainage	45	0.34	1.4	0.064	0.058
Yadkin River drainage	2	0.00	1.0	0.000	0.000
Southern lineage	19	0.05	1.1	0.004	0.004
Northern lineage	5	0.20	1.2	0.053	0.036
Introgressed	32	0.48	1.5	0.099	0.091
Atlantic Ocean drainages	5	0.05	1.1	0.004	0.007
Gulf of Mexico drainages	51	0.33	1.4	0.068	0.063

Based on analysis at four polymorphic allozyme loci (*CK-A2*, G3PDH*, GPI-A*, sMDH-B1,2**). Abbreviations: number of populations per group (*N*), proportion of polymorphic loci (*P*), mean number of alleles per locus (*A*), expected heterozygosity (*Ho*), and observed heterozygosity (*He*).

Table 2. Genetic diversity of brook trout populations, variously grouped by drainage, lineage, and geographic location relative to the eastern continental divide.

(P = 0.08, H_0 = 0.007), New River (P = 0.34, H_0 = 0.064), and Holston River (P = 0.29, H_0 = 0.100) populations. Atlantic-slope populations exhibited lower mean percent polymorphic loci and heterozygosity values (P = 0.05, H_0 = 0.004) than Gulf of Mexico drainage populations (P = 0.33, H_0 = 0.068). Analysis of molecular variance showed that approximately 34% of the total genetic diversity resulted from variation within populations, 18% among populations within drainages, and 48% among drainages. Most of the total limiting variance was attributed to the *CK-A2** locus, meaning that most of the variance that we measured with allozyme markers was due to differentiation among northern and southern lineages of the species.

There was no apparent pattern regarding where populations characterized as southern, northern, or introgressed were located geographically within the New, Holston, Yadkin, and James drainages (**Figure 1**). Cluster analysis of unbiased genetic distances [38] among all populations showed that all populations of northern origin or with a high frequency of the *CK-A2**78 allele clustered together; these included populations from the James River drainage (Barbours Creek, Ewin Run, and Pickles Branch), the Holston drainage (Henshew Creek), the New River drainage (Pearis Thompson and Little Indian Creek), and Paint Bank Hatchery. The Roaring Fork population in the Holston drainage had a high frequency of the northern allele, but did not cluster closely with the other northern populations due to a high frequency of a rare allele at the *GPI-A** locus. Cluster analysis of unbiased genetic distances [38] among populations showed no geographic patterns of genetic variation among the populations of putative southern Appalachian origin.

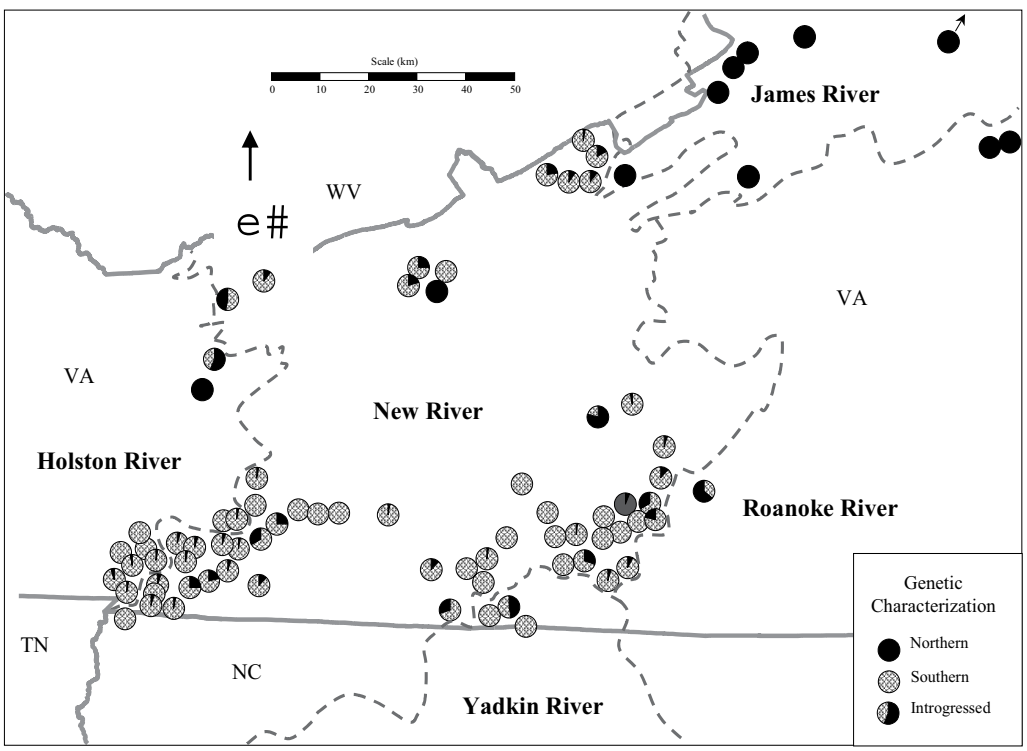

Figure 1. Genetic characterization at the *CK-A2** locus for 83 wild brook trout populations in southwest Virginia, including 56 populations characterized in this study and 27 populations characterized previously.

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4. Discussion

4.1. Decline of brook trout

We sampled 78 streams that historically contained brook trout populations, but found the species in only 56 of them [30]. The range of brook trout is shrinking [39] for several reasons, including habitat alteration, overexploitation, competition with introduced rainbow trout (*O. mykiss*) and brown trout (*S. trutta*) and more recently, climate change.

4.2. Duplicated isozyme loci in brook trout

Certain allozyme markers posed complications to interpretation of underlying genotype. Brook trout show a high incidence of duplicated enzyme loci due to the tetraploid ancestry of salmonids [40]. Duplicated loci (termed isoloci) are genetically independent, but exhibit alleles of similar electrophoretic mobility that cannot be unambiguously assigned to either locus. Three of the five enzymes that we screened were encoded by isoloci (i.e., *MDH-B1,2**, *sAAT-1,2**, and *GPI-B1,2**). Ambiguous interpretation of the banding patterns of two of these isoloci, *sAAT-1,2** and *GPI-B1,2**, led us to eliminate them from statistical analysis [30]. Precise estimation of genetic diversity and differentiation metrics require data from many loci [41, 42]. Information from only four markers clearly limited the power of statistical analysis of genetic differentiation, especially with small sample sizes for some of the populations [43]. Genotypic data from more markers likely would reveal genetic differentiation not detected with only four loci. Ongoing screening of additional, more highly polymorphic markers, such as microsatellite DNA markers, will increase the ability to quantify population genetic differentiation.

4.3. Geographic distribution of SABT in southwest Virginia

Based on fixation for the diagnostic allele at the *CK-A2** locus and allele frequency differences at three other marker loci, 34% (n = 19) of the brook trout populations analyzed in this study were of southern Appalachian origin, 9% (n = 5) were of northern origin, and 57% (n = 32) were of mixed genetic origin (**Tables 1** and **2**). The level of certainty for precise characterization of a population is directly related to sample size. That is, any population observed to be fixed for the common allele actually may harbor the alternate allele at a low, undetected frequency. For example, with a sample size (s) of 20, our likelihood (p) of detecting an allele with a frequency (p_a) of 5% is 36% (i.e., $p = (1-p_a)\, s = 0.9520$, [44]). Therefore, there is a non-zero likelihood that some populations characterized as "pure" southern Appalachian are of mixed genetic origin. Similarly, sample size also affects estimation of within-population diversity statistics such as P and H_0. Sampling of a limited number of populations in a watershed also would affect estimates of between-population genetic variability.

Of the six populations from the Holston drainage, four were of mixed genetic origin, with the southern allele at frequencies ranging from 0.44 to 0.95. The Grassy Branch population was characterized as southern Appalachian, and the Henshew Branch population was characterized as pure northern. Results from earlier genetic studies [8, 11, 14] and its geographic location suggest that the Holston River historically contained the southern Appalachian lineage, so the presence of the northern allele is likely due to stocking.

The Yadkin (upper Pee Dee) River is an Atlantic-slope watershed. Despite the common presumption that Atlantic-slope drainages would contain native northern-form brook trout [8, 12, 15], two pure southern Appalachian populations (Pauls Creek, South Fork Stewarts Creek) were found in the Yadkin drainage. Although no early sampling efforts are known from the upper Pee Dee in Virginia [45], the section of the river that flows through North Carolina was excluded from the range of brook trout originally described by Smith [46]. However, several stream capture events have been inferred in this region, suggesting that these populations are descendants of brook trout captured from the New River [45]. Inspection of stocking records showed that both Pauls Creek and South Fork Stewarts Creek were stocked in the recent past, implying that the "native" southern strain persisted despite stocking.

Earlier genetic study [14] and geographic location suggest that the James River historically contained northern-form brook trout. Three populations from the James River screened in this study were characterized as northern form. This finding leaves little doubt that the New River is the boundary between northern and southern Appalachian brook trout populations.

In this study, 16 populations from the New River drainage (36%) were characterized as southern Appalachian brook trout. No geographic patterns of genetic variation were observed among the populations of putative pure southern origin. Interestingly, two of these "pure southern" populations (Crooked Creek and West Fork Dry Run) were stocked in the recent past with northern-derived hatchery fish. Crooked Creek is a "put-and-take" fishing area, and 5000 brook trout are stocked annually, yet it maintained an apparently pure southern population. Sixty-three percent of the populations from the New River drainage were of mixed origin, with the southern allele at frequencies ranging from 0.21 to 0.98. Although stocking records are limited, only two of these (Howell Creek and Little Indian Creek) are known to have been stocked with northern-derived hatchery fish. Only one population (Pearis Thompson Branch) in the New River was characterized as pure northern.

In addition to the 56 populations characterized in this study, we compiled data from all known genetic studies of brook trout populations in southwest Virginia [12, 14, 15]. Forty-seven percent (n = 39) of all 83 populations characterized in southwest Virginia were of mixed genetic origin (**Table 3**); however, many of these introgressed populations were largely southern. In addition, the "pure" southern populations (n = 26) that remain provide opportunities for restoration of southern Appalachian brook trout in Virginia.

4.4. Range-wide geographic distribution and genetic affinity of New River brook trout populations

With the zone of contact between the northern and southern forms lying roughly at the New River watershed, it is unknown whether the New River historically contained the pure southern Appalachian form, or whether it was a zone of intergradation among southern and northern Appalachian lineages. Interpreting data across this study and the three studies noted above [12, 14, 15], the New River drainage contains 20 pure southern populations, suggesting that the presence of northern alleles could be due to either stocking or stream capture events. However, a large proportion (64%) of populations from the New

Stream	River drainage	County	*N*	% Southern allele	Source
Green Cove Creek	Holston	Washington	19	95	[15]
Grindstone Branch	Holston	Smyth	16	97	[14]
Houndshell Branch	Holston	Smyth	12	100	[14]
Jerry Creek	Holston	Smyth	11	100	[14]
Little Laurel Creek	Holston	Smyth	16	100	[14]
Johns Creek	James	Giles	23	0	[14]
Shawvers Run	James	Giles	23	0	[14]
Spy Run	James	Augusta	21	0	[14]
Valley Branch	James	Craig	15	0	[14]
Burks Fork	New	Floyd	15	67	[15]
Cox Branch	New	Tazewell	15	53	[15]
Dry Creek	New	Smyth	24	100	[14]
Hanks/EF Chestnut Creek	New	Grayson	10	70	[14]
Helton Creek	New	Grayson	21	79	[15]
Jerry Creek	New	Grayson	15	67	[15]
Killinger Creek	New	Smyth	12	88	[14]
Laurel Branch	New	Floyd	15	97	[14]
Laurel Fork	New	Floyd	7	79	[12]
Lewis Fork	New	Grayson	21	79	[15]
Middle Fork Helton	New	Grayson	20	100	[14]
NF Elk Creek	New	Grayson	19	100	[14]
NP Buckhorn Creek	New	Carroll	25	100	[14]
Wilburn Branch	New	Grayson	21	75	[15]
Big Stony Creek	Roanoke	Bedford	10	0	[12]
Little Stony Creek	Roanoke	Bedford	6	0	[12]
Rock Castle Creek	Roanoke	Patrick	25	36	[14]
Turkey Creek	Yadkin	Carroll	15	47	[15]

N = number of individuals per sample.

Table 3. Genetic characterization at the *CK-A2** locus for southwest Virginia brook trout populations not sampled in this study, compiled from both published and unpublished data sources.

River are of mixed genetic origin, suggesting either that hatchery fish persisted in the New watershed or that the New River is a zone of natural intergradation. To gain a better understanding of the geographic distribution of southern Appalachian brook trout, we compiled

allele frequency data from all known genetic studies of brook trout populations throughout the native range (**Table 4**). Frequencies of the *CK-A2**100 (i.e., southern) allele were weighted based on sample size and averaged across all populations in each river drainage. **Figure 2** shows the frequency of the southern allele in each of the major river drainages from which data were collected.

River drainage	State	Position[1]	# of streams	# of individuals	% Southern[2]	Source(s)
Susquehanna	PA/MD	East	4	145	0	[7, 9]
Ohio	MD	West	3	110	0	[9]
Gunpowder	MD	East	1	40	0	[9]
Patapsco	MD	East	1	40	0	[9]
Potomac	MD/VA	East	6	190	0	[9, 14]
James	VA	East	7	142	0	[14, current]
Rappahannock	VA	East	1	25	0	[14]
Roanoke	VA	East	3	41	22	[12, 14]
New	VA/NC	West	101	1999	85	[14, 15, current]
Yadkin	VA/NC	East	37	691	58	[8, 12, 15, current]
Holston	VA/TN	West	24	320	91	[8, 11, 14, current]
Nolichucky	NC/TN	West	51	1058	64	[7, 8, 11]
French Broad	NC/TN	West	80	1281	73	[8, 11, 16]
Little Tennessee	NC/TN	West	49	886	82	[8, 13]
Watauga	NC/TN	West	44	691	88	[8, 11]
Broad	NC	East	3	41	29	[8, 11]
Hiwassee	NC	West	6	146	76	[8, 11]
Cheoah	NC	West	10	210	80	[8, 11]
Little	TN	West	8	90	80	[8, 11]
Tellico	TN	West	5	64	42	[11]
Savannah	NC/GA	East	27	533	63	[10, 16]
Chattahoochee	GA	West	1	21	31	[10]
Tennessee	GA	West	7	145	93	[10]
Coosa	GA	West	1	12	100	[10]

[1]Relative to eastern continental divide.

[2]Allele frequency based on number of individuals analyzed per stream and averaged across all populations in each drainage.

Table 4. Genetic characterization of brook trout populations in regional river drainages, based on frequency of the diagnostic *CK-A2*100* allele using data gathered from all available published and unpublished studies.

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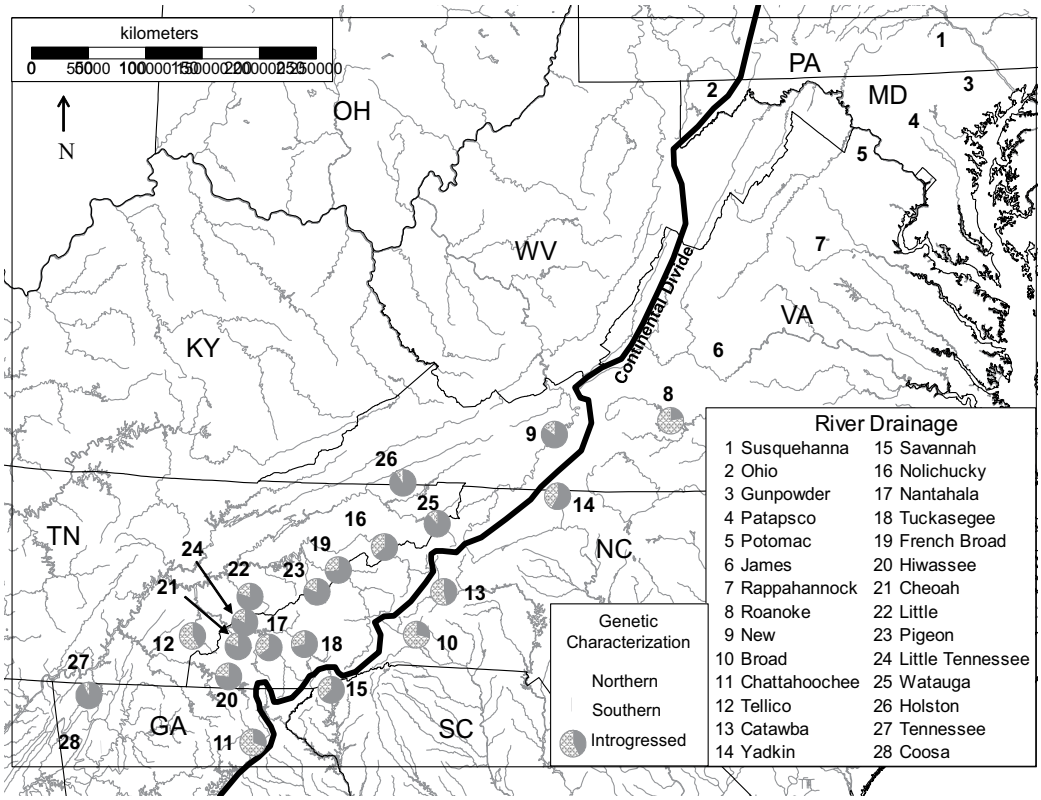

Figure 2. Genetic characterization of brook trout populations in major river drainages, based on the *CK-A2** locus, using data compiled from all known genetic studies of brook trout populations throughout the native range. See **Table 4** for details.

All river drainages north of the New River were characterized as pure northern, with the exception of the Roanoke River drainage that contained a single population with a low frequency of the southern allele, likely due to the transfer of individuals from another location or stream capture. The frequency of the southern allele in river drainages south of the New River ranges from 29% in the Broad River of North Carolina to 100% in the Coosa River of Georgia. Genetic characterization of individuals from 111 populations in the New River drainage showed an 85% frequency of the southern-form allele. **Figure 2** shows that the south/north break is sharp and that this break occurs at the New/Roanoke-James watershed divide. This weakens the hypothesis that the New River is a zone of natural intergradation between the southern and northern forms of brook trout, and supports the hypothesis that the presence of northern alleles is due to either stocking or stream capture. However, it is important to qualify this inference by noting that genetic characterization is based on variation at a single locus. Ongoing screening of New River populations using microsatellite DNA markers will provide further insights into patterns of population genetic differentiation, shedding light on the native character of New River brook trout populations. In particular, microsatellite variation may clarify whether northern alleles observed in populations examined are characteristic of particular hatchery stocks or of native regional variation.

4.5. Management implications

Brook trout is the only salmonid native to the southern Appalachian region. The American Fisheries Society Southern Division Trout Committee developed a position statement [22] expressing the importance of SABT and presenting recommendations for conservation-oriented management of this regional resource. Our results contribute to the recommended completion of genetic inventory of critical populations using non-lethal sampling methods. In this context, we frame the management implications for management of SABT populations.

Results from this and other studies demonstrate that stocking of non-native genotypes poses long-term genetic impacts and interferes with efforts to conserve southern Appalachian brook trout. Although negative effects of stocking have become well known, some fisheries management agencies maintain imprecise stocking records. Further, hatchery personnel often substitute one brook trout stock for another based on availability. We recommend that all stocking and transfers of brook trout be well planned with cognizance of genetic conservation objectives and thoroughly and accurately documented.

Management units—that is, populations that are demographically independent of one another—may be defined functionally as populations that have substantially divergent allele frequencies at many loci [47]. We had but limited ability to estimate levels of genetic diversity and differentiation among regional brook trout populations using allozyme markers. The results of ongoing screening of microsatellite DNA markers will be used to quantify differentiation among native populations, providing the basis for defining defensible management units. Results to date support the view that southern Appalachian brook trout populations should be managed on a stream-by-stream basis.

Those populations characterized as pure SABT should be given conservation priority. The stocking and transfer of non-native genotypes into these populations should be prohibited. Harvest should be allowed only in those populations that are demographically able to sustain themselves. We recommend that introgressed populations that contain less than 5% admixture from northern-strain brook trout be treated as 'pure' southern. However, we caution that the level of introgression in these populations may be higher than allozyme frequencies suggest; hence, individuals from these streams should not be transferred into streams that contain pure SABT populations. Hatchery brook trout should be stocked only into those streams that contain pure northern-strain populations and those with greater than 5% admixture.

We caution that any negative consequences of stocking also would apply to native northern-strain populations (i.e., in the James and Roanoke river drainages). Allozyme markers do not provide enough resolution to differentiate between native northern and hatchery populations, and so we recommend that all brook trout populations should be screened and characterized using microsatellite or single nucleotide polymorphism markers. Until we know more about the genetic composition of these populations, it may be wise to stock only infertile triploid brook trout [48].

Southern Appalachian brook trout hatchery stocks are being established in conservation-oriented hatchery programs ([49], https://brooktrouthatchery.wordpress.com/, http://archive.

knoxnews.com/news/aquarium-helping-to-restore-native-trout-ep-510367109-355447741.html). SABT can be stocked to re-establish populations in streams where they have been extirpated. Also, while we do not recommend eradicating non-native or introgressed populations in watersheds where brook trout are native, we recommend stocking southern-strain hatchery fish into these populations to shift allele frequencies toward those of native populations. Progress in re-establishing native brook trout populations should be monitored using genetic markers every few generations.

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Chapter 4

Alien Fish Species in France with Emphasis on the Recent Invasion of Gobies

Fabrice Teletchea and Jean-Nicolas Beisel

Additional information is available at the end of the chapter

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Abstract

Introduction of alien species constitutes worldwide one of the major threats to biodiversity, particularly in freshwater ecosystems. In France, the number of alien aquatic plant and animal species has increased exponentially over time in freshwater ecosystems and shows no sign of decreasing. For fish only, more than 40 alien species have been either voluntary or involuntary introduced in the past decades. About two-thirds are still present today and at least 26 are naturalized. As in many European countries, the fish introduction history in France switched from voluntary introduction in the nineteenth century (aquaculture, sport fishing, and management of ecosystems) to unintentional but human-aided introductions (aquarium trade and global ship transport). The negative impacts of alien species on native species and ecosystems are most often unknown in France and needs further studies to develop a functional policy on alien species introductions and the protection of aquatic ecosystems integrity. The information gathered allow discussing the possible reasons explaining whether an alien species is able or not to establish sustainable populations in France and thereafter became invasive, such as gobies recently arrived.

Keywords: inland waters, invasive species, gobies, climate change

1. Introduction

Introduction of alien species (also sometimes called non-native, transplanted, or exotic with a slightly different meaning, see **Table 1**) constitutes worldwide one of the major threats to biodiversity, with alteration or destruction of habitats, pollution, overexploitation, and climate change [1, 2]. In freshwater ecosystems, the introduction of fish is considered as a significant component of human-caused environmental changes [3]. The rate of introductions has strongly

Term	Definition
Non-native or foreign	Species not occurring naturally in a geographic area
Exotic	Species introduced from other biogeographic realms
Indigenous or native	A species occurring naturally in a specific geographical area without human intervention
Introduced population	Population that arrives at locations not normally achievable by that species, with intentional or accidental human assistance
Naturalized	Self-sustaining populations in the wild of a non-native species
Invasive	Non-native species that spread and cause significant ecological changes or cause severe economic losses
Translocated	Species that is transported from a region where it is native to another part within the same country

Table 1. Definitions of the main terms used in the literature on fish introduction (modified after [16, 18, 34].

increased in the past century [4–7]. In France, for instance, the number of alien aquatic plant and animal species has increased exponentially in freshwater ecosystems from less than 10 prior to the beginning of the nineteenth century up to 148 today and shows no sign of decreasing [7].

For fish, the main causes of introduction of alien species are aquaculture [3, 6, 8], commercial and recreational fisheries [9, 10], aquarium fish market [11], and management of aquatic ecosystems [3, 12]. Introductions could also result from a spread following the human modifications of hydrosystems, such as the construction of canals [3, 13] or dams [14] that allow species to disperse by their own means or transported by ship ballasts [3, 15]. Today, more than 600 freshwater fish species have been introduced into areas outside their native range globally [16], which resulted in that more than half of the river basins across the world host at least one alien fish species [17]. Among these 600 species, Toussaint et al. [18] found that 14 are present into at least one of the 1054 river basins studied in the 6 biogeographic realms defined by [19]: Afrotropical, Australian, Nearctic, Neotropical, Oriental, and Paleartic. Three species are present in all six realms: rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus carpio*), mosquitofish (*Gambusia affinis*), and three more, goldfish (*Carassius auratus*), sea trout (*Salmo trutta*), and Eastern mosquitofish (*Gambusia holbrooki*) in five realms [18]. These few alien species contribute the most to the global homogenization pattern, whereas most introduced species have low impact on the global change in dissimilarity, i.e., beta-diversity [18]. The authors thus concluded that focusing conservation efforts in controlling the spread of these few species may be more relevant to counteract the global homogenization trend [18].

Most often, exotic species freshly introduced are not able to survive in their new environment and it is generally considered that only a low percentage succeeds to establish sustainable populations and become invasive [1, 20]. However, García-Berthou et al. [21] found that the average percentage established of the 123 alien aquatic species into six European countries (United Kingdom, France, Spain, Sweden, Germany, and Italy) is 63% (167 of 264 introductions), much higher than the 5–20% suggested by Willamson's "tens" [21]. Once established, the eradication of a freshwater non-native species is almost impossible [17], which is a real problem because it is still today very difficult to prevent new introductions and to predict the success and effects

of invading species [20]. There is indeed no consensus about what makes a successful invader, even though some biological attributes have been proposed, among which a high environmental tolerance (e.g., eurythermal and euryhaline), a high genetic variability, a short generation span, a rapid growth, an early sexual maturity, a high reproductive capacity, and a broad diet [22]. Based on the analysis of 10 life-history traits between 13 non-native and 46 native freshwater fish species inhabiting the Central European biogeographical region, Grabowska and Przybylski [23] found that the former were significantly different from the latter. Non-native species tend to be small or medium in size, have a short longevity and mature early, a rather low fecundity but with large eggs, spawn at least twice each year over an extended reproductive season, and exhibit some form of parental care. MacDougall et al. [24] assume that the divergence in at least one biological attribute (on the basis that it results in a "fitness difference") can better explain invasion success than a particular suite of specific life-history attributes [23]. For instance, the gibel carp (*Carassius gibelio*) possess a unique reproductive attribute (the eggs can be activated by the sperm of other cyprinid species, allowing the production of progeny in the absence of conspecific males), which probably partly explain why it is one of the most successful invader in Poland and more generally in Central Europe [23]. More broadly, the theory of MacDougall et al. [24] belongs to a vein of interest related to the ecosystem naiveté, with at least two hypotheses that can be extended to freshwater fish even if the latter has been mostly tested for terrestrial plants. Ricciardi and Atkinson [25] proposed the phylogenetic distinctiveness hypothesis: larger impacts are caused by exotic species that add novel taxa to the community. The evolutionary naïveté hypothesis [26, 27] assumes that impact of exotic species depends on the recipient community's evolutionary experience with functionally similar species. Both hypotheses have originally been produced to explain the ecological impact of exotic species but can also be used to investigate their success in terms of establishment.

The success or failure of an alien species relies probably partly on its biological attributes (or on one or a few specific attributes) but also depends on the recipient ecosystem characteristics, including both biotic and abiotic factors [20, 28, 29]. All aquatic ecosystems seem potentially colonizable even though some might be more susceptible to invasion: simple systems (i.e., with rather low native species richness) or complex systems (i.e., species-rich communities) [17], geographically and historically isolated environments (e.g., islands), disturbed or anthropogenic habitats, or regions where no co-adapted foes, including competitors, predators, parasites, or diseases are present [30]. At last, the human-mediated propagule pressure (the number of individuals introduced as well as the frequency of introductions in a given area) is positively correlated with the establishment of alien species [1, 17]. Commensalism with human activity has also been found as one of the most consistent attribute of the success of invasive species [22, 30].

Most alien fish species have generally low impacts on native species and ecosystems [1], but high-impact invaders comprise at least 10% of the total number of invaders [31]. The main consequences of alien species on native species and ecosystems are varying and non-exclusive: hybridization, predation, competition, extirpation, dissemination of diseases and parasites, habitat change, and food web alteration [3, 12, 15, 32, 33]. In metropolitan France, more than one-third of the freshwater fish species are alien [7, 34]. Even though none has yet been documented in France as the cause of native species extinction, several of them are well known as causing major ecosystem disturbances.

The aim of the present chapter is first to reassess the knowledge acquired on alien fish species in France in the past years, and second to focus on the recent invasions of gobies in the north-east of the country [35] to dissect possible factors enhancing successful invasion.

2. Alien species in Europe and France

Several atlas and field guides have been written on the European fish fauna, among which the last and most completed was published 10 years ago [36]. It appears that this fauna is one of the poorest across the world as these authors only recognized 579 species in European freshwaters west of the Urals, particularly in comparison with the 13,000 freshwater fish species described in the world [19]. Among these 579 species, 33 have been introduced from regions outside Europe (North America and Asia in a large majority [37]), of which 28 are established [36]. Besides, much more species have been moved between European countries [34]. Consequently, Leprieur et al. [17] have highlighted that western and southern Europe regions are among the six areas where introduced species represent more than a quarter of all species. A database developed from the project DAISIE (Delivering Alien Invasive Species Inventories for Europe) was launched about a decade ago [38], and includes now more than 12,000 alien animals and plants in Europe (http://www.europe-aliens.org/). A total of 162 alien fish species (including diadromous) are listed in European freshwaters (an update of the 136 analyzed in [39]), among which 3: the round goby (*Neogobius melanostomus*), the pseudorasbora (*Pseudorasbora parva*), and the brook trout (*Salvelinus fontinalis*), are classified within the 100 of the worst alien species.

The French fish fauna has also been extensively studied in the past decades (e.g., [40–43]. Traditionally about 80 species were recognized [34], yet with the advent of the DNA barcoding and integrative taxonomy [44] several taxonomic revisions have been done: some new species have been described [45–47], others have been invalidated [48]. For instance, it was thought that the Northern pike (*Esox Lucius*) was the only species present in Europe, but recent integrative analyses based on both morphological and molecular characters concluded that three species are actually present in France, the Northern pike (which is the most common), *E. aquitanicus* (from the Charente to the Adour drainages), and *E. cisalpinus* (mostly in the Lake Geneva). Besides, more than 40 alien species have been either voluntary or involuntary introduced in the past decades in France [42]. Nearly one-third are no longer present in France (or their presence is very doubtful), among which several salmonids (*Oncorhynchus tschawytscha*, *O. kisutch*, and *Coregonus* spp.) and centrarchids (*Pomoxis* spp., *Lepomis* spp.) [42]. Among those that are still present today, most have established self-sustaining populations (**Table 2**); even though some occupied a very restricted area, such as the rock bass (*Ambloplites rupestris*) [49]. Few other alien species are probably not established, such as the rainbow trout *Oncorhynchus mykiss* or the grass carp *Ctenopharyngodon idella*. In total, the fish species richness has increased in the past decades [49] and there are today more than 100 species inhabiting France, which belong to 26 families. The two most speciose families are Cyprinidae (n = 40) and Salmonidae (n = 9), whereas 12 are monotypic, among which Gadidae [50].

The timing and reasons of introductions of alien species in France are, in general terms, similar to other European countries [3, 16, 34], such as in Belgium [51], Germany/Austria [52, 53],

Latin name	Date of first observation in France	Vectors of introduction	Native range	French name	References
Ambloplites rupestris	1904	Release: recreational fishing		Crapet des roches	[5, 41]
Ameirus melas	1871	Escape: from the "Museum National d'Histoire Naturelle"	North America	Poisson chat	[5, 40, 42]
Aspius aspius	1976	?		Aspe	[5, 40, 42]
Carassius auratus	Around 1750	Release: ornamental purposes		Carassin doré	[5, 40]
Carassius carassius	Around 1750	Release: aquaculture		Carassin commun	[5, 40]
Carassius gibelio	Around 1850	Release: aquaculture		Carassin argenté	[5]
Cobitis bilineata	Around 1995	Unintentionally introduced		Loche transalpine	[42]
Coregonus albula	1860	Release: recreational fishing		Petite marène	[5, 40]
Cyprinus carpio	Around 1250	Release: aquaculture		Carpe commune	[5, 42, 40]
Gambusia affinis	1924	Release: anti-mosquito biological control		Gambusie	[5, 42, 40]
Gambusia holbrooki	1924	Release: anti-mosquito biological control		Gambusie	[5, 42, 40]
Lepomis gibbosus	1977	Release: recreational fishing	North America	Perche soleil	[5, 42, 40]
Leuciscus idus	Around 1950	Dispersal: unintentionally introduced during stock enhancement		Ide mélanote	[5, 42, 40]
Micropterus salmoides	1890	Release: recreational fishing		Black-bass à grande bouche	[5, 42, 40]
Neogobius fluviatilis	2014	Dispersal: shipping	Ponto-caspian	Gobie fluviatile	Unpublished data
Ponticola kessleri	2011	Dispersal: shipping	Ponto-caspian	Gobie de Kessler	[35, 42]
Neogobius melanostomus	2011	Dispersal: shipping	Ponto-caspian	Gobie à taches noires	[35, 42]
Pachychilon pictum	Around 1980	?		Epirine lippue	[5, 42]
Proterorhinus semilunaris	2007	Dispersal: shipping	Ponto-caspian	Gobie demi-lune	[35, 42, 90]
Pseudorasbora parva	Around 1978	Escape: from aquaculture production unit		Pseudorasbora	[5, 42, 91]
Salvelinus fontinalis	1876	Release: recreational fishing		Omble de fontaine	[5, 42, 40]

Latin name	Date of first observation in France	Vectors of introduction	Native range	French name	References
Salvelinus namaycush	1886	Release: recreational fishing		Cristivomer	[5, 42, 40]
Sander lucioperca	Around 1880	Release: recreational fishing		Sandre	[40–42]
Silurus glanis	1857	?		Silure glane	[5, 40, 42, 92]
Umbra pygmea	1910	Release: aquaculture		Umbre pygmée	[5, 42, 93]
Vimba vimba	1989	Dispersal		Vimbe	[5, 42]

Silurus glanis is included in this table but its alien status remains questionable. Vectors of introductions have been classified into: Dispersal (range expansion by active or passive means from populations of neighboring countries. It includes accidental transport by human means), Escape (escaped from captivity) or Release (deliberately released into the wild)

Table 2. List of alien fish considered as naturalized in French inland waters.

Bulgaria [55], Poland [56], or Norway [57]. The first species that was introduced in France is the common carp *Cyprinus carpio* in roman times, followed by the goldfish *Carassius auratus* [41, 42]. Nevertheless, this is only during the second half of the nineteenth century that more frequent introductions occurred under the auspices of the Imperial Society of zoological acclimatization ("Société impériale zoologique d'acclimatation") [41, 42], which was established in 1855 [34]. Introductions were first motivated by research curiosity and to improve fish stocks for fishery. Introductions concerned exclusively European and North American fish, among which various salmonids and centrarchids [41, 42]. Then, new species were deliberately introduced to improve the fish market economy by diversifying the market of native species, for sport fishing, to act as biological control agents of algal blooms in eutrophic ecosystems, or to control mosquitoes [3]. More recently, because of stricter legislation and change in fisheries management practices away from stocking with non-natives [34], the main pathways for alien fish introduction are via either the ornamental trade and subsequent unintentional introduction [6, 11] or angling practices, such as for the asp (*Aspius aspius*), which is one of the three alien species that showed the most spectacular colonization in France during the past decades [49]. Besides, several introductions were accidental (e.g., during stocking events), which is probably the case for species not favored for fishing, such as *Pseudorasbora parva*, *Pachychilon pictum*, and pumpkinseed *Lepomis gibbosus* [49]. Other introductions result from natural species range expansion accelerated by several human activities and infrastructures, such as fluvial transport and artificial canals [56]. In conclusion, the attitude to the introduction of non-native fish have changed over time from efforts made to seek out and introduce new species actively to the protection of hydrosystems face from all new species [33, 34].

The negative impacts of alien species on native species and ecosystems, as for other countries [16], such as Belgium [51], Norway [57], Bulgaria [55], or Poland [56], are most often unknown in France and needs further studies [5, 41, 42] to develop a functional policy on alien species introductions and the protection of aquatic ecosystems integrity [51]. The recent European Union legislation addressing the problem of invasive alien species or IAS (EU Regulation No. 1143/2014)

identifies different types of intervention including prevention, early warning, and rapid response. It required member states to develop a list of invasive alien species of concern in addition to a list of Union concern (see EU 2016/1141 and 2017/1263). These lists are dynamic at the Member State and EU levels and need scientific evidences to identify and prioritize IAS of regional and indeed global concern. We still need a quantitative methodology to assess potential impacts of invasive fish even if some recent proposals have been done [58, 59].

2.1. Two alien fish species intentionally introduced but not acclimatized: the rainbow trout and grass carp

The rainbow trout (*Oncorhynchus mykiss*), which is a salmonid originating from the west coast of North America, was one of the first fish species to be domesticated and introduced globally: today it is present in more than 90 countries [60, 61]. In France, it was first introduced in the beginning of 1880 for angling [41]. Thereafter, with the control of artificial production, it has become one of the leading species in inland European aquaculture [62] and accounts for more than three-quarters of the French fish production [61]. Yet, rainbow trout is still considered non-established in France [49], and in most European countries, except in few Norwegian drainage basins where only six self-reproducing populations are confirmed by the mid-1990s [57], as well as in few Alpine rivers in Austria, Slovenia, Switzerland, and Italy [36]. The failure of rainbow trout to establish in most parts of Europe may to a large extent be caused by its susceptibility to whirling disease, a myxozoan parasite *Myxobolus cerebralis* [57]. Nevertheless, even though it is not established, it is still very common in France, because of escapees from aquaculture and intentional releases in lakes, rivers, and particularly private ponds for sport fishing [16, 51]. The impacts of rainbow trout on native species and ecosystems is poorly documented in France [5], but it is generally considered that this species do not show severe environmental impacts across Europe [3].

The grass carp (*Ctenopharyngodon idella*) is native from East Asia and was first introduced in France in the end of 1950s [41], similar to other European countries [34]. As rainbow trout and two Asian carps (silver carp *Hypophthalmichthys molitrix* and bighead carp *H. nobilis*), the grass carp does not breed in natural conditions in France because it requires very specific conditions that are not met in this country: large rivers with a strong current (1 m/s), important and rapid water level variations (1–2 m), water temperatures comprised between 20 and 25°C during several weeks in summer, and long unregulated water courses in which the pelagic eggs can incubate [63]. Yet, easy artificial reproduction has allowed this species to be spread in numerous countries [55], even though it is only very occasionally found in open waters in France [63] or Belgium [51]. However, it is present in numerous ponds across France [63]. The impact of grass carp is poorly document in France [41, 63]. Yet in other countries, it is considered that several parasites were transferred, which infested the common carp in Bulgaria [55] and Poland [56]. More generally, it is considered that grass carp can significantly influence native ecosystems because of their prevalence in some water bodies [56]. For instance, they are reported to destroy the spawning grounds of native phythophilous fish species through foraging on macrophytes, which could led to the decreased of fishing of some species in several lakes or depletion of wild fowl fauna, particularly those feeding on soft aquatic vegetation,

e.g., coot, *Fulica atra*, and swan, *Cygnus* sp. [56]. Nevertheless, it is important to mention that the use of grass carp has allowed in certain cases to effectively control macrophyte development while avoiding the use of more costly and environmentally unacceptable alternatives such as insecticides or herbicides [16].

These two examples illustrated what has occurred in the past in France (and more generally in Europe) to improve angling, aquaculture production, and management of ecosystems. Today, it would be a futile and potentially a controversial exercise to try to eradicate these two species and more generally already established alien fish species because of high expense, difficulty of success, and the likelihood of imposing substantial collateral damage [56, 64]. However, as the possible outcomes of introductions are still very poorly documented, the precautionary approach ("guilty until proven innocent") is most appropriate for dealing with new alien species introductions [3, 51]. Because, nowadays, aquaculture is the main pathway of initial introduction of new fish species in Europe [6], one possible way to decrease risks while increasing production [16] would be therefore to rely more on the production of local species with valuable qualities such as pikeperch (*Sander lucioperca*) or European perch (*Perca fluviatilis*) ([54], Teletchea et al. 2009).

2.2. One unwanted invasive species: *Pseudorasbora parva*

The topmouth gudgeon is a small cyprinid originating from East Asia, including Japan, the Korean section of the Amur River Basin, China (basins of the rivers Yangtze and Hoanghe), and Taiwan [65]. It was accidentally released in Europe in early 1960s with stocking material of Asian herbivorous cyprinids [51, 56, 65–67]. Then, because of both stocking and natural range expansion, it has rapidly spread across Europe [51, 56, 67] and more generally in numerous countries in the world, being now classified as a worldwide pest [66]. It still continues today to expand its range, and represent one of the most common alien species in France [49]. Similarly, following its introduction into lakes in the UK in 1996, populations appear to establish rapidly and become dominant in the fish community (often >97% by number) [64]. The reason for its success is its very high reproductive rate, which gives rise to dense populations of fish that compete with fry of other species [65]. Besides, this species is opportunist and has a wider ecological and physiological tolerance than many European fish species and can survive to a moderate degree of pollution, elevated temperatures, and low water levels. The ability to spawn on any smooth-surfaced object, such as branches, leaves, and artificial substrata, is another important factor likely to have contributed to the rapid dispersal of this species [65]. The impact of the topmouth gudgeon is poorly documented in France [42]. Yet, in other countries, it has been shown that it can compete for food with other species such as *Aphanius anatolie* and *Orthrias* sp. [65]. It was also described that populations of *Leucapius delineatus* decreased when topmouth gudgeon increased, the latter being a vector of a lethal pathogen for the former [49, 64, 65]. More generally, their high abundance provokes concerns of detrimental ecological impacts through, for example, high competition for resources such as food and spawning habitat, and they become a pest species to anglers [64]. The topmouth gudgeon is included in the list of exotic species of concern in the framework of European Union legislation addressing the problem of invasive alien species (EU Regulation No. 1143/2014).

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3. Recent invasion of gobies: why?

The recent, spectacular invasion of French hydrosystems by gobies is a good medium to discuss about what makes the success of an invasion. Since 2007, four freshwater Gobiidae species have been introduced in French hydrosystems: the tubenose goby (*Proterorhinus semilunaris*) in 2007, the bighead goby (*Ponticola kessleri*) in 2010, the round goby (*Neogobius melanostomus*) in 2011, and the monkey goby (*Neogobius fluviatilis*) in 2015. All that Ponto-Caspian species have moved in Europe with a contiguous East to West range expansion, with a spread from the Black Sea to the Rhine Delta observed as early as in the 1960s for the tubenose goby. Here, we focused on the round goby that reach locally high densities in many locations of the Upper Rhine and the Moselle River [35, 68]. This species has begun to spread in the 1990s [69]. It was observed for the first time in a downstream section of the Upper Rhine in Germany (between Düsseldorf and Cologne) in 2008, upstream of the confluence with the Neckar in 2010, in the French Upper Rhine (the Gambsheim fishway) in 2011 and in Basel harbor, 143 km upstream the Gambsheim fishway, in 2012 [35, 70]. Five years after its first observation, the round goby represented in several locations along the Upper Rhine more than 80% of the total catch by electrofishing (100 fishing points). The relative density of the round goby never fall below 25% of the total catch 1 year after its first observation, with a maximum value reaching 90% in a location dominated by rip-rap embankment [68]. This population dynamic is an amazing success that we dissected considering first the species bio/ecological traits and secondly the characteristics of its recipient ecosystems.

3.1. A profile of invader

Potential reasons for the proliferation of the round goby include (1) its reproductive success, (2) its singular behavior by comparison with native species, and (3) its tolerance to a wide range of physicochemical conditions. The fecundity per round goby female during a reproductive season ranges between some hundreds and a maximum of 5200 eggs that are divided in up to six spawns per year (unpublished results and values reported in [71, 72]). This number of eggs is not important by comparison with native species but the round goby exhibits two characteristics that make them prolific: multiple spawning combined to a protracted reproductive season and some forms of parental cares [73, 74]. The male occupies and defends a nest—an enclosed cavity—to which females are attracted to spawn adhesive eggs on the undersize of rocks [75–77]. In laboratory experiments conducted in Canada, up to three females were selected by a male and spawned sequentially in a nest [78], but field observations reported that up to 15 different females could enter a nest to spawn [76]. Inside the nest, eggs are regularly inspected by males and constantly ventilated using pectoral and caudal fins. In Europe, gobies are the most typical species of guarders—nest spawners according to the typology of parental investment recently used by [23] in a comparative study between exotic and native fish.

Gobies lack a swimbladder, which makes their positioning in the water column predominantly benthic. They stand at the bottom and are considered bad swimmers in that they cannot fight against an important current or make jumps. A consequence is that at any stage of their biological cycle the gobies need numerous shelter and hiding places in their environment.

Mineral structures (pebble, stones, and blocks) or macrophytes are useful habitats but in a given environment they would be more frequent in hard substrates as typically rip-rap embankments [79–81]. Few other native species, such as the European bullhead (*Cottus gobio*), the freshwater blenny (*Salaria fluviatilis*) or the ruffe (*Gymnocephalus cernua*), have these characteristics [43, 82]. *Neogobius melanostomus* inhabits a wide range of temperate freshwater and brackish-water ecosystems [72]. It has also demonstrated its capacity to adapt to local conditions in terms of prey availability [72]. This species exhibits a wide thermal tolerance, ranging from −1 to 30°C, but its energetic optimum temperature is estimated to be 26°C [72]. They would also be fairly little sensitive to pollutions. The distinguishing ecological features of the round goby by comparison with native ones make them singular in the range of bio/ecological profiles of species in place, a distinctiveness that could promote its success [25].

3.2. Hydrosystems prone to invasions

There are multiple potential and non-exclusive hypotheses to explain the gobie's success from the hydrosystem point of view. Among these, we emphasize the ideas (1) that the environments are not saturated in species and (2) that the rivers were man-modified in a way favoring the installation of exotic species.

The environments invaded by exotic species are not saturated in species for two main reasons. First, they correspond to hydrosystems that were largely defaunated during the Würm glaciation (80,000–10,000 BP). At the end of this period, the Rhine basin was recolonized by fish species from refuge areas that were outside ice range extension [83]. This recolonization by natural process takes a long time in the Rhine River considering the isolation of this basin and its geographical orientation with the downstream part to the North. The process was artificially accelerated these last centuries by human-aided introduction and the opening of the hydrographic basin with canals. Nowadays, the Upper Rhine is the main navigating way in Europe with two-third of goods transported on that fluvial road (330 millions of tons per year). Man activities allowed species from refuge area during the last glaciation, in particular the Ponto-Caspian area, to reach this unsaturated ecosystem. The Rhine has hence become the main entrance point for the dispersal of many invasive aquatic animal species in France over recent decades [7]. Another reason why ecosystems are not saturated in species is that pollution and human activities have profoundly modified natural communities, leaving vacant ecological niches within the hydrosystem. The decline of the Atlantic salmon (*Salmo salar*) or the European eel (*Anguilla anguilla*) in French inland waters are for example well documented. In conclusion, the ecosystems are not saturated because the post-glaciation process of recolonization is not achieved and several native species in place have already declined.

Second, the river stretches that served as entrance point in French hydrosystems are highly modified in terms of structure, quality, and functioning. The alteration of their habitats has placed the native species in a situation of anachronism: they are un-adapted to their own natural environment. The changes of habitats were too fast since the nineteenth century to allow a real adaptive response from species in place. Most of them disappeared, and the others

can have a declining level of competition. The resulting consequence is that the remained native species in place is not efficient to compete with some euryecious and prolific species. Furthermore, the rule of biotic factors in the success of gobies can be explained from a theoretical point of view by the invasional meltdown [84] and the enemy release hypotheses [85, 86, 87]. To be explained, the invasional meltdown can be drawn schematically. The propagule pressure received by a navigated and highly modified hydrosystem, such as the Rhine, is so important that a first exotic species always finished successfully. This one became a factor favoring a second exotic species, for example, because it will decrease the pressure of a potential predator. The two exotic species can then pave the way for a third exotic species and so on. This concept could probably be applied to the round goby in that it was preceded by the invasion of crustaceans and molluscs fed massively by this fish [88]. The enemy release hypothesis assumes the advantage of the loss of the original parasite burden of an invader. A recent study [89] revealed that 3 years after its first observation, the round goby hosted only one macroparasite in the French Upper Rhine, whereas in all other locations along its invasive pathway or its native range a minimum of three macroparasites were reported. This is typically an example of the enemy release an introduced species can benefit at least at the beginning of the invasion process.

4. Conclusions

The main goal of the present chapter is to give an update picture of alien fish species in France and their fate in the past decades. We dissected how the fish introduction history in France switched from voluntary introduction in the nineteenth century to unintentional but human-aided introductions (aquarium trade and global ship transport). The 28 alien fish established represent one-third of the fish species in France and >25% of the European exotic fish. Four species of our list are included among the 100 worst invasive species of Europe (DAISIE) and three others among the 100 worst invasive species of the world (IUCN). The information gathered will allow discussing the possible reasons explaining whether an alien species is able or not to establish sustainable populations in France and thereafter became invasive, such as gobies. Now and in the near future, natural resource managers have no other choice than to deal with them because no invasive fish have spontaneously collapsed up to a local extinction in France.

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Chapter 5

Ice Age Terrestrial and Freshwater Gastropod Refugia in the Carpathian Basin, Central Europe

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Abstract

Thanks to its unique microclimatic, geomorphological , hydrological conditions forming a mosaic-like environment present at all scales, numerous Late Tertiary and Pleistocene warmth-loving gastropod taxa managed to find refugee within the Carpathian Basin during the major-minor cold spells of the Ice Age. This complex system of refugia have been continuously functioning and evolving since the Late Tertiary through the entire Pleistocene and the Holocene. To understand the spatial and temporal evolution of refugia, detailed paleoecological investigations have been implemented, results of which are summed here. The high-grade fractal-like complexity of the environment led to the emergence of a so-called dual refugia, which is a unique feature of the Carpathian Basin. This temporally parallel but spatially differing presence of habitats for taxa of contrasting ecological needs was noted for paleotemperature gradients and temperate woodland and steppe habitat types as well. Furthermore, detailed geological and paleoecological analysis of a small Pleistocene hot-spring fed pond revealed information about the evolution of endemic thermophylous freshwater gastropod taxa within this microrefugia. This chapter is aimed to give an overview of the nature, evolution of temperate terrestrial and freshwater gastropod refugia present in the Carpathian Basin during the Ice Age.

Keywords: gastropods, refugia, evolution of mollusk biota, ice age, Carpathian Basin, Central Europe

1. Introduction

Embraced by the rugged peaks of the Carpathians, Alps, and Dinaride Mts lies the Carpathian Basin covering an area of ca. 300,000 km^2 at the boundary of Central and Southeast Europe

(**Figure 1**). During the ice ages, glaciers were strictly restricted to the Alps with only sporadic occurrences in the adjacent Carpathians leaving the entire basin ice-free [1]. Moreover, permafrost was restricted to the northern rim alone [2–4]. Numerous scientific postulations emerged from as early as the late nineteenth and early twentieth centuries, according to which several Late Tertiary and Pleistocene warmth-loving (thermophilous) gastropods could have found refugee within the Carpathian Basin during the major-minor cold spells of the ice age. This proposed system of refugia must have been continuously functioning and evolving from the Late Tertiary through the entire Pleistocene up to the Holocene. Most of these postulations were made about a single member of a gastropod family, whose representatives were widespread in Europe during the Tertiary: the Melanopsidae. This taxon was first reported during the mid-nineteenth century by the Austrian geologist, von Bregrath Franz Ritter Hauer, from a hot-water spring-fed thermal lake (Lake St. Ladislaus, Püspökfürdő) (**Figure 2**) found near the city of Nagyvárad (today Oradea) [5]. It must be noted though that the German natural scientist Phillippi who gave the first taxonomic description of the gastropod taxon *Melanopsis parreyssii* in his seminal volume from 1847 [6] (page 176 and Table 4, Figure 15) mentions Hungary as the type locality and the Austrian naturalist von Mühlfeld as the collector. Unfortunately, not a single word is given about the exact locality or the time and area of von Mühlfeld's visit. No further details on how the specimen described were attained.

Although there is no exact way to prove that the referred specimen was collected from Püspökfürdő (Baia Mai 1, Romania today), this assumption cannot be fully refuted either, as modern occurrences of this taxon are strictly restricted to a single locality, the referred thermal lake, with no others known. The first detailed geological description of the lacustrine deposits and the embedded Melanopsis taxa comes from the Austrian geologist von Heinrich Wolf [7].

His profile, located at the lakeshore, starts with an embryonic humus layer [7]. The lack of a map displaying the exact location, orientation of his work, as well as the environmental changes of the past centuries hampered the later comparative use of his first description. Yet his work is by no means futile as the major geological units identified and described by him were clearly traceable in later works on the lacustrine deposits as well. So, the referred gastropod taxa appear in later publications from the late nineteenth century as well [8]. Despite the promising start, the first detailed presentation on the Quaternary mollusk fauna of Püspökfürdő, including morphological changes observed on the shells of Melanopsidae, was given only in 1890 by the Hungarian amateur naturalist Mihály Tóth on the 25th Congress of Hungarian Medical Doctors and Naturalists held in Nagyvárad (Oradea) [9]. He presented a series of mollusk shells collected via singling from a 2-m-deep profile. In addition, based on the observed variations, he lined up an evolutionary series with numerous members. In his view, the lake harboring the *Melanopsis* taxa is older than the so-called Old Alluvial (presently known as the Holocene epoch). Thus, these gastropods must derive from a Tertiary ancestor, whose descendants managed to survive the cold periods of the Diluvial (Pleistocene epoch). His work is the first presentation of the referred ice age refugia hypothesis in Central Europe. Considering the formerly accepted relatively short time

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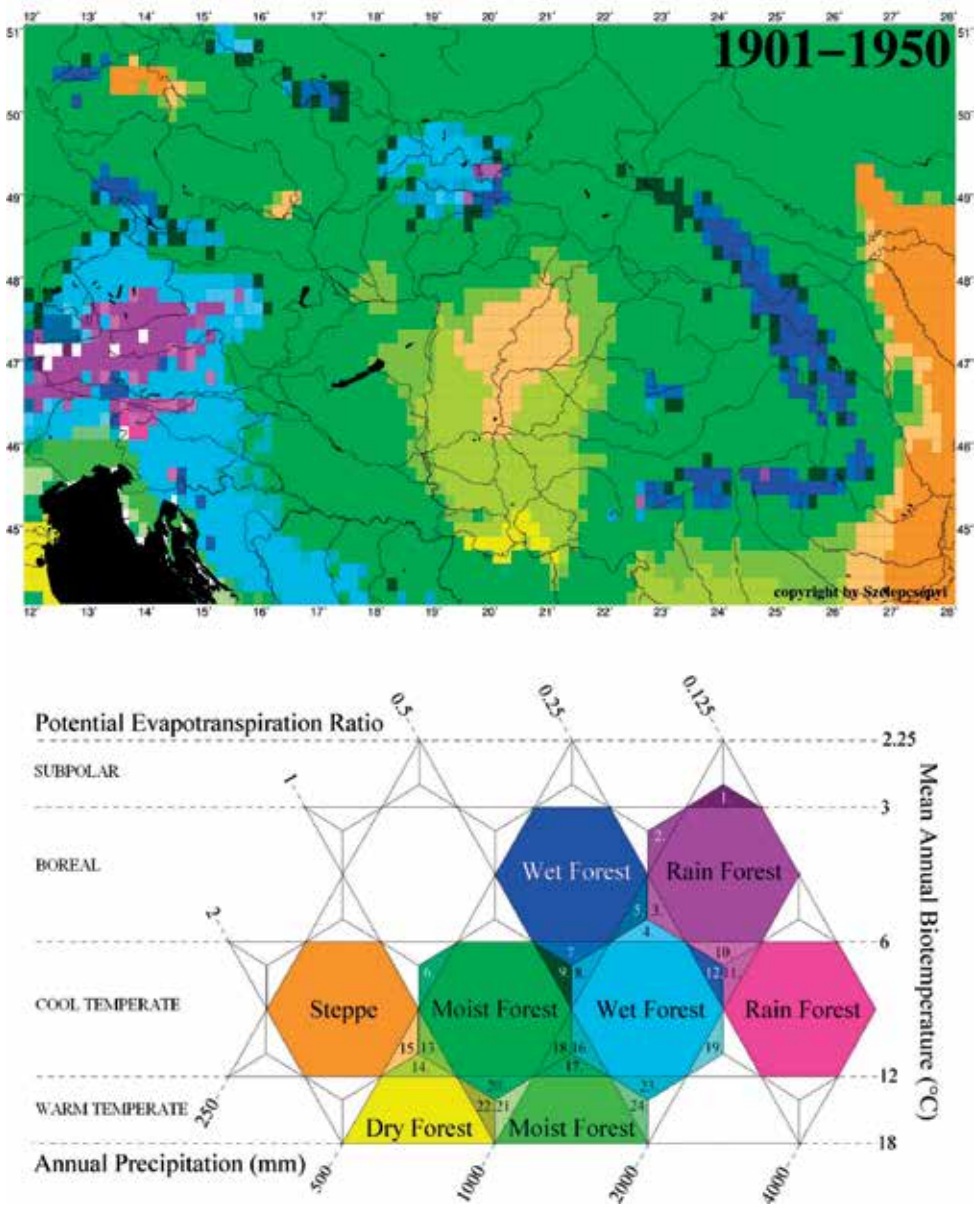

Figure 1. Location of the sites mentioned in this study on the Holdridge-type bioclimatological map of Szelepcsényi et al. [69]: (1) Lake St. Ladislaus, Püspökfürdő-Nagyvárad (Baia Mai 1, Oradea, Romania); (2) Petény rock shelter (Bükk Mts, Hungary); (3) Rejtek rock shelter (Bükk Mts, Hungary); (4) protected marshland, Bátorliget (Nyírség, Hungary); (5) Selyemrét, Ócsa (Danube-Tisza interfluve, Hungary); and (6) brickyard (Crvenka, Serbia).

span of the Pleistocene at ca. 600 Ky [10–11] compared to the modern known value of 2.58 My [12–16], his postulation is rather remarkable considering our modern understanding of speciation and macroevolution.

Figure 2. View of thermal Lake St. Ladislaus near Nagyvárad-Püspökfürdő (Oradea, Bai 1 Mai, Romania) in 1999 preceding its complete desiccation.

Tóth's work gave a major impetus to further detailed malacological studies on the mollusk fauna of the lake in the forthcoming decades involving the two internationally known malacologists of the Austro-Hungarian Monarchy: the Croatian Spiridon Brusina and the Hungarian Tivadar Kormos as well [17–21]. Their work included the genera *Melanopsis* and *Theodoxus* as well. Brusina examined the shells collected by Tóth, in addition to the ones collected by himself from the surficial deposits alone [17]. No profiles or boreholes were deepened to yield him stratigraphically reliable data and samples, which had serious consequences leading to erroneous observations and statements in the future. In his work Brusina identified eight different taxa of the *Melanopsis* genus, including the first described *Melanopsis parreyssii*. In addition, 23 varieties were mentioned. Unfortunately, his work is lacking a detailed taxonomic description and figures, having only a short diagnosis of each taxa in two or three sentences [17]. Brusina, based on his readings of the works of a paleobotanist Móric Straub [22, 23] describing a special waterlily from the thermal lake, considered the mollusks as representatives of a remnant Subtropical Tertiary oasis. Tivadar Kormos on the other hand prepared a new 11-m-deep composite profile near the

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one by Tóth after careful consideration of the geology and geomorphology of the area and the available publications [8–9]. Kormos gave a detailed description of the stratigraphy and geology in addition to the identified *Theodoxus* and *Melanopsis* taxa including the ones described by Brusina [18–21]. What is more a geological compilation of regionally available boreholes is also given along with maps displaying the exact location of these including his own and Tóth's profile as well. Thus, Kormos' seminal work could have been considered as a reliable foundation for our future research. The most important bullet points of his work are:

- Taxa of the genera *Melanopsis* and *Theodoxus* (*Neritina)* identified in the lacustrine deposits must have a Tertiary common ancestor. In his view, this ancestor must be found in the Late Tertiary mollusk fauna of the southern parts of the Carpathian Basin.
- Some taxa of the genus *Melanopsis* from Lake St. Ladislaus have clear evolutionary relationship with certain taxa of the genus *Fagotia* (considered as *Melanopsis* by him), especially the ones of *Fagotia acicularis* and *Fagotia esperi* [20]. These latter two taxa now known as *Esperiana* (*Microcolpia*) *daudebartii acicularis* (Férussac, 1823) and *Esperiana* (*Fagotia*) *esperi* (Férussac, 1823) are considered to be Pontian from a biogeographical point of view by certain researchers [24]. However, cyclical recurrence of these taxa in the malacofauna of the Carpathian Basin is connected to the warmer periods of the Pleistocene, that is, interglacials, and by no means to the Neogene Period (Tertiary) [25, 26].

It must be emphasized though that the presence of a special waterlily *Nymphaea lotus thermalis* in Lake St. Ladislaus leads numerous botanists to the conclusion that the area is a true subtropical relict of a former geological period [22, 23, 27, 28] despite a clear objection of other botanists, who were highly skeptic regarding the origin and age of immigration of the referred plant taxon [29, 30]. So Kormos simply adopted the generally accepted scientific notion of his era along with another famous malacologist studying the modern mollusk fauna of the region: Lajos Soós [31, 32].

WWI brought a sudden halt to malacological investigations. The only exception is perhaps an article published by Pauca [33] following the 1933 conservation of the site. Pauca kept only two taxa *Melanopsis parreissi* and *Melanopsis sikorai* from the ones listed by the former researchers providing highly questionable justification for his choice. In addition, no reason was given why these two were considered to be relict taxa from the Late Tertiary. His views were systematically adopted in later studies on the Romanian mollusk fauna [34] as well as the malacofauna of the region [24, 35] despite Diaconesa and Popa's works from the 1960s [36, 37], who clearly justified a Holocene timing of waterlily the invasion into the thermal lake. The origin and evolution of the Melanopsidae of Lake St. Ladislaus are far from settled and are a constant subject of scientific debates [38–42]. Yet, as it is an important hallmark in the question of Carpathian Basin refugia, a brief discussion based on our latest findings is presented in the next chapter.

WWI meant an end not only to malacological research done on the unique Melanopsidae of Lake St. Ladislaus but on the question of refugia in Hungary and Central Europe as well. Not

long after, however, studies implemented on the woodland mollusk fauna of a species-rich marshland near Bátorliget, NE Hungary initiated another debate regarding a potential refugia for temperate mollusk taxa in the basin during the ice age [43, 44]. Evidence for the survival of temperate woodlands in the Carpathian Basin during the Pleistocene was first presented between 1956 and 1969 by the paleobotanist József Stieber based on his detailed anthraconomical studies of wood remains deriving from cave sites in the Bükk Mts and loess/paleosol profiles from different parts of Hungary [45–48]. Thirty years after Stieber's seminal work, members of a British-Hungarian research group managed to independently corroborate the idea of Stieber on the presence of temperate woodland refugia in the Carpathian Basin [49–56]. Besides the presence of woodland refugia, ice age refugia for several temperate grassland elements have also been recently identified [57–61]. Detailed malacological studies starting from the 1980s and the accompanying reconstruction of Late Glacial and Holocene vegetation changes have brought the question of ice age refugia into focus again [54, 55, 62–65]. Complementing comprehensive paleoecological investigations of loess/paleosol sequences from various parts of the basin using numerous biotic proxies (mollusks, phytoliths, alkanes) clearly highlighted the presence of temperate grassland refugia in the southern parts of the Great Hungarian Plain and its wider surroundings [53, 54, 66–70]. In the following chapters, a short overview of the results of these works is also presented in addition to those of freshwater and woodland mollusk refugia.

2. Ice age refugia in hot-water springs and lakes: Lake saint Ladislaus, Nagyvárad-Püspökfürdő (Lake Petea, Oradea region)

In 1999, following the works of Kormos [18–21] and Tóth [9], a new 2-m-deep geological profile was created in the littoral part of Lake St. Ladislaus adjacently to the one of Tóth by members of our Geoarcheological and Quaternary Paleoecological research group. In addition, numerous undisturbed cores were also taken to delineate the spatial distribution of the identified stratigraphic horizons. Another 8.4-m-deep profile was dug next to that of Kormos in 2012 as part of a bilateral Romanian-Hungarian project, this time in the fully dried-up lakebed. The first geological profile spans the interval from the Late Glacial, while the 2012 one dates back to the last glacial maximum (LGM) of the last glacial.

Lithological observations made on the shorter profile are fully congruent with the one made by Tóth [9]. The profile starts with coarse silt-rich clayey lacustrine silts followed by lacustrine carbonate muds ending in highly altered peats representing the final desiccation of the lakebed. The stratigraphy of the longer, more recently established profile is much more complex recording several sedimentary changes. Nevertheless, the ice age part here is likewise represented by clayey, fine-silty coarse silts similarly to the bottom part of the shorter profile. A significant increase in the carbonate content is recorded only from the Late Glacial part of the profile, which also means that the more complex lithology is restricted to the upper Holocene parts. Samples from both profiles are fossiliferous yielding representatives of the

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gastropod taxon *Melanopsis*. However, for our final evaluation, the material of the longer profile was used. Here a larger volume of samples (ca. 30 l) yielded several thousand shells yielding better representativity of the original mollusk fauna. The forms and varieties described by Tóth [9], Brusina [17], and Kormos [18–21] were all present in the studied samples from both profiles [38, 39]. The identified taxa were further investigated using X-ray photographs of the shells (**Figure 3**) as this way even minor morphological and size differences could have been noted as well.

After a meticulous study of shell variations from different parts of the profile, a clear evolutionary lineage could have been outlined. In the layers corresponding to the LGM as well as the Heinrich 1 event [71–73], smooth shelled forms prevailed, displaying a close affinity to the taxon *Fagotia acicularis* [*Esperiana* (*Microcolpia*) *daudebartii acicularis*]. Other morphological varieties or taxa were clearly missing from these horizons. This horizon and its dominant taxon were missed in the works of Neubauer and his colleagues [41, 42], because their analysis was restricted to museum specimens representing those of the surficial collections made by Brusina [17] and the 2-m-deep profile of Tóth [9]. So the projection of their findings to our 8.4-m-deep profile [38, 39] is highly misleading, similarly to their delineation of the Pleistocene–Holocene boundary [41]. According to the recorded 14C age of a charcoal piece from the depths of 596–600 cm in our profile (10,789–11,185 cal BP years), the majority of the profile can be dated to the Holocene. The Pleistocene/Holocene boundary could have

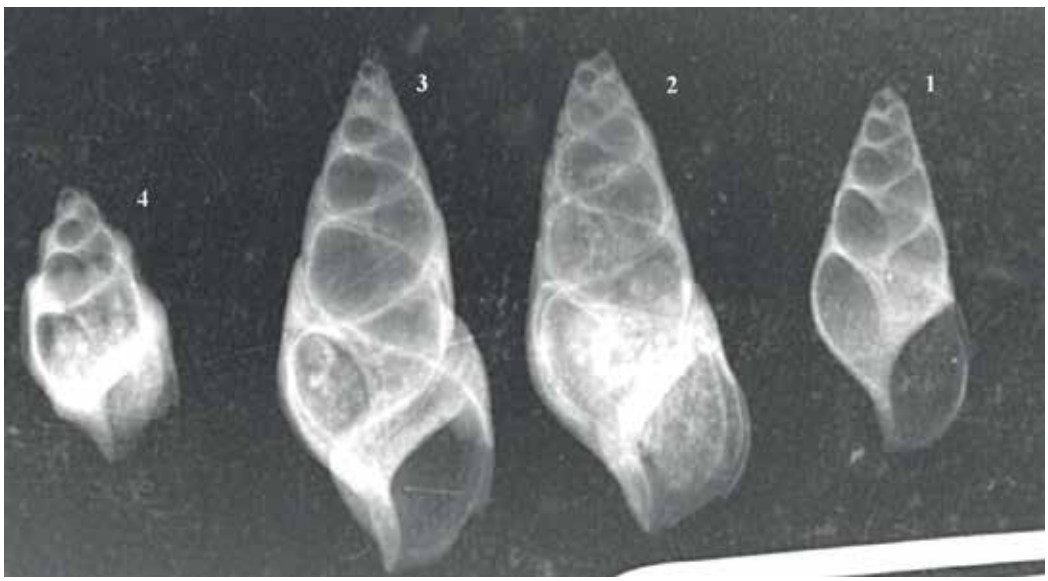

Figure 3. X-ray photographs of shells of various *Esperiana* (Microcolpia) taxa identified from Lake St. Ladislaus, Nagyvárad-Püspökfürdő (Oradea, Baia 1 Mai, Romania): (1) *Esperiana* (*Microcolpia*) *daudebartii acicularis* [*Fagotia acicularis*] (Férussac, 1823), (2) *Microcolpia parreyssii sikorai* (Brusina, 1903), (3) *Esperiana daudebartii daudebartii* (Prevost, 1821) [*Esperiana daudebartii acicularis F. thermalis*], and (4) *Microcolpia parreyssii parreyssii* (Philippi [6]).

been placed between 600 and 620 cm in our 8.4-m-deep profile. So, during the LGM, forms of the *Melanopsis* taxa displaying a close affinity to *Fagotia acicularis* [*Esperiana (Microcolpia) daudebartii acicularis*] were present. A longer type of this taxon with a thicker shell turns up in the Late Glacial part of the profile termed as *Fagotia acicularis F. thermalis* besides another smaller type which was described as *Melanopsis hazayi* by Brusina [17]. In addition, several other forms, taken to represent individual taxa by researchers during the late nineteenth and early twentieth centuries, turn up here as well (*sublanceolata, szontaghi*, *mucronifera*, *tothi*, *hazayi*, *staubi*, *franciscae*, *vidovici*, and *hazayi*) [9, 17, 18–21]. The appearance of these various new forms is clearly connected to a major environmental change noticed in a sudden increase in the carbonate content and water-soluble Ca and Mg content of the samples. This marked change in the geochemistry and the lithological character was so strong that it must signal a significant increase in the water temperature of the hot spring and the lake during the Late Glacial following the H1 event compared to temperatures characteristic for the LGM. It is also the horizon, where the first smooth, keeled, and shouldered shell forms of *Melanopsis* are recorded, though subordinately. These smooth, keeled, and shouldered shells having spiral striae running parallel with the suture are similar to the ones of the taxon *Melanopsis sikorai* (Brusina [17]). On the basis of these observations, we can postulate that the ecophenotypes leading to the evolution of the endemic taxon *M. parreyssi* inhabiting the modern lake must have emerged even during the Late Glacial. The opening of the Holocene marked the appearance of further sculpted, shouldered forms (*M. sublanceolata*, *M. staubi*), but the Late Glacial ecophenotypes were also preserved. The Early and Middle Holocene are characterized by an outstanding variety of shell forms. The Holocene also marks the disappearance of the smooth forms displaying close affinity to *Fagotia acicularis* [*Esperiana* (*Microcolpia*) *daudebartii acicularis*] including the taxa determined as *M. hazayi* and *Fagotia acicularis F. thermalis*. The Late Holocene corresponding to the last 2000 years is characterized by an increase in the organic content of the lacustrine deposits marking the transition to a marshland at the end of the Iron Age. By this time the presence of ribbed and shouldered forms can be attested representing the so-called *Melanopsis hungarica* assemblage (*M. hungarica*, *M. themaki*). It is also the time when the first representatives of the modern endemic taxon *Microcolpia* (*Melanopsis*) *parreyssii parreyssii* (Phillipi [6]) turn up. This endemic taxon represents the final member of an evolutionary lineage starting from the Late Pleistocene ancestor *Esperiana* (*Microcolpia*) *daudebartii acicularis* through various Late Glacial and Holocene ecophenotypes. Findings of detailed genetic analysis have independently corroborated our assumptions regarding the evolution of this taxon [74].

The final part of the story is rather heartbreaking as this unique Middle Pleistocene refugee forming a cradle for various endemic gastropod taxa, including *Theodoxus prevostianus*, is bound to fully disappear in a couple of years. The last members of the endemic *Microcolpia* (*Melanopsis*) *parreyssii parreyssii* (Phillipi [6]) are living among artificial conditions in the aquarium of a Hungarian research institute thanks to the quick response of dedicated zoologists to the human-induced desiccation of the lakebed of Lake St. Ladislaus. Although there have been measures taken to restore the previous conditions, the newly created artificial thermal lake system may not fully be capable to fulfill its preservational role as refugia in the future. Yet our records have clearly pointed to the importance of hot-water spring-fed

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thermal lakes in the preservation of mollusks even during the coldest periods of the ice age in the area of the Carpathian Basin. As several such systems are known from various parts of the basin as well as the transitional zone to the Carpathian Mts, one can presume that these also could have a significant role in the survival of warmth-loving mollusk taxa during the cold spells of the ice age.

3. Ice age woodland refugia in highlands between the elevations of 500–700 m ASL: Petény cave and Rejtek 1 rock shelter, Bükk Mts, NE Hungary

Excavations starting in the 1950s as part of a collaborative work of vertebrate paleontologists, anthracologists, and archeologists in two cave systems found in the Bükk Mts in NE Hungary yielded outstanding records regarding the presence of ice age woodland refugia in a highland setting of the Carpathian Basin [45–48, 75–78]. One of the studied caves was formed in Triassic limestone at an elevation of 735 m ASL (Petény Cave) (**Figure 1**). The other, the Rejtek rock shelter, found at an elevation of 500 m ASL was formed in Jurassic limestone. Both cave systems are facing south. According to the retrieved vertebrate fauna and recovered archeology, sediment accumulation must have initiated from the terminal part of the last glacial in these karstic depressions. Fine-stratigraphic sampling of the identified lithological horizons yielded numerous charcoal pieces. The taxonomic composition of these charred plant remains indicated the emergence of mixed taiga woodland at this elevation during the last stage of the last glacial [45–48]. Besides the clear dominance of spruce and Scots pine, scattered patches of deciduous elements like elm, oak, lime, maple, beech, ash, as well as hazel were also present in these woodlands. On the basis of this information, József Stieber came up with the idea regarding the presence of ice age woodland refugia for temperate thermomesophylous trees in the Carpathian Basin for the first time [45–48]. Unfortunately, his observations were by no means welcomed by the majority of Hungarian botanists of his time. It was only in 1999 when a British-Hungarian research group managed to corroborate his presumption independently [49–52, 55, 56]. The samples taken by Stieber and his colleague Dénes Jánossy yielded numerous terrestrial mollusk shells, which were handed over for further evaluation to the late Endre Krolopp, an outstanding Quaternary malacologist of the late twentieth century. The malacological remains of both caves have been scientifically evaluated along with a revision of the charcoal remains complemented by 14C dating of the major stratigraphic units sampled in the 1950s [55, 56]. According to the results of these investigations, Central European woodland mollusk elements prevailed in this area from even the last glacial onward (**Figure 4**).

The prevailing taxon of the Late Glacial horizon is *Cochlodina cerata*. The modern distribution of this taxon's habitat is found in the area of the Northern, Northeastern Carpathian Mts ranging from the lower alpine woodlands of the foothills to the upper alpine woodlands to a height of ca. 1000 m [79–84]. Consequently, the studied region in the Bükk Mts, NE Hungary, between the elevations of 500–750 m ASL must have acted as an ice age

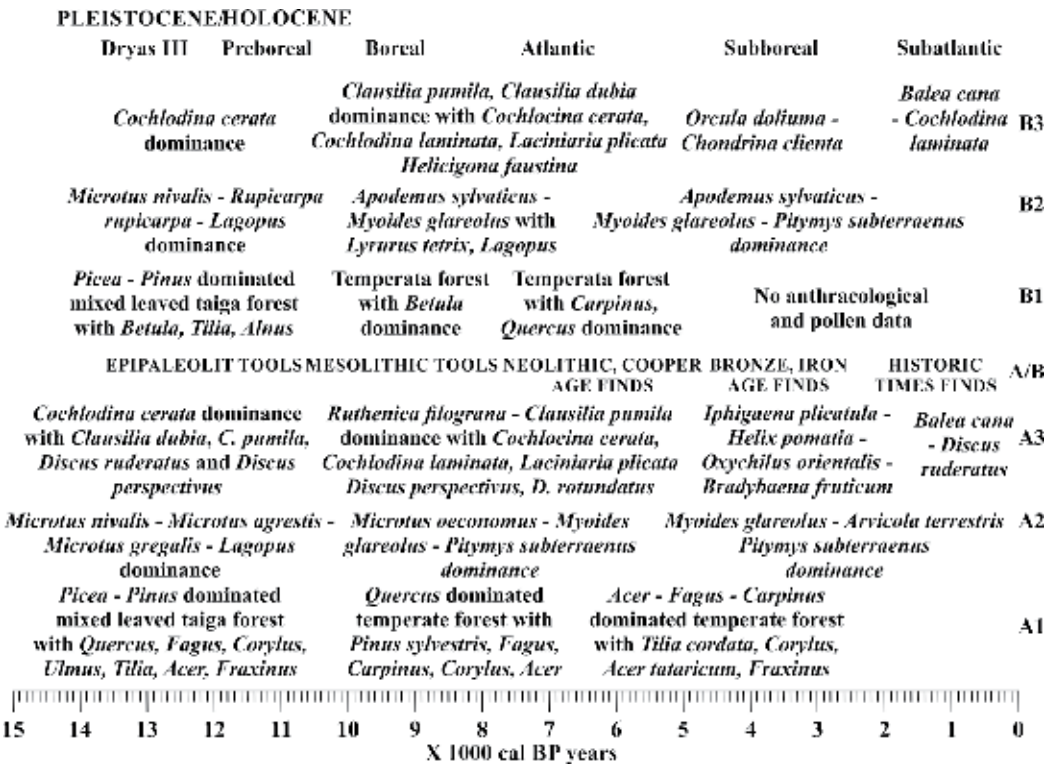

Figure 4. The radiocarbon-dated palaeoecological data from Petény (Peskő II) and Rejtek rock shelters A1 = anthracological-based vegetation phases of the sequence in the Rejtek site, A2 = mammalian phases of the sequence in the Rejtek site, A3 = local malacological zones of the sequence in the Rejtek site, A/B = archeological finds and times in the analyzed sites, B1 = anthracological- and pollen-based vegetation phases in the Petény site, B2 = mammalian phases of the sequence in the Petény site, and B3 = local malacological zones of the sequence in the Petény site.

refugia for woodland mollusk taxa [49–55, 64]. These findings have been corroborated by later studies on the Quaternary mollusk fauna of the Hungarian Highland and the Western Carpathians [85–90]. It must be noted that in Ref. [90], radiocarbon results presented span the Holocene Epoch alone. Cold-loving and cold-resistant elements in the accessory fauna of the *Cochlodina cerata* horizon are represented by a single taxon, presently dwelling in beech and pine woodlands: *Discus ruderatus*. However, warmth-loving elements like *Bradybaena fruticum* and *Euomphalia strigella* have also been recorded in this part of the profiles. Consequently, the general composition of the mollusk fauna is clearly in line with the observations and conclusions made on the basis of charcoal remains. Namely, the presence of refugia for temperate thermophilous woodland taxa within a Late Glacial mixed taiga forest [45–52]. The Late Glacial presence of *Discus perspectivus* as well as the records of the mollusk taxa *Cochlodina cerata*, *Cochlodina laminata*, *Clausilia dubia*, *Clausilia pumila*, and *Laciniaria plicata* in the layers representing the Pleistocene/Holocene transition is a highly outstanding feature of the sites. The collective appearance of *Discus perspectivus* and *Discus ruderatus* indicates the development of a dual refugia in the study area [49], i.e., the collective presence of Pleistocene cold-resistant and cold-loving elements with warmth-loving

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Holocene taxa during the Late Glacial in the Carpathian Basin as postulated earlier on the basis of paleoecological, geological, geochemical, and sedimentological observations made at the environmental historical site of Bátorliget marshland discussed in the next chapter [55]. According to our findings, rock walls and rock surfaces with a southern exposure in the inner Subcarpathian zone of the Carpathian Mts at suitable heights between 800 and 500 m ASL must have had special microclimatic conditions (angle of incoming radiation, minimal height for air humidity condensation), which allowed for the emergence of temperate woodland refugia [53–56, 67], even during the coldest periods of the ice age [91, 92]. Similar woodlands could have developed at the ecotone of floodplains and foothill areas of island-like hills in the heart of the basin as well [93, 94].

4. Ice age refugia at the interface of windblown sand dunes and floodplains: examples from the Bátorliget marshland, Nyírség region, NE Hungary, and the Ócsa marshland, Danube-Tisza interfluve, Central Hungary

The highly protected area of the Bátorliget marshland is situated on the NE part of the Great Hungarian Plain (GHP) approximately 2 km west of the state border with Romania in the area of the Nyírség alluvial fan covered by windblown sands (**Figure 1**). The Ócsa marshland is on the other located at the NW part of the GHP at the fringe of the Danube Lowland in an area of scattered windblown sand dunes. Both sites are located in an interdune depression hosting initially an oligotrophic lake during the ice age, which developed into a calcareous lake system finally evolving into a eutrophic lake then a marshland. According to the available absolute chronology, the emergence of an oligotrophic lake initiated around 20–22 ka during the coldest period of the last glacial: the last glacial maximum (LGM). However, the first mollusk remains turn up in deposits dated to the Late Glacial only. Shells of the Pontian *Pomatias rivulare*, the Central European woodland dweller *Discus perspectivus*, as well as the Boreal woodland dweller *Discus ruderatus*, presently populating the alpine woodlands of the Carpathian Mts, turn up as early as the transitional zone between the Late Glacial and the Postglacial in the profile of the Bátorliget marshland. This paleoassemblage composed of cold-loving and thermophilous elements can be clearly synchronized by the findings of pollen analysis indicating the presence of a mixed taiga hosting stands of pine and various temperate arboreal elements [55, 95]. These records thus indicate the development of a mixed taiga woodland at the interface of windblown sand and floodplain areas in the NE part of the GHP during the ice age, whose composition was the same as the mixed taiga woodlands of the foothill areas mentioned in the previous chapter. A significant difference in the mollusk faunas is the appearance of a Pontian gastropod taxon, which evolved during the Tertiary in the Carpathian Basin [96, 97], in the marshland sequence. This gastropod taxon, the *Pomatias rivulare*, has a clear preference for milder climatic conditions (**Figure 5**).

A similar paleoassemblage was identified in the Postglacial/Holocene deposits of the Ócsa marshland. However, in this latter profile, the Pontian *Pomatias rivulare* is substituted by

Figure 5. The European distribution of *Pomatias elegans* and *Pomatias rivulare* and their Hungarian fossil, pre-modern and modern occurrences. Horizontal black line = European distribution of *Pomatias elegans*; vertical black line = European distribution of *Pomatias rivulare*; white cross = Hungarian Pleistocene distribution of *Pomatias elegans*, Vértesszőlős, Tata, and Budai Várhegy; white stars = Hungarian modern distribution of *Pomatias elegans*, Bérbaltavár, Tihany, Őrtilos, Zákány, and Zákányfalu; white circle = Hungarian Holocene distribution of *Pomatias elegans*, Szurdokpüspöki, Esztergom, Budapest—Rákos-patak, Ócsa, Kiskörös, Fehérvárcsurgó, and Keszthely—Fenékpuszta, Kisapáti, Tapolca, Celldömölk, Ménfőcsanak, Balf, and Fertőboz; white pentagon = modern Hungarian distribution of *Pomatias rivulare*, Bátorligeti marshland, Szekszárd Sötét-valley, and Nagymányok; white rectangle = Hungarian Holocene and subrecent distribution of *Pomatias rivulare*, Bátorligeti marshland, Bátorliget Fényi-forest, and Vámospércs Jónás region.

its Atlanto-Mediterranean vicariant counterpart: *Pomatias elegans*. The radiocarbon-dated appearance of these latter taxa in the Ócsa profile is the oldest recorded one in Europe several thousand years before the first Atlantic distribution records of the taxon [98, 99]. On the basis of this record, the assumption that the Atlantic and Central European distribution of this taxon is connected to the Middle Holocene [100] must be refuted. The appearance of *Pomatias*

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elegans coevally with our Hungarian records was noted in the Early Mesolithic cultural layer of the Italian Grotta di Latronico Cave as well. Shells of this taxon have been retrieved in deposits older than 9000 cal BP years and those dated to the Postglacial and Early Holocene as well [101]. Representatives of this taxon with similar ages have been reported from Iberia [102] as well as Southern France [103]. In these latter examples, the first representative of the gastropod taxon *Pomatias elegans* likewise appears in layers older than the Mesolithic horizon dated at 9000 cal BP years.

To sum up briefly, the mollusk fauna dated to the opening of the Holocene in the areas of the Ócsa and Bátorliget marshland was a woodland dweller one characterizing temperate closed woodlands. This Early Holocene fauna is marked by the appearance of such taxa, which are restricted to the areas of the Carpathians, the Transylvanian Mid-Mountains, and the Hungarian Mid-Mountains and unique to the Great Hungarian Plains, serving as outstanding indicator species in both the stratigraphy and the paleoenvironmental conditions because they tended to appear in the mildest interglacial periods enjoying the greatest rainfall during the Pleistocene [25, 26]. The exuberant woodland malacofauna is made up of highly tolerant species appearing in uniform quantities in the closed woodland environment, which inhabit the mountains of Central Europe and are widespread in the area extending from the Balkan Peninsula up to the Baltics. The most characteristic elements of this community are the following: the Carpathian-Baltic *Ruthenica filograna*, *Macrogastra latestriata*, and *Bulgarica cana*; the Carpathian *Perforatella vicina*; the Central European *Acicula polita*, *Laciniaria plicata*, and *Perforatella bidentata*; the Central and Southern European *Discus perspectivus* and *Oxychilus glaber*; the European *Carychium tridentatum*, *Vertigo pusilla*, *Ena obscura*, *Vitrea crystallina*, *Aegopinella pura*, and *Cochlodina laminata*; and the Euxinic *Pomatias rivulare* or Atlanto-Mediterranean *Pomatias elegans*.

Both regions hosting the referred marshland had direct connections with the foothill areas of the surrounding mid-mountains, the south facing escarpments via the river valleys based on our subfossil distribution data of the mentioned woodland elements. It was these foothill areas which functioned as refugia for woodland elements from the Postglacial onward [55, 56].

5. Ice age refugia of temperate forest steppe and steppe gastropods: The results of quaternary malacological investigations on the loess/paleosol sequence of the Crvenka brickyard, northern Vojvodina, Serbia

The findings of Quaternary mollusk analyses implemented on radiocarbon-dated loess/paleosol sequences from the eastern half of the Carpathian Basin in the 1980s have clearly revealed that the Late Pleistocene environment of the Carpathian Basin was far from being uniform [54, 55, 64]. These observations gave an impetus to further comparative paleobiogeographical investigations using our malacological records deriving from different parts of the basin and covering the period of the Late Pleistocene. To highlight the environmental conditions characteristic of the southern part of the basin, our findings on the mollusk fauna of a Northern

Vojvodinian loess/paleosol sequence at the brickyard of Crvenka are presented in the following part [69, 104, 105]. The observed features of the malacofauna are in line with the ones made at other loess/paleosol profiles from the Southern GHP, spanning the same time period [54, 55, 67, 68]. A comparative analysis of these profiles with that or Crvenka enabled us to reconstruct the Late Glacial paleobiogeography of the southern part of the basin and tackle the spatial distribution of refugia for temperate forest steppe and steppe gastropod elements. Changes observed in the mollusk fauna of the Crvenka loess/paleosol profile are in line with climatic fluctuations recorded in the NGRIP Greenland ice core. The presence of numerous interstadial horizons representing the Greenland Interstadials between GI17 and GI2 could have been identified (**Figure 6**). The relatively low resolution of sampling hampered the identification of all GI events at the centennial scale. Based on our malacological data, the milder periods were characterized by higher temperatures and decreasing humidity resulting in a fixation and expansion of short grassland vegetation and the accompanying steppe-dwelling mollusk elements, while the cooler periods experienced only a minor drop in temperature and increasing humidity favoring the expansion of high grasses and some arboreal elements (scattered trees and bushes).

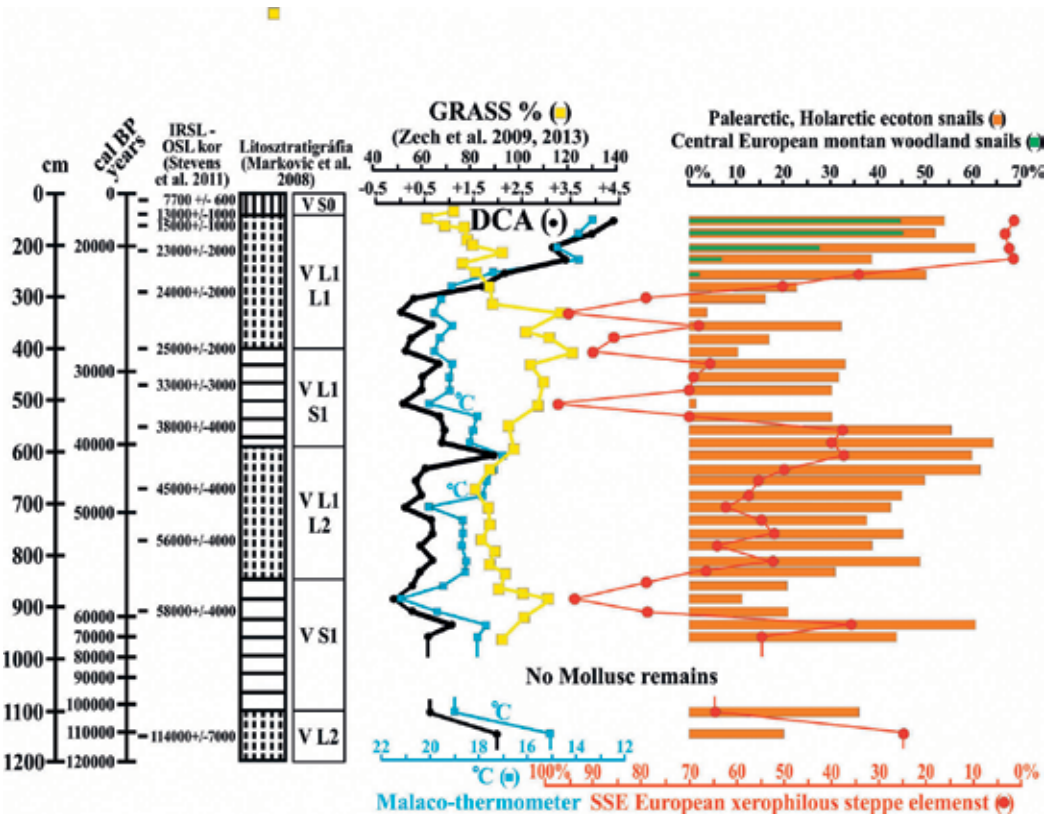

Figure 6. Fluctuations of different palaeoecological indicator groups on the late Pleistocene loess/paleosol sequence of the brickyard at Crvenka (northern Vojvodina, Serbia).

This type of change is especially pronounced in the horizon corresponding to the last glacial maximum. During this time an expansion of cold-loving, tundra-dweller mollusks is recorded in the northern and western ice-free areas of Europe. Conversely, this period is characterized by the appearance of closed woodland dwelling mollusks coevally with the cold-loving Arcto-Alpine elements. According to the trends observed, the southern parts of the GHP were characterized by fluctuating expansions and retreat of short grass and high-grass grasslands during the warmer and cooler periods of the late glacial. Thanks to the mosaic-like complexity of these habitats, mollusk taxa with contrasting ecological preferences regarding humidity, temperature, and vegetation cover could have existed side by side during the Late Pleistocene. Humidity increases during the coolings favored the expansion of mollusks resulting in a highly diverse fauna. Conversely, the warmer periods creating drier conditions decreased the diversity.

This unique feature of the southern part of the Carpathian Basin is by no means a newly described phenomenon. It has been known for ca. 30 years that compositional changes in the mollusk fauna of the southern parts of the basin are utterly different from those recorded in the other parts of the Carpathian Basin as well as in northern, western, and eastern Europe [54, 64, 67, 94, 106]. The most important difference is seen in the constant presence of xerothermophylous grassland elements (*Cochlicopa lubricella*, *Granaria frumentum*, *Pupilla triplicata*, *Chondrula tridens*, and *Helicopsis striata*) during the entire Late Pleistocene and their clear dominance in periods corresponding to the interstadials [67, 69]. It must be noted that the dominance peaks of the individual taxa in different profiles of the Southern GHP may not be fully coeval thanks to variations in local environmental and microclimatic conditions. Yet the general fluctuations of the similar warmth-loving paleoecological groups in time are synchronous between the individual profiles [67, 69].

It is also important to note that during the stadials characterized by only small temperature drops, the dominant elements of the mollusk fauna were those of the Holarctic forest steppe and high-grass-steppe dwellers, primarily members of the taxon *Vallonia costata*. During the interstadials, the fauna was prevailed by taxa characteristic of short grass Pontian grassland dwellers, which occupy the central driest Pannonian forest steppes of the Carpathian Basin (*Cochlicopa lubricella*, *Granaria frumentum*, and *Helicopsis striata* paleoassemblage). At the same time, elements of Holarctic forest steppes were also present in the malacofauna, although highly subordinately. Similarly, the Pontian grassland elements were likewise present in the mollusk assemblages of the stadials.

All these data indicate the presence of an ecotone of temperate Pannonian forest steppe-steppe areas composed of highly complex patches of short and high grassland types between 60,000 and 24,000 cal BP years. The recurring macroclimatic changes of the Late Pleistocene controlled a cyclical expansion and retreat of these environmental mosaics [67]. The best indicator element of temperate grasslands is *Granaria frumentum*. The largest aerial distribution of this taxon is recorded during the Late Glacial ca. 60 ka in the Carpathian Basin. During this time the presence of this taxon could have been observed in the areas of the Transdanubian Mid-Mountains in Western Hungary and the Sub-Carpathian alpine region as well besides that of the GHP. This spatial distribution must have developed during the last interglacial

(MIS 5), when this taxon expanded to almost all areas of Central Europe, including the Czech Basin [107], the Vienna Basin [108], as well as the Alps and the Carpathian Mts to a height of ca. 1000 m ASL [25, 26, 109]. All these indicate the expansion of temperate forest steppes to the foothill areas and the lower highlands during the drier periods of the last interglacial characterized by higher temperatures as well. After 60 ka, marking the onset of the last glacial, there is a gradual retreat in the distribution of *Granaria frumentum* to scattered habitats characterized by favorable microclimatic conditions. There is another major expansion of the referred taxon which can be dated between 40 and 30 ka. The highest dominances are recorded in the southern parts of the GHP with gradually decreasing northward trends. There is a major retreat between 30 and 24 ka to scattered refugia found in the southern part of the Carpathian Basin again, which hallmarked the start of the coldest phase of the last glacial. According to the findings of comprehensive studies done using our own mollusk data compiled into a database (Hungarian Quartermalacological Database), the southern part of the GHP, including the area of Vojvodina, Serbia, as well, was a transitional fluctuation zone between the refugia of the southern foothills of the Carpathians, the marginal area of the Dinarides and its northern island hills. This zone harbored an ecotone of temperate Pannonian grassland and forest steppe during the warmer, drier periods of the Late Pleistocene. Conversely, this vegetation complex was replaced by a boreal forest steppe during the cooler periods, similar to the taiga steppes of Southern Siberia today. This special evolution of the vegetation is utterly different from that of the northern parts of the Carpathian Basin as well as Northern, Western, and Central Europe. The difference is attributable to the regional and local higher temperatures of the ice age resulting in drier conditions in the former areas. Thus the most important ecological driver, regarding the evolution of both the vegetation and the mollusk fauna, in the southern areas of the basin was humidity. The higher aridity of this area during the Late Glacial is attributable partly to the high distance from the seas and oceans. In addition, the intensification of the so-called basin effect as a result of the uplift of the surrounding mountains.

6. Concluding remarks

The area of the Carpathian Basin is characterized by a large-scale environmental mosaicity present on the scale of the basin. This complexity increases downward to the regional and local scales. The major driving factor on the scale of the basin is the regional overlap of various climatic influences ranging from the Atlantic, Alpine, and Continental influences to the Sub-Mediterranean-Pontic climatic effects. According to our data, this type of mosaic-like complexity of the environment developed even during the ice age and controlled the evolution of the mollusk fauna both on the regional and local scales. Geomorphology, bedrock, groundwater table, exposition, as well as soil conditions further increased the macro-scale mosaicity on the regional and local scales. These regional environmental mosaics functioned as the ice age refugia for terrestrial and freshwater warmth-loving gastropods. Long-term conservation of these is the key to the preservation of the natural biota of the basin among changing climatic conditions.

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Chapter 6

Biodiversity of Gastropod in the Southeastern Gulf of California, Mexico

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Abstract

Currently, studying the environment is important because of the phenomena that take place on the earth every day. That is why it is a priority to carry out studies that relate environmental changes to the biology of organisms. This allows us to know the interactions with the environment, and in this way solve, reduce or prevent ecological and economic problems, if they are organisms with a commercial value. The objective of this investigation is to determine ecological parameters of the gastropod community from the intertidal zone on five islands from the Gulf of California, México, to model the diversity, distribution and abundance of malacological fauna. We considered to evaluate the Shannon-Wiener diversity (H'), Pielou's of evenness (J) and the Margalef species richness indexes, in order to evaluate through an analysis from biotic and abiotic factors, the species status that was collected from the exposed and non-exposed zone tidal. The generated data were contemplated from a year-based biodiversity project (2016–2017) on the following islands: Patos, Bledos, Bleditos, Tunosa, and Mazocahui which belong to the Ohuira lagoon in Ahome, Sinaloa, southeast of the Gulf of California, México. Likewise a status about the importance of gastropods is mentioned for the study area.

Keywords: mollusks, Gulf of California, Muricidae, Melongenidae, tourism, fisheries, aquaculture

1. Introduction

The Gulf of California is one of the most important marine ecosystems in Mexico and one of the most productive and biodiverse in the planet, as well as being one of the least disturbed. There we find 922 islands [1] that stand out for their high diversity in species, its high level of endemic species and a great biological richness, features that have allowed these places to be considered as natural evolutionary laboratories [2]. Mollusks within the marine ecosystem play a big role in the energy flux and community structure, due to the fact that many of them work as ecological regulators [3, 4] and indicators of disruptions that take place in these systems [5]. Besides, they constitute an abundant and ecological important group due to the functions performed by each one of their members within the food web, nutrient recirculation and energy flux [6].

Mollusks are mainly used in benthic studies to relate their presence/absence and/or dominance, with the aim to set their relationship with types of seabed and substrate [7]. Furthermore, they help to establish a baseline for future follow-up and evaluation programs [8]. Life cycles, a high level of stress tolerance [7], an intimate relationship with the sediment and a high response toward disturbances [9] make them ideal organisms to study natural and anthropogenic environmental changes [10, 11].

As a framework, most of the published research about Mollusks in the Mexican Pacific has to do with faunistic studies and taxonomy, whereas others talk about diversity aspects and variation through time [12–17]. Additional studies relate to distribution and abundance [18–24] and ecology [25–27]. Based on these previous studies, there is a lack of current information about the biology and ecology of the community of Mollusks in the intertidal zone from the islands of the Gulf of California; henceforth, it is necessary to do research that can increase and deepen the knowledge about the composition, abundance, and diversity of Mollusks.

1.1. Geography and oceanography in the Gulf of California

The Baja California Peninsula encloses the Gulf of California and is one of the most remote peninsular areas of the world. The gulf is a big semi-enclosed sea with more than 1100 km in length, 100–200 km in width, and with 258,593 km (99,843 mi^2) of surface which comprises more than 9° of latitude which cross the Tropic of Cancer in its southern part, extending to Cabo Corrientes (Jalisco, Mexico). It is the home of more of 900 islands and islets; it gives place to a highly rich and diverse habitat region for the evolutionary forces which in turn shape its flora and fauna. The northeast part of the gulf covers around 60,000 km^2 (24,000 mi^2) of sea surface, it extends to 3° latitude, and it is a unique water body in many ways. The weather is very dry, with an annual rainfall of less than 100 mm. The array of average monthly air temperatures in the northern gulf is of 18°C. The northern gulf presents some of the biggest tides in the world. The annual tide (amplitude) in San Felipe and Puerto Peñasco comprises around 7 m and in the Delta of the Colorado River, at the highest part of the gulf reaches almost 10 m [28].

1.2. Geography and oceanography of the Ohuira lagoon

The Ohuira lagoon connects itself with a 700-m-width cannel at Topolobampo Port. The Ohuira lagoon, with 125 km^2 (12,500 has) of surface, was the river basin of an ancient canal of the Fuerte River, which extended through the Topolobampo Bay, discharging its waters into this port. It is an area of shallows that during the rain period presents a deep zone of variable location depending on the tides and sediment dragging and presents a branch system that connects it with Navachiste Bay. In total, the system has eight islands: five within the Ohuira lagoon: Patos, Bledos, Bleditos, Tunosa, and Mazocahui (I and II) [29]. The circulation of the maximum currents in the lagoon's mouth is of 1.15 m s^{-1}, and in the channels of 1.10 m s^{-1} [30].

1.3. Biodiversity of mollusks

1.3.1. Current status

Mollusks are one of the zoological groups with more biological success, as much for its number of living species as for the habitat diversity they colonize [31]. Within the marine ecosystem, Mollusks play an important role in the energy flux and the community structure, due to the fact that many of them work as ecological regulators [32] and as disturbance indicators inside these systems [5]. In addition, they constitute an abundant and ecologically important group because of the role that each member performs within the food web, nutrient recycling and energy flux [6]. Inside this group, there are primary consumers, both herbivores and detritivores, second-level predators and specialized parasites, as well as opportunistic species, which indicates different answers to habitat modifications and pollution [33]. These organisms possess one of the most widespread distributions in the planet, ranging from the coastline to great sea depths [34]. The highest ability of Mollusks to adapt has given them a huge success along their evolution, and they have colonized terrestrial, damp and freshwater habitats [35], as far as deserts and polar areas, as well as the tropics and great sea depths [36], being widely studied due to their social and economic importance, as well as their commercial and nutritional values [37].

Many diverse studies about Mollusks have been undertaken in the Gulf of California. Nevertheless, the available information regarding the community structure of the group is scarce. Such investigations contribute important information because density variations from specific populations can be known in a specific period of time and locality, as well as the abundance and composition of a community within a natural gradient or when pollution or illness problems exist in the environment [38, 39]. In 2008, in the Guasave municipality, Sinaloa, Mexico, a Mollusk census was performed in the intertidal zone from La Mapachera, Tesobiate, La Huitussera, San Lucas, Guasayeye, Nescoco, El Metate and Las Chivas islands from the lagoon system known as Navachiste, in order to elaborate taxonomic lists and an intertidal species diagnosis. The collected Mollusks were located systematically in four classes (Gastropod, Bivalvia, Polyplacophora and Cephalopoda), 40 families and 81 species. Gastropods represented 59% with 24 families and 46 species, bivalves constituted 43% with 14 families and 34 species, polyplacophora comprised 3% with 2 families and 2 species and the remaining 1% corresponded to cephalopods with 1 family and 1 species [40].

In previous studies held in 2014 in the intertidal rocky zone (beach and mangrove area) from the Ohuira and Topolobampo Bays (Ahome), Sinaloa, the collected organisms represented a highly important trophic phase. The biodiversity and distribution of the community of epibenthic invertebrates was composed by a specific richness (S) of 168 species, divided in 10 taxonomic groups: 3 porífera, 2 cnidarians, 2 platyhelminths, 35 annelids, 2 sipunculids, 74 mollusks, 46 crustaceans, 1 pycnogonida, 1 ectoprocta, and 2 echinoderms, where Mollusks were the most predominant group with 74 species. The dominant Mollusks species were *Neritina* sp., and *Cerithium stercusmuscarum*. The epibenthic distribution was influenced by salinity and organic matter, enhancing the differences in the Ohuira lagoon [41].

1.3.2. Biodiversity of gastropods

The interest in studying biodiversity is linked to the lack of knowledge that exists over its magnitude, the processes that determine it and the constant loss due to human actions or climate change effects; thus, it is important to know and understand the processes that determine the abundance and distribution of biodiversity under different spatial and temporal scales in the gastropod species, as well as their transformation due to the environment [42–45].

2. Biodiversity of gastropods in Ohuira lagoon

Considering the period between October 2016 and 2017, organisms were collected by some of the authors from this present chapter in order to evaluate the biodiversity of Mollusks within the project named "Community structure of the Mollusks found in the islands of the north of Sinaloa, México" (Register number DSA/103.5/16/10277). Sampling stations were established in Patos (25°20′450″ N, 109°00′531″ W), Bledos (25°18¨350″ N, 109°00′458″ W), Bleditos (25°14′566″ N, 109°00′664″ W), Tunosa (25°15′785″ N, 109°00′924″ W) and Mazocahui (25°34′154″ N, 109°00′855″ W) islands in the Ohuira lagoon. To collect gastropod Mollusks we took as reference six quadrants of 1 m^2 dimension in the zone exposed to the waves and three quadrants in the area not exposed to the tides. The organisms were collected from the sand, mud, silt-clay and rocky soil, which were representative from the study area. Thereupon, in soft substrates the harvest was made manually, and those organisms that were found adhered to rocky substrates were removed with a scraper, chisel or hammer. In addition, those organisms that were found at a greater depth were collected by snorkeling. The collected Mollusks were stored in plastic bags with their corresponding label, according to the type of sample method. The organisms were conserved in ice to be transferred to the biology lab at the Universidad de Occidente Unidad Los Mochis, Ahome, Sinaloa, Mexico. Taxonomic keys were used to identify the gastropod Mollusks [46–53].

2.1. Alpha diversity measurement

The analysis of the community structure of gastropod Mollusks was based on ecological indexes that quantified the information given by the lagoon system, which were applied

based on each island and whether the organisms were exposed or not to the tide. To represent the biodiversity of gastropods we used the following indexes:

The species richness (S) was estimated by counting the number of species because it is the easiest way to measure biodiversity, since it is based on the number of species that are present without considering their importance. The abundance (A) was estimated by counting the number of organisms that were registered in each sampling station. The relative abundance (Pi) represented the existing relation between the organisms of a single species and the total number of organisms from all the species encountered, by using the following equation (Eq. (1)):

$$Pi = \frac{\mathrm{ni}}{N} \tag{1}$$

where ni is the number of organisms from the "i" species and N is the total number of organisms from all gastropod species.

To identify the dominant species from the community we used the community dominance index (ID) (Eq. (2)) [27, 54]:

$$\mathrm{ID} = \frac{\mathrm{Y1} - \mathrm{Y2}}{\mathrm{N}} * 100 \tag{2}$$

where Y1 is the abundance of the most common species, Y2 is the abundance of the species that occupied the second place in abundance, and N is sum of the abundance of all species.

In accordance with the estimators, Pi, ID, H′, as well as with the dominance level, the main species for each island was determined. To represent the sui generis characteristic from the community, we analyzed jointly the abundance (A) and frequency (F) to establish four categories of species which are cataloged as (AF)—highly abundant and very frequent, (aF)—less abundant and highly frequent, (Af)—highly abundant and less frequent, and (af)—less abundant and less frequent [27].

The Shannon-Wiener diversity ecology index measured the average uncertainty degree to predict to which species a randomly chosen individual could belong to within a collection (Eq. (3)) [27, 38, 55, 56]:

$$\mathrm{H}' = \Sigma Pi * lnPi \tag{3}$$

where Pi is proportional abundance of the "i" species.

Pielou's equity measured the proportion of the observed diversity from the maximum expected diversity. Its value ranges from 0 to 1, where 1 corresponds to those situations in which all species are equally abundant (Eq. (4)) [38]:

$$\mathrm{J}' = \frac{\mathrm{H}'}{H' max} \tag{4}$$

where H′: Shannon-Wiener's diversity and H′max: maximum diversity.

Species diversity under conditions of maximum equity, in other words, the species diversity from a sample if all the species (S) had the same abundance equity (Eq. (5)):

$$H' max = ln S \tag{5}$$

The diversity index or Margalef's richness (D_{mg}), transformed the number of species per sample into a proportion in which species are added by the expansion of the sample. This index assumes that there is a functional relation between the number of species and the total number of organisms [38]:

$$S = k\sqrt{N} \tag{6}$$

where k is the constant.

If the constant is not maintained, then the index varies with the sample size in an unknown manner. By using s-1 instead of S, we get $D_{mg} = 0$, when there is only one single species (Eq. (7)):

$$Dmg = \frac{S-1}{lnN} \tag{7}$$

where S: number of species; and N: total number of organisms.

In order to calculate these indexes the abundance data were transformed into a natural algorithm [27].

2.1.1. Richness (S), abundance (A), and relative abundance (Pi)

At the Ohuira lagoon we collected a total of 5431 gastropods, being Patos Island the one with the highest abundance of 2135 organisms (39.35%), followed by Bleditos Island with 1471 (27.12%), Tunosa Island with 768 (14.14%), Bledos Island with 649 (11.95%), and Mazocahui Island with 408 organisms, representing 7.44%.

In general, within all the islands that were studied in Ohuira lagoon a total of 22 species of gastropods were collected. In those areas where there was nonexposure to tides, the species that were found were: *Cerithium stercusmuscarum* (n = 333, Pi = 0.0613), *Neritina* sp. (n = 208, Pi = 0.0383), *Nerita scabricosta* (n = 301, Pi = 0.0554), *Nerita funiculata* (n = 207, Pi = 0.0381), *Nassarius luteostoma* (n = 22, Pi = 0.0041), *Nassarius gallegosi* (n = 20, Pi = 0.0037), *Crucibulum spinosum* (n = 217, Pi = 0.040), *Eupleura* sp. (n = 5, Pi = 0.000921), *Crepidula onix* (n = 1, Pi = 0.000184), *Crepidula rostrata* (n = 6, Pi = 0.001105), *Fisurella* sp. (n = 1, Pi = 0.000184), *Littorina aspera* (n = 5, Pi = 0.000921), *Littorina modesta* (n = 14, Pi = 0.0026), *Crepidula lessoni* (n = 10, Pi = 0.00184), *Tegula corteziana* (n = 5, Pi = 0.000921), *Diodora* sp. (n = 3, Pi = 0.00055), *Scurria mesoleuca* (n = 3, Pi = 0.00055), *Diodora digueti* (n = 3, Pi = 0.00055), *Crucibulum scutellarum* (n = 7, Pi = 0.00130), *Murex (Recurvirostris) lividus* (n = 3, Pi = 0.00055), *Terebra* sp. (n = 10, Pi = 0.00184), and *Hexaplex (Muricanthus) nigritus* (n = 10, Pi = 0.00184).

In the areas where there was tidal exposure a total of 19 gastropod species were found: *Cerithium stercusmuscarum* (n = 704, Pi = 0.130), *Neritina* sp. (n = 271, Pi = 0.050), *Nerita scabricosta* (n = 319, Pi = 0.059), *Nerita funiculata* (n = 505, Pi = 0.0930), *Nassarius luteostoma* (n = 11, Pi = 0.0021), *Crucibulum spinosum* (n = 14, Pi = 0.0026), *Crepidula onix* (n = 10, Pi = 0.00184), *Crepidula rostrata* (n = 8, Pi = 0.0015), *Littorina aspera* (n = 1, Pi = 0.000184), *Littorina modesta* (n = 50, Pi = 0.0092), *Crepidula lessoni* (n = 69, Pi = 0.0127), *Diodora digueti* (n = 4, Pi = 0.00074), *Murex (Recurvirostris) lividus* (n = 11, Pi = 0.0021), *Turritella gnostoma* (n = 1, Pi = 0.000184), *Thais biceralis* (n = 1, Pi = 0.000184), *Hexaplex (Muricanthus) nigritus* (n = 557, Pi = 0.1030), *Hexaplex eristrosthomus* (n = 76, Pi = 0.0140), *Phyllonotus brassica* (n = 15, Pi = 0.0028), and *Melongena patula* (n = 10, Pi = 0.00184).

2.1.2. Biodiversity in Patos Island

A total of 11 gastropod species were registered in Patos Island, where *Cerithium stercusmuscarum* showed the highest abundance with 214 organisms in the zone that was not exposed to the tide, whereas a total of 395 organisms were found in the area exposed to the tide. The least abundant species were *Crepidula onyx*, *Crucibulum scutellatum*, *Nassarius gallegosi*, *Nassarius luteostoma*, *Littorina modesta*, and *Murex (recurvirostris) lividus*, which were present with one single organism collected, both in the area exposed to the tide and the one not exposed to it (**Figure 1**).

2.1.3. Biodiversity in Bledos Island

Bledos Island was the one that showed a higher mollusk diversity with 13 species in which *Cerithium stercusmuscarum* was the most abundant with 41 organisms, whereas *Nerita funiculata* was the least abundant, being represented by only one collected individual (**Figure 1**).

2.1.3.1. Biodiversity in Bleditos Island

Bleditos Island had a gastropod diversity of 12 recorded species. *Cerithium stercusmuscarum* and *Nerita scabricosta* were the most abundant species in the intertidal area, being represented by 130 organisms. *Crucibulum spinosum* had 180 organisms in the area not exposed to the tide (**Figure 1**).

2.1.3.2. Biodiversity in Mazocahui Island

On Mazocahui Island a total of 9 species were registered from which *Nerita funiculata*, and *Neritina* sp. had the highest abundance with 109 and 124 organisms, respectively in the intertidal area. On the other hand, *Nassarius gallegosi* (17 organisms) and *Cerithium stercusmuscarum* (26 organisms) had the highest abundance in the area not exposed to the waves (**Figure 1**).

2.1.4. Biodiversity in Tunosa Island

Tunosa Island had a diversity of 10 species, from which *Nerita funiculata*, *Nerita scabricosta*, and *Hexaplex (Muricanthus) nigritus* were the most abundant in both zones, the one exposed

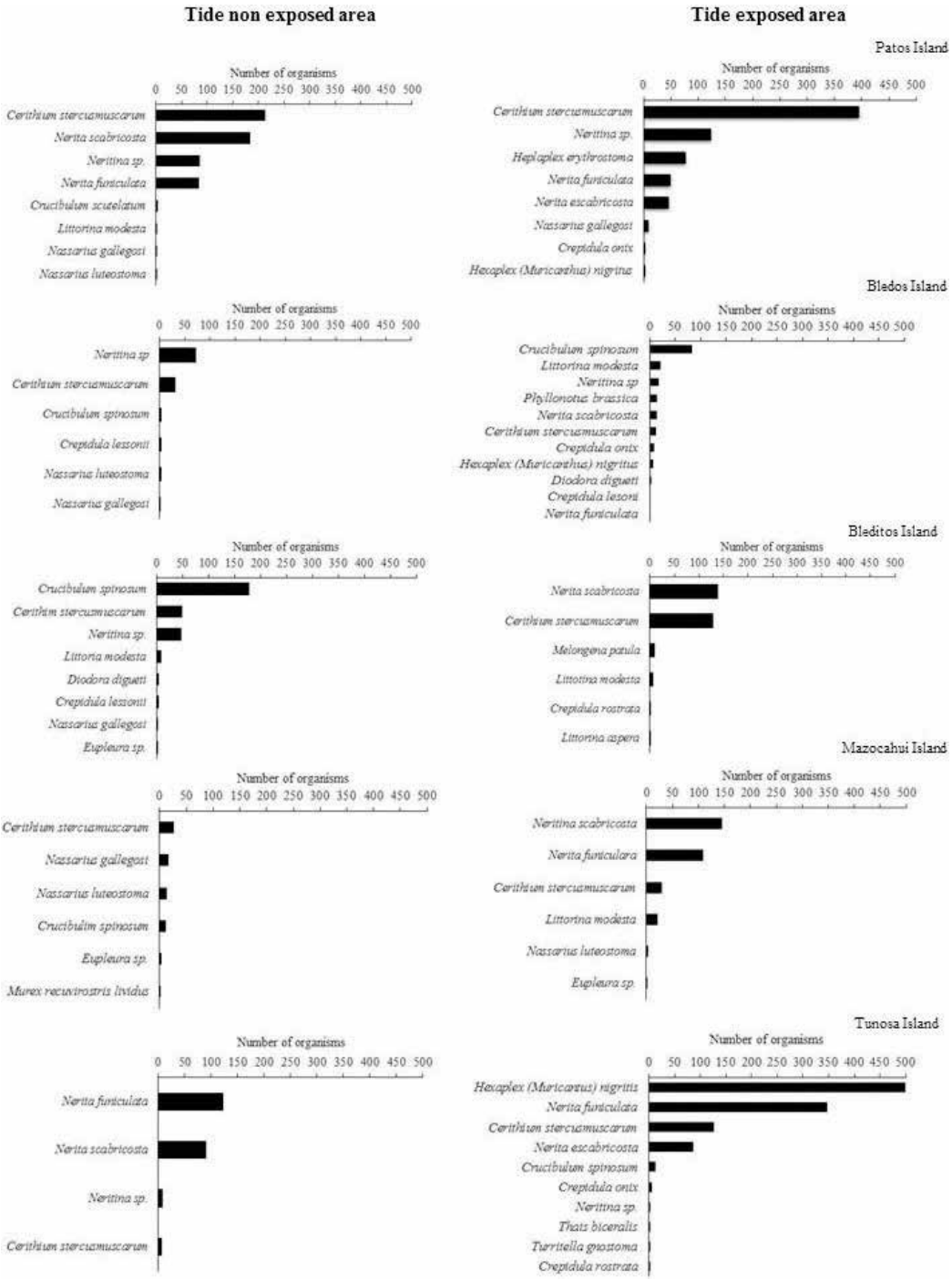

Figure 1. Diversity and abundance of gastropod mollusks on islands of Ohuira lagoon.

to the tide and the one not exposed to it, while *Turritellla gnostoma*, *Thais biceralis*, *Hexaplex (Muricantus) nigritus*, *Crepidula onix*, *Crucibulum spinosum* and *Neritina* sp. were the least abundant species in the area exposed to the tide (**Figure 1**).

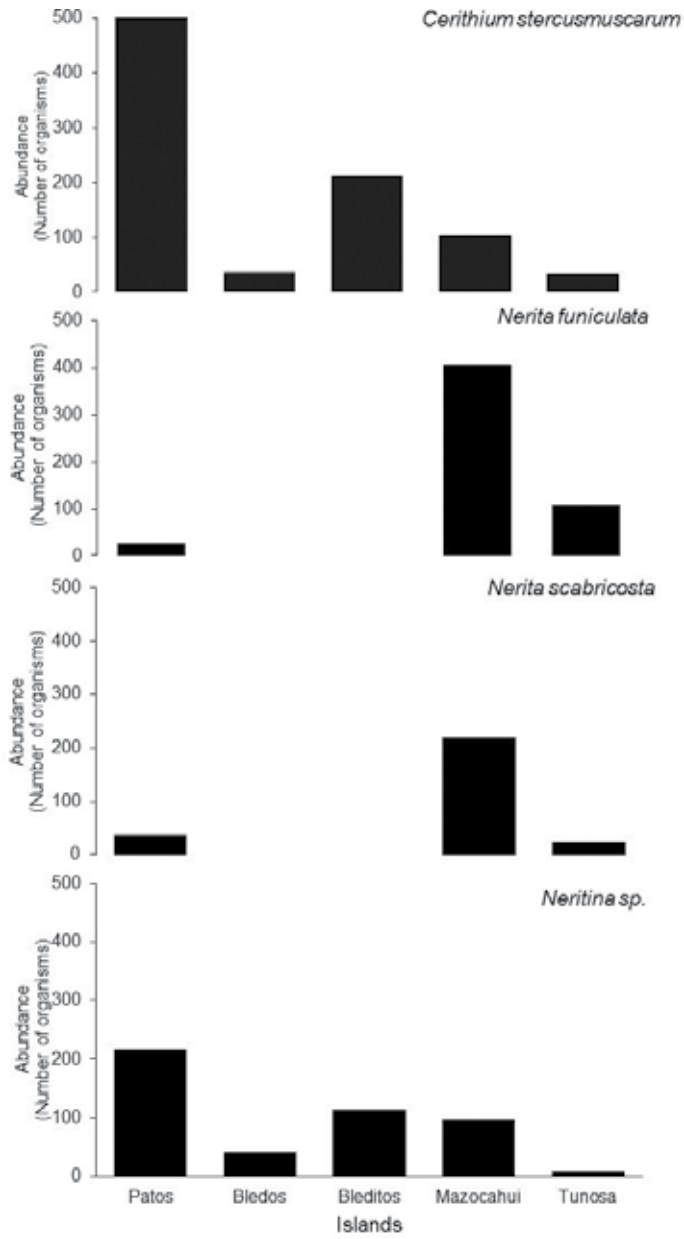

Figure 2. Dominance of *Cerathium stercusmuscarum*, *Nerita funiculata*, *Nerita scabricosta*, and *Neritina* sp. on the islands of Ohuira lagoon, Ahome, Sinaloa, Mexico.

2.2. Ecological indexes

2.2.1. Dominant species (ID) and Shannon-Wiener's diversity (H´)

Cerithium stercusmuscarum (ID = 32.20%, AF, H´ = 0.3161) was found on five islands with 1037 collected organisms, being Isla Patos the island with the highest abundance and Mazocahui the one with the lowest abundance (35 organisms). *Nerita funiculata* and *Nerita scabricosta* were

the most abundant species on Tunosa Island with 712 (ID = 24.53%, Af, H′ = 0.2662) and 620 (ID = 21.67%, Af, H′ = 0.2478) collected organisms, respectively, in the intertidal zone. *Neritina* sp. was the dominant species and presented the highest abundance on Patos Island with 479 collected organisms (ID = 13.07%, aF, H′ = 0.2142), the sum of the dominance of these species was of 91.47%, where 8.53% corresponds to the rest of the remaining gastropod species. The biometrics of the dominant species on the islands of the Ohuira lagoon, Ahome, Sinaloa, México were as follows: *Cerithium stercusmuscarum* with 26.65 ± 0.146 mm in length, *Nerita funiculata* with 16.03 ± 0.118 mm in length, *Nerita scabricosta* with 39.03 ± 1.46 mm in length, *Neritina* sp. with 8.9 ± 0.205 mm in length (**Figure 2**).

2.2.2. Pielou's equity

2.2.2.1. Comparison between dominant species in tide exposed areas (J'_1) and non-exposed areas (J'_2)

The gastropods *Cerithium stercusmuscarum* (J'_1 = 0.5413, J'_2 = 0.8390) was recorded on five islands with 1037 collected organisms, being Patos Island the one with the highest abundance and Mazocahui Island the one with the lowest (35 organisms), finding a higher tendency of the proportion of the observed diversity in those nonexposed areas. *Nerita funiculata* and *Nerita scabricosta* were the most abundant species on Tunosa Island with 712 (J'_1 = 0.4677, J'_2 = 0.8298) and 620 (J'_1 = 0.6469, J'_2 = 0.6739) respectively, recording the highest equity index in the areas exposed to the tides. *Neritina* sp. dominated with the highest abundance on Patos Island with 479 collected organisms (J'_1 = 0.5831, J'_2 = 0.6993) showing the same tendency of high equity in the nonexposed areas.

2.2.2.2. Diversity index or Margalef's richness (D_{mg})

On Patos Island we found D_{mg} = 1.80, on Bledos Island D_{mg} = 1.68, Bleditos Island D_{mg} = 1.53, Tunosa Island D_{mg} = 1.95, and on Mazocahui Island, D_{mg} = 1.81, which suggests that on Patos and Mazocahui Islands, the diversity or richness is similar, being higher on Tunosa Island and lower on Bleditos.

3. Importance of gastropod biodiversity in the study area

Due to the lack of biological information from the Gulf of California islands, specifically from the ones found in Ohuira lagoon and their ecological and economic importance, it is necessary to perform research to increase the knowledge about them and to contribute on the elaboration of management methods and alternatives for the sustainable use the marine resources found on the islands. While it is true that in the past years the number of investigations have increased, the marine studies that have been performed on the islands of the Ohuira lagoon do not provide enough information about the species that inhabit the area, such as their biology, ecology, reproduction, physiology and taxonomy. This is why this current research pretends to set a baseline for future studies of the gastropod mollusk community from these islands throughout time, in order to evaluate possible environmental changes whether they are natural or anthropogenic.

3.1. Importance in ecology, biology and taxonomy

In Ohuira lagoon, Ahome, Sinaloa, the ecological importance in particular of the species of gastropod Mollusks is related to trophic levels, since there are organisms that are of carnivorous feeding habits such as the black murex *Hexaplex (Muricanthus) nigritus*, the prince murex *Hexaplex (Muricanthus) prínceps*, the cabbage murex *Phyllonotus brassica*, the pink-mouthed murex *Phyllonotus erythrostoma*, Regal murex *Phyllonotus regius*, the Pacific melongena *Melongena patula*, the giant Eastern Pacific conch *Strombus galeatus*, and the Pacific cask shell *Malea ringens*, which can feed on other smaller gastropods such as *Cerithium stercusmuscarum*, the onyx slipper shell *Crepidula onyx*, *Terebra armillata, Hormospira maculosa*, and *Fusinus (Barbarofusus) colpoicus*, to mention some species. It is important to emphasize that both gastropods, the black murex *Hexaplex (Muricanthus) nigritus* and the ambiguous murex *Hexaplex (Muricanthus) ambiguus*, are sometimes considered as northern subspecies of the species radish murex *Hexaplex (Muricanthus) radix* [52]. Considering the gastropod species with the greatest economic-commercial importance in the study area, the biology is described for each case [41, 52].

3.1.1. Gastropods black murex Hexaplex (Muricanthus) nigritus (Philippi, 1845)

The distinctive features of the gastropod *Hexaplex (Muricanthus) nigritus*, are that it belongs to the family Muricidae, it has a relation of synonymy with *Murex nigritus* and *Muricanthus nigritus*; it has a very large, robust, bulbous shell with a moderately prominent conical spiral and a wide body turn. It presents six to nine strong spinal mandibular varices in the back of the body, crossed by spiral ribs intermixed with smaller ribs. It has relative scarce thorny acute varices, and those located on the shoulder and on the basis of the longer shell. It has a wide oval opening with a small rear channel and a wide siphoned channel, fairly well developed and slightly curved. It presents a strongly crenulated outer lip and an internal lip with a columellar adherent callus and a spiral crest on its back, and the nucleus of the operculum presents an anterior position. The color of the outer surface is white opaque, with a blackish-brownish dye on the ribs, spirals and thorns, and the opening is porcelain white. The maximum size reported was 150 mm, although the most common size is 120 mm. The reported habitats of the species were reefs or sandy bottoms in the intertidal zone and subcoastal shallow waters [52].

3.1.2. Pink-mouthed murex gastropod Phyllonotus Erythrostoma (Swainson, 1831)

It belongs to the Muricidae family, the synonyms used are *Murex erythrostoma*, *Chicoreus erythrostoma*, and *Hexaplex erythrostoma*. The distinctive features are a large, robust, globose-oval shell, with a short conical spiral and a wide body turn; four or five thick axial varices around the body, alternating with tubercular axial ridges; six to seven spiral crests that form nodules in the intervarical ridges and become open and sharp spines on the varices, being stronger in the shoulder; oval opening with a small posterior canal and a wide siphonel canal, relatively short and curved; an external erect and crenulated lip; an internal lip with a thin, expanded columellar callus and the nucleus of the operculum with an anterior position. The outer surface is opaque white and it has a bright pink opening. The maximum size reported was 150 mm,

while the most common size is up to 100 mm. The habitat of the species was reported in sandy and muddy bottoms, both at low levels of the intertidal zone and offshore, up to 50 m deep [52].

3.1.3. Pacific melongena gastropods Melongena Patula (Broderip and Sowerby, 1829)

With only a single species in the study area, it belongs to the Melongenidae family. It presents a large and heavy, piriformed shell, the most recent rounds gradually enveloping the oldest, forming an irregular and deeply grooved suture and a very small spiral coil. Young organisms (less than 60 mm in length) are therefore more fusiform. The sculpture consists of a single-spaced row of short spines (although this feature might be absent) on the rounded shoulder, as well as numerous fine spiral grooves, mainly in the lower part of the shell. It has a rather smooth and thick periostraco, a very large opening, with a short and wide channel, an internal smooth and satin lip and a simple outer lip. The corneum operculum has a claw-like shape, with a terminal nucleus. The color is dark brown with cream spiral bands, just below the widest part of the last lap. The opening's color is yellowish to pinkish. The families of gastropods with similar appearance present in the area are: Fasciolariidae, with more fusiform shells, longer and narrow siphon canals, with few folds sometimes present in the columella. The maximum reported size was 260 mm; nevertheless, the most common size was up to 160 mm. Its habitat is in sandbars and mud from the high levels of the intertidal zone. It is a carnivorous species that especially feeds on other gastropod Mollusks [52].

3.2. Elaboration of crafts (tourism)

Several gastropods are used to create crafts (Port of Mazatlán, Sinaloa) such as jewelry boxes, picture frames, key holders, reliquaries, candles, lithographs, among others, by using shells of the gastropods belonging to the genus *Hexaplex*, *Melongena*, *Phyllonotus*, *Strombus*, *Turritella*, *Crucibulum*, *Crepidula*, and *Cerathium*. Mentioned artisan products are acquired by domestic and foreign tourists in local sales outlets established in municipal markets and in touristic areas. The elaboration of handcrafts has been carried out for decades mainly in the port of Mazatlán, where in many cases it becomes the livelihood-sustaining asset for a large number of families that take advantage of a waste product (shells) once the organism has been extracted for consumption and commercial importance. The same situation takes place with the smaller gastropods whose shells are collected on beach areas for these same purposes [10] (**Figure 3a–d**).

3.3. Fisheries

3.3.1. Current status

Some organisms of the gastropod are a very important fishery resource worldwide and have a significant economic impact through the generation of resources at the level of artisanal fishermen, local trade and the export of fishery products of international value. The gastropod fishery destined for human consumption in the study area is based on the black murex *Hexaplex (Muricanthus) nigritus,* prince murex *Hexaplex (Muricanthus) prínceps,* cabbage murex *Phyllonotus brassica,* pink-mouthed murex *Phyllonotus erythrostoma,* Regal murex *Phyllonotus regius,* Pacific melongena *Melongena patula,* giant Eastern Pacific conch *Strombus galeatus,* and the Pacific cask shell *Malea ringens* [52].

Figure 3. (a–c) Decorative articles made with shells of gastropods, simulating flowers; (d) jewelry boxes made with different species of gastropods; (e) capture of black murex *Hexaplex (Muricanthus) nigritus* by snorkeling on a working day in the lagoon Ohuira, Ahome, Sinaloa, and also the adhesion of masses of embryos (Me) on the shells of the organisms captured; (f) capture of gastropods *Melongena patula* (Mp), *Hexaplex (Muricanthus) nigritus* (HMn); and *Phyllonotus erythrostoma* in the study area; (g) "Chipped" processing of the capture of the gastropod *Hexaplex (Muricanthus) nigritus* in the study area.

3.3.1.1. Gastropods black murex Hexaplex (Muricanthus) nigritus (Philippi, 1845)

The fishery of the gastropods black murex *Hexaplex (Muricanthus) nigritus* (*H. M. nigritus*) is the one of greatest effort considering the abundance of the species, incidentally including another gastropod (prince murex *Hexaplex (Muricanthus) prínceps*) of the family Muricidae which has similar morphometric characteristics that go unnoticed to fishermen. Current official data from the port of Topolobampo, Sinaloa, on *H. M. nigritus* catches in the study area, with reference to the year 2008 with a catch of 8000 kg, and by 2014 of 4063 kg in live weight, which represented an income of $21,196.35 Mexican pesos [57, 58]. The exploitation and effort applicable to this gastropod in each season requires previous evaluations for each season according to the availability of the resource for each catch zone, due to their eating habits, the evaluation method can be by marking and recapture of marked organisms. The fishing effort is very variable, since not every year the catches are recorded. A fisherman by snorkeling can capture approximately 700 organisms in 4 h of work (**Figure 3e**). A minimum size for catching 90 mm of the shell [52] is contemplated in the Mexican legislation (**Figure 3f–g**). The average shell length recorded during the period 2016–2017 was 100 ± 2.53 mm, with a total weight of 104.45 ± 19.34 g. With regard to fisheries, growth with respect to the length ratio of the shell (mm)-total weight (g) was evaluated in black murex *H. M. nigritus* in the Ohuira lagoon and was represented by the potential model $TW = 4E\text{-}06SL^{3.7956}$, with $R^2 = 0.8317$ (**Figure 4a**).

3.3.1.2. Pink-mouthed murex gastropod Phyllonotus erythrostoma (Swainson, 1831)

The pink-mouthed murex gastropod fisheries *Phyllonotus erythrostoma* (*P. erythrostoma*) in the study area is complemented by the gastropod cabbage murex *Phyllonotus brassica* and regal murex *Phyllonotus regius* which have similar morphometric characteristics that go unnoticed by fishermen [52]. There are currently no official data in the office of the Port of Topolobampo, Sinaloa on the catches of *P. erythrostoma* in the study area, the closest reference is to the year 2008, with a catch of 8000 kg, which represented an income of $ 11,096.21 Mexican pesos [57–59]. The exploitation and effort applicable to this gastropod in each season of capture, as in black murex *H. M. nigritus*, requires previous evaluations for each season according to the availability of the resource for each catch zone. Due to their eating habits the evaluation method can be made by labeling, and the recapture of marked organisms. The fishing effort is very variable, since not every year has a recorded catch. A fisherman by snorkeling can capture approximately 150 organisms during 4 h of work (**Figure 3f**). Growth with respect to the length ratio of the shell (mm)-total weight (g) was evaluated in pink-mouthed murex *Phyllonotus erythrostoma* in the Ohuira lagoon and was represented by the potential model $TW = 0.5596SL^{1.2287}$, with $R^2 = 0.4442$ (**Figure 4b**).

3.3.1.3. Pacific melongena gastropod Melongena patula (Broderip and Sowerby, 1829) and incidentals gastropods

Captures in the study area of the Pacific melongena gastropod *Melongena patula* (*M. patula*), considering the official records in the Port of Topolobampo, Sinaloa, amounted 21,695 kg for the year 2014 with a value of $ 130,432 Mexican pesos. In contrast, the black murex gastropod

H. M. nigritus has a lower abundance than *M. patula* but it is compensated with the longer shell length and total weight that also has its commercial value (**Figure 3f**). FAO in 1995 [52] reported a maximum shell length of 260 mm, with a common length of 160 mm. There are gastropods that have a shell length and total weight similar or superior to *H. M. nigritus*, *P. erythrostoma*, and *M. patula* like the giant eastern Pacific conch *Strombus galeatus* (Swainson, 1823) and the Pacific cask shell *Malea ringens* (Swainson, 1822). However, their catch is incidental because of their low abundance. For these species of gastropods, there are no official regulatory standards for their fisheries, it is only mentioned those non-updated catch volumes, as well as the recommended catch sizes in the national fisheries charter issued by the National Fisheries Institute, Mexico.

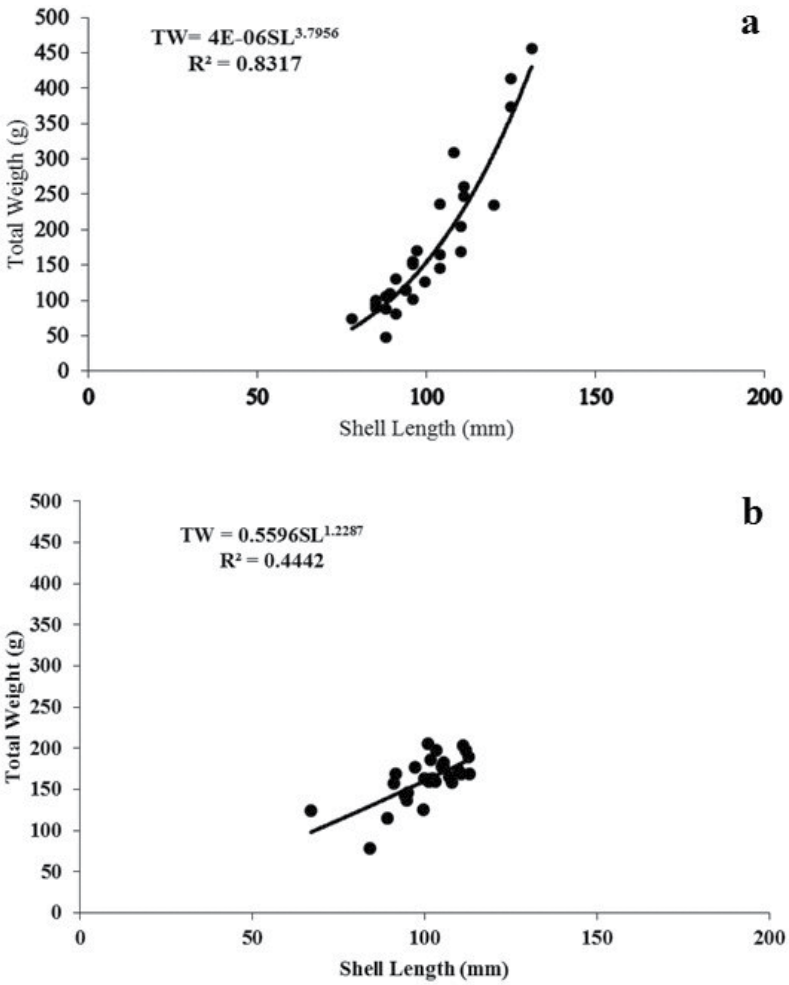

Figure 4. Growth in length of the total weight (g)-shell of the (a) black murex *Hexaplex (Muricanthus) nigritus* and (b) pink-mouthed murex gastropod *Phyllonotus erythrostoma* in Ohuira lagoon, Ahome, Sinaloa, Mexico.

3.4. Aquaculture

3.4.1. Current status

The reproductive cycle of the snail *Hexaplex (Muricanthus) nigritus*, was studied in 2011, under lab conditions. A total of three females and two males were collected in Macapule lagoon, Guasave, Sinaloa, Mexico. After being held at water exchange regimes a total of 24 eggs masses were collected. The average number of capsules found within an eggs mass was of 150.75 ± 37.23. The estimated height and width for the capsules averaged 15.05 ± 1.21 and 4.93 ± 0.58 cm, respectively; the average number of embryos found per capsule was of 1583 ± 149, obtaining a total of 238,626 ± 3457 embryos in the egg mass. The obtained results were considered as useful tools to estimate the reproductive potential of *H. M. nigritus* for commercial and repopulation purposes [60].

4. Discussion

A difference was found among the ecological indexes (H′, J′, D_{mg}) from the intertidal Mollusks community between the present study from the Ohuira lagoon, Ahome, Sinaloa (2016–2017 period) and previous studies undertaken at the Guasave municipality in Sinaloa (Navachiste lagoon) [15] and in the Topolobampo and Ohuira lagoons (mangrove zone) [16]. The abundance distribution of organisms and species on the collecting sites was heterogeneous. In the gastropod mollusk community in Ohuira lagoon, Ahome, there was a certain type of association with the type of substrate, which is composed of rocky and sandy zones (beach), zones with small rocks and in less proportion mangroves. The sampling methods showed that the gastropod *Littorina modesta* associates with the mangrove. In a previous study about epibenthic invertebrate communities associated with hard substrates in the intertidal zone in the Ohuira and Topolobampo lagoons, Sinaloa, the authors mentioned that the rocky intertidal zone and its organisms represent a very important trophic phase, and they recognize the importance of getting to know more about the biodiversity and distribution of the epibenthic invertebrate community of that study area. In their study, they performed 4 samplings with 50 × 50 cm quadrants at 5 stations from August 2011 to February 2012. The results showed that the community presented a specific richness (S) of 168 species, where 74 of them corresponded to Mollusks. The gastropod *Cerithium stercusmuscarum* was found within the dominant species, which matches with the results reported in the present study on the Ohuira islands [52]. The species *Cerithium stercusmuscarum, Neritina* sp., *Nerita funiculata, Nerita scabricosta, Crucibulum spinosum, Nassarius luteostoma* and *Crepidula onix* could be considered as representatives of the malacological fauna of the Ohuira lagoon, Ahome, Sinaloa, Mexico.

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Chapter 7

Marine Snails of the Genus *Phorcus*: Biology and Ecology of Sentinel Species for Human Impacts on the Rocky Shores

Ricardo Sousa, João Delgado, José A. González, Mafalda Freitas and Paulo Henriques

Additional information is available at the end of the chapter

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Abstract

In this review article, the authors explore a broad spectrum of subjects associated to marine snails of the genus *Phorcus* Risso, 1826, namely, distribution, habitat, behaviour and life history traits, and the consequences of anthropological impacts, such as fisheries, pollution, and climate changes, on these species. This work focuses on discussing the ecological importance of these sentinel species and their interactions in the rocky shores as well as the anthropogenic impacts to which they are subjected. One of the main anthropogenic stresses that affect *Phorcus* species is fisheries. Topshell harvesting is recognized as occurring since prehistoric times and has evolved through time from a subsistence to commercial exploitation level. However, there is a gap of information concerning these species that hinders stock assessment and management required for sustainable exploitation. Additionally, these keystone species are useful tools in assessing coastal habitat quality, due to their eco-biological features. Contamination of these species with heavy metals carries serious risk for animal and human health due to their potential of biomagnification in the food chain. Thus, the use of these species as bioindicators is warranted to the establishment of conservation measures targeting marine coastal environments. Climate change increases the level of environmental stress to which intertidal organisms are subjected to, affecting the functioning of biological systems at different levels of organization. *Phorcus* species have been widely used as indicators of the effect of climate change on local disturbances of intertidal ecosystems and geographic distribution shifts of these organisms. Further studies concerning biological parameters of *Phorcus* species and how they react to exploitation, pollution, and climate change will consolidate these species as indicators of large-scale ecological impacts of anthropogenic activities.

Keywords: *Phorcus*, topshells, life history traits, fisheries, pollution, climate change

1. Introduction

Topshells are marine gastropods that inhabit the rocky shores. These marine snails together with limpets and winkles are the most successful algal grazers present in the intertidal of the Northeastern Atlantic and Mediterranean Sea [1]. Topshells occupy the rocky sea shores from the supratidal to the subtidal, one of the most extreme, heterogeneous, and dynamic environments in nature that expose these organisms to different levels of thermal and hydric stresses [2, 3]. These unpredictable environmental conditions are therefore responsible for many of their peculiar morphological and biological characteristics that can be perceived as adaptations to the intertidal environment [4]. The marine snails of the genus *Phorcus* are ecologically important algae grazers that play a major role in regulating the ecological balance of their habitat and have often been used as biological indicators in evaluating the consequences of anthropogenic impact on this ecosystem [4, 5].

The diversity and ecological importance of the genus *Phorcus* prompted intensive research over the past years. Recently, this genus was redefined by Donald et al. [5] to include species previously under the genus *Monodonta* Lamarck, 1799, or *Osilinus* Philippi, 1847, allowing to trace the biogeographic history of this genus' origin to 40–20 Ma, prior to the closure of the Tethyan Seaway.

Intertidal invertebrates' life history traits vary inter- and intraspecifically because of genetic differences and environmental effects. Growth, reproductive strategy, and mortality depend on a complex combination of selective forces and are fundamental to understand the distribution and abundance of these species along the intertidal [6, 7]. As such, knowledge of life history traits of *Phorcus* populations provides important information required to understand how these species adapt to an ever-changing environment, whether because of human activities, such as fisheries, habitat disturbance, pollution and climate change, or natural causes.

One of the main causes of disturbance in the intertidal ecosystem is the harvest of gastropods in the rocky shores, which has occurred since prehistorical times, resulting in shifts in abundance and/or size structure of these species. Another cause of disturbance is the contamination of coastal waters, by the presence of unnatural chemicals, as a result of industrial spillage and sewage discharges among others. Gastropod molluscs are frequently used as bioindicators to assess the health status of the coast and determine the effect of marine pollution [8]. Walsh et al. [9] recorded that these sentinel species have the potential to act as a useful biomonitoring system of pollutants in the marine environment. As such, they act as pollution indicators by tracing metals, providing information required for the establishment of protective measures of the ecosystem.

Phorcus species are recognized as good bioindicators of water quality due to their reduced mobility, easy sampling, adequate size for tissue analysis, widespread distribution, abundance all year-round, and ability to accumulate high metal concentration in their shell and tissues, reflecting heavy metal availability in coastal waters [10, 11].

Global climate change also causes disturbance in the intertidal ecosystem that results in changes in the geographical distribution of marine gastropods. Intertidal invertebrates are

known to respond to climate change through alterations in biogeographic distributions following a latitudinal gradient, from warmer towards cooler regions. *Phorcus* species are bioindicators and changes in their distribution have been successfully linked to hypothesis of climate change on Northeastern Atlantic shores, particularly in species presently at their northern limits, which may be expected to move further north as the coastal waters continue to warm, as has happened in the last decades.

The aim of this work is to compile and review a wide array of subjects related to *Phorcus* species biology and ecology, comprising anatomy, growth, reproduction, mortality, behaviour, and ecological role and also to evaluate and discuss the consequences of anthropological impacts such as fisheries, pollution, and climate changes on these keystone species and their potential as bioindicators of the effect of human activities on coastal marine environments.

2. Biology and ecology of topshells

2.1. Anatomy

Gastropods are comprised essentially of two main parts: the shell and the body. These asymmetrical molluscs have a twisted, spirally coiled shell around its body, which protects them from biotic and abiotic factors present in their environment, and a corneous or calcareous operculum, a flat plate that rests on the upper dorsal side of the foot that acts as a supporting pad for the shell. When the snail actively moves or blocks the aperture, the body withdraws, protecting the animal from predators and preventing water leakage in exposed rocky shores [12, 13].

In topshells, the shell is complete and usually pyramidal, moderately large, conical to globose in shape, with rounded to angular body whorls and often with a flattened base and an interior consisting of mother-of-pearl. This structure is formed in the embryonic stage, with the secretion of protein fibres from the outer skin of the visceral mass and from the mantle, while they are free-swimming larvae and they are followed by the secretion of calcium carbonate from the same cells. Posterior to the embryonic phase, the shell continues to grow through the addition of a protein mesh and calcium carbonate mostly on its margins but also on its interior. Shell growth is not continuous and it frequently leaves different growth lines since maturity and adverse environmental conditions may cease growth. The shell offers refuge both from predators and from desiccation being impervious to gasses and liquids and resistant to crushing [12–14]. Colour patterns of the shell are usually highly variable in topshells and are mostly related to diet rather than to genetic control (**Figure 1**) [12].

The soft body consists of two compartments connected by a waist and present a dark ash colour with a greenish tint [15]. The lower compartment encompasses the muscular foot and the head. The foot is used for locomotion over the substrate, swimming, jumping, and returning the animal to an upright position when overturned. Also, it helps to detect food. The upper compartment is used for respiration, digestion, excretion, gamete production, and shell secretion. The body of these organisms comprises a head with a short snout, a pair of conical

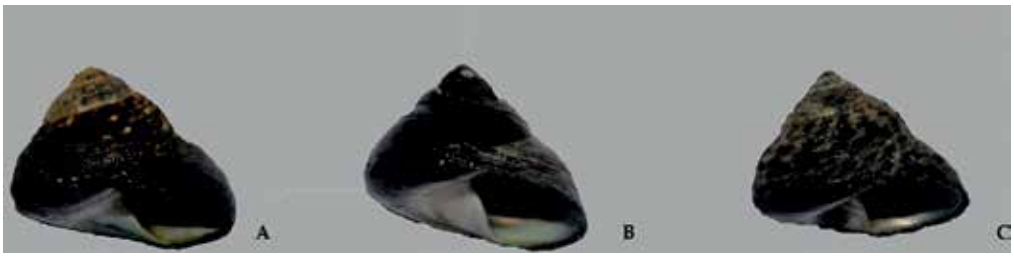

Figure 1. Shell phenotypic variability of *Phorcus sauciatus*. A – Portugal mainland, B – Madeira Island, C – Gran Canaria Island.

and papillate tentacles, cup-shaped open eyes on distinct stalks, a foot, a muscular ventral organ with a flattened base used for locomotion, and a visceral mass, which fills dorsally the spire of the shell and contains most organ systems and the mantle, a collar-like tegument, which lines and secretes the shell, and forms a mantle cavity normally provided with respiratory gills for breathing in water and a well-vascularised mantle cavity, which allows the animals to breathe in air [13, 14].

2.2. Taxonomy and geographic distribution

Phorcus Risso, 1826, are herbivorous marine snails (Gastropoda: Prosobranchia) belonging to the family Trochidae Rafinesque, 1815, that inhabit rocky shores from the Mediterranean Sea through the Northeastern Atlantic Ocean including the Macaronesian Archipelagos of Madeira, Canaries, Azores, and Cape Verde [14].

This genus of gastropod grazers is currently represented by nine recognized living species [5, 6] and is comprised of *Phorcus articulatus* (Lamarck, 1822), *Phorcus atratus* (Wood, 1828), *Phorcus lineatus* (da Costa, 1778), *Phorcus mariae* Templado & Rolán, 2012, *Phorcus mutabilis* (Philippi, 1851), *Phorcus punctulatus* (Lamarck, 1822), *Phorcus richardi* (Payraudeau, 1826), *Phorcus sauciatus* (Koch, 1845), and *Phorcus turbinatus* (Born, 1778) [1, 5].

There is a clear separation between the species of *Phorcus* that occur in the Atlantic and the Mediterranean. This split distribution is thought to result from the barrier imposed by the Strait of Gibraltar, since there is no species overlap in the adjacent area, and the nearby Alboran front that act as biogeographic breaks for animals with short larval stages, such as *P. lineatus*, whose lecithotrophic veliger larvae remain in the water column for, at the most, 6–7 days [5, 14, 16]. As such, four species of this genus are restricted to the Mediterranean Sea, specifically *P. turbinatus*, *P. mutabilis*, *P. articulatus* and *P. richardi* and the remaining five species occur in the Northeastern Atlantic Ocean, namely *P. lineatus*, *P. sauciatus*, *P. atratus*, *P. punctulatus*, and *P. mariae* (**Figure 2**) [5].

In the North Atlantic Ocean, *P. lineatus* is the species that reaches the northernmost geographic limits of the genus *Phorcus* in North Wales and Ireland and *P. punctulatus* the southernmost limits in Senegal. *P. mariae* is restricted to Cape Verde archipelago, *P. atratus* to the Canaries archipelago and Selvagens Islands, and *P. punctulatus* to Senegal [1, 5, 14]. *P. lineatus* has a wide distribution ranging from North Wales and Ireland to Morocco and *P. sauciatus* includes

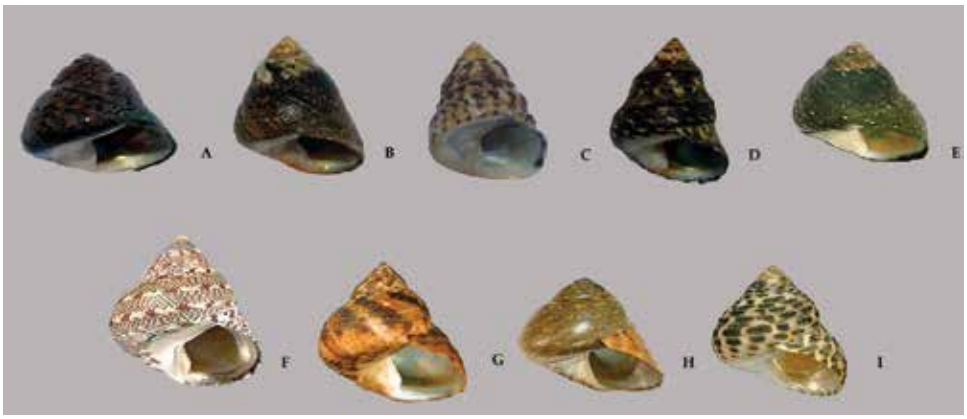

Figure 2. Shells of the nine species of the genus *Phorcus*. A – *Phorcus sauciatus* from Madeira archipelago, B – *Phorcus lineatus* from mainland Portugal, C – *Phorcus atratus* from Selvagens Islands, D – *Phorcus mariae* from Cape Verde archipelago, E – *Phorcus punctulatus* from Senegal (NMR 36429) [17], F – *Phorcus articulatus* from Spain (NMR 36447) [17], G – *Phorcus mutabilis* from Greece (NMR 36658) [17], H – *Phorcus richardi* from Greece (NMR 36669) [17], I – *Phorcus turbinatus* from Greece (NMR 36606) [17]. Images E, F, G, H, and I by Joop Trausel and Frans Slieker and available online at NMR – Natural History Museum Rotterdam [17].

the Macaronesian archipelagos of Madeira, Canary, and Azores with its northern boundary in the Iberian Peninsula and its southern limit in the African mainland, with negligible genetic differentiation between them, suggesting either recent or continuing dispersal among these areas [5, 18, 19].

Concerning the geographic distribution of the genus *Phorcus* in the Mediterranean Sea, *P. turbinatus* occurs from Spain to Cyprus, *P. articulatus* from Spain to Tunisia, *P. richardi* from Spain to Croatia, and *P. mutabilis* from Italy to Turkey [5].

Topshells as limpets are subject to an array of environmental stresses due to their extended vertical distribution, which ranges from the upper to the lower shore levels. Thus, these organisms can exhibit varying degrees of structural adaptations since their position relative to the shore influences their exposure to desiccation, hydrodynamic action of the waves, temperature variation, and tidal width [20–23], resulting in a wide array of intraspecific phenotypic variability.

2.3. Respiratory system

Marine snails of the genus *Phorcus* have a gill for water breathing and a well-vascularised mantle cavity, which allows the animal to breathe in the air [14]. The mantle cavity placed between the body and its overhanging mantle skirt is constituted by a single gill in the front part of the mantle cavity and thin-walled organs that absorb oxygen from the sea water [12].

The marine snails' blood, the haemolymph, contains haemocyanin, a copper-containing protein that can fix and transport two to three times more oxygen, from the gills to the heart, than organisms without this protein. The heart pulsations push the oxygen-rich blood over a closed system of arteries that lead the blood to a system of open arteries, without epithelial

walls, that surround the viscera and the muscles covering all organs with oxygen-rich blood. The body organs receive the oxygen from the haemolymph and release carbon dioxide into it, which then returns to the gills, via a system of veins, where it releases the carbon dioxide and again receives oxygen [12].

2.4. Feeding habits, behaviour, and ecological importance

Molluscan grazers are known to have an important influence on the overall structure of benthic marine communities, because of the influence and control they exert on algae [24, 25]. Removal of grazers often leads to an imbalance on the population dynamics of the species involved on the rocky shores ecosystem, due to a dramatic development of seaweed beds [25].

Topshells, winkles, and limpets form a guild of microphagous herbivores that feed on microbial biofilms, by grazing the rocky substrate with the radula, a specialized rasping organ unique to molluscs, on which successive rows upon rows of backwards-pointing teeth are placed. The teeth crack, break, and wear away during use, by the food or the hard substrate from which the sea snail scrapes [12]. Marine snails can all be found together, grazing on the open shore, and it is probable that these various snail species do not feed in exactly the same place, at the same time, in the same manner, or on exactly the same food [14] in order to avoid interspecific competition. The feeding adaptations between these species can be behavioural through spatial differentiation or anatomical through adaptations in the radula. Among these species, radulae show different hardness and patterns, being multi–fine-toothed rhipidoglossan in topshells, less complex taenioglossan in winkles, and simple docoglossan in pattelid limpets; therefore, it is easy to conclude they feed in different ways [14].

In several species of sea snails, the digestive fluids contain the cellulase enzyme that breaks down cellulose. This is one of the very few cases throughout the animal kingdom of an animal producing an enzyme capable of breaking down cellulose [12]. Feeding behaviour in topshells is assumed to occur at night or during high tide as stated by Crothers [14] for *P. lineatus*. Food particles are gathered by the radula, squashed by the jaws, and then transported inward into the mouth where the digestive track begins, in the front of the body, and then transported back along the body through the oesophagus to the stomach where most of digestion occurs, and finally, digested food loops and descends forwards to the intestine where faeces are formed and expelled by the anus, which drains into the mantle cavity, at the front of the body [12].

Common topshells and edible winkles swing their head from side to side while crawling and may leave grazing tracks on the rock surface and visible slime trails. Usually, the more active species secrete a thicker layer on which to crawl and this may show up as a pale band over the rock surface. Trail-following, namely the crawling over existing mucus trails, will reduce the expense of producing a mucus trail. These trails might also be used to locomote back home, to find mates, and to assist in feeding, by trapping food particles from the water column [12]. Marine snails crawl by squeezing the front end of the foot against the substrate and by means of a ripple of muscle contraction, pass that point of contact forcing the mass of the snail forwards. In topshells, the two halves of the foot work independently of each other, out of phase, producing a characteristic slime trail [26].

Contrary to limpets, topshells are active at low tide and respond very rapidly to changes in weather conditions, moving out into the open when the sun shines and hiding from rain or cold winds in crevices or under boulders [26]. These species are limited in their vertical zonation by their tolerance to temperature variation; as such, they undertake vertical migrations up and down the shore over the seasons [27].

Wave action also acts as a limiting factor on suspension feeders and on semisessile and sessile organisms that are favoured on exposed conditions, since the water movement allows the flow of food, propagules, nutrients, and preys to these organisms. However, in these habitats, the increase of exposure to wave action involves an increase on the risk of dislodgement and physical damage, limiting the range of susceptible and physically fragile species [2]. In order to overcome the adverse conditions of the exposed areas, intertidal gastropods inhabiting these areas have a thin and smooth shell with large aperture due to the large foot required to cope with the higher risk of wave displacement and to be able to maintain a firm hold on rocky surfaces [28, 29]. In dangerous circumstances, a snail withdraws into its shell and adheres firmly to the substrate, so as to not be detached by waves or predators [12]. In the Northeastern Atlantic, *P. lineatus* is usually used as an indicator of sheltered rocky shores [30] contrary to *P. sauciatus* that seems to be more tolerant to wave action being found lower on the shore but also able to establish on sheltered zones [18]. The anatomic features of these two species corroborate this hypothesis since *P. sauciatus'* thinner shell, larger foot, and consequently large aperture imply that this species is more tolerant to wave action than *P. lineatus* with thicker shell and smaller aperture. On the other hand, these anatomic differences result in *P. sauciatus* being less tolerant to desiccation than *P. lineatus*.

2.5. Growth

Growth is a key variable in determining the survivability of any given animal, and it is important to understand the factors that drive it [31]. Biological parameters such as growth rate, asymptotic length, longevity, and age structure reflect the overall state of health of a population and are commonly used as stock assessment tools of exploited marine organisms [4]. In gastropods, growth rates have been determined through several features such as growth lines and rings in shells [32, 33], opercula [34], and statoliths [35]. Size and age of topshells are positively related, thus allowing to investigate population structure [36].

Size and growth rates in the species of the genus *Phorcus* are influenced by fluctuations in food supply [26, 37], competition [38], and wave action [39], while population density is mainly controlled by the successful settlement of larvae and predation [26, 38]. The oceanographic current systems are known to be largely responsible for the water temperature and nutrients of the coastal ecosystems, which mark the distribution and behaviour of organisms throughout the coastlines [2]. As such, temperature also influences growth in the species of the genus *Phorcus*. For instance, Crothers [14] and Mannino et al. [40] observed that a decrease in water temperature promotes a metabolic deceleration, resulting in the interruption of growth during the winter in *P. lineatus*. However, after this season, growth continues rapidly through the year, slowing only in the next winter. In general, in the first year, the growth rate of this species is high and decreases thereafter [14] as a possible result of achieving sexual maturity.

In the first six months postsettlement, specimens can grow up to 8 mm diameter, reaching 11–15 mm by the end of the year [41]. Although the growth rates slow down dramatically after the achievement of sexual maturity, since energy is mostly directed towards reproduction, growth continues throughout the life cycle of this species. In habitats with low abundance, *P. lineatus* grows rapidly to a large size and reaches maturity early but has lower longevity. While individuals that live in habitats where they are more abundant grow slowly, they do not achieve great size and may live to an older age. These differences in growth are likely related to different levels of food availability depending on population density, which in turn is related to settlement success and predation evasion [26]. The specimens of this species have been known to reach a size of 34 mm in shell height and a longevity of 15 years of age in southern Britain [36]. *P. sauciatus* have approximately the same size range of *P. lineatus.* For instance, in the Madeira archipelago, *P. sauciatus* size ranges from 2 to 28 mm (pers. obs.); in the Canary Islands, this species size ranges from 5 to 26 mm [42]; and in the Portuguese mainland coast, its size ranges from 7 to 24 mm (pers. obs.). There is, however, a great gap in knowledge concerning life history parameters of *Phorcus* species. Most studies focused on *P. lineatus* due to their wide geographical distribution spanning from Morocco to North Wales/Ireland. Life history parameters such as growth rates, asymptotical length, size at first maturity, recruitment patterns, and mortality of *Phorcus* species are likely to differ inter- and intraspecifically as a result of different biotic and abiotic factors. Further studies on the biology and population dynamics of *Phorcus* are therefore required in order to guarantee the implementation of successful conservation strategies and a sustainable exploitation based on effective management measures.

2.6. Reproduction

Topshells' reproductive system is usually strikingly simple, with a genital duct opening into the mantle cavity through the right kidney. Sea snails commonly have separate sexes but these species are not externally sexually dimorphic and sex determination is only possible through macroscopic observation of the gonads. Internally, the most reliable character for sorting them is the appearance of the urogenital aperture. In males, the lips of this organ are unpigmented and smaller, while in females, the lips are yellow and swollen. Nevertheless, in the ripe state, males have cream testis and females greyish-green ovary covering the digestive gland and viscera [43, 44], being therefore easily differentiated in the breeding state. The lobes of the gonad, whether ovary or testis, lie near the apex of the visceral hump, among the lobes of the digestive tube, and they drain into the pericardium [12].

Prior to the breeding season, adults migrate up shore to the high eulittoral zone. It seems that this migration brings the animals into a region of higher temperature required for spawning. An increase in temperature may stimulate spawning as suggested by Desai [44] who observed that adults that have migrated furthest up shore were the first to spawn.

In fact, spawning in intertidal organisms seems to be promoted by environmental triggers such as temperature, high wind speed, and wave action. Biological factors as an increase in phytoplankton concentration may also stimulate spawning as occurs in limpets [38, 45]. As such, breeding stages of a given species may differ according to their geographical position. In fact, in

the northernmost range limit, breeding seasons are shorter with a single spawning period, while in southern regions, the breeding season is longer with multiple spawning events [46, 47]. For instance, in *P. lineatus* from Austurias, Spain, the gonadal development occurs from November to June and the breeding stages from June to September and may last until November in some specimens [46]. Spawning occurs between May and August [48]. Further north in Wales, the same species is reported to have a shorter spawning season, lasting from July to August [14]. On the other hand, *P. turbinatus* that occurs in the Mediterranean Sea appears to have a longer breeding period with two spawning events in spring and autumn [49].

Fertilisation is external, with both sexes releasing their gametes into the sea and the whole process occurs directly in the water. During the reproductive season, males and females approach each other and then females send out chemical signals, leading to sperm being discharged in the water by males, which in turn stimulates females to release the oocytes [12]. According to Desai [44], males discharge clouds of spermatozoa that become very active 2 or 3 minutes after being released, and females liberate oocytes separately, a few at each spasm. This process of external fertilisation, regarded as a primitive trait in snails, becomes a high-risk strategy and improbable to succeed unless the species is locally common [14]. The fertilised egg develops within approximately a day and becomes trochophore larvae, which are capable of independent locomotion. The veliger larvae enclosed in a tiny shell develop in one or two days. At metamorphosis, the veliger turns upside down with the foot becoming ventral and the shell dorsal. Posterior to the snail's development, the back dorsal rotates in 180° anticlockwise in relation to the head and foot. Veliger larvae remain in the water column for at most 6–7 days [5, 14, 16], and at settlement, the shell measures a little over 1 mm across [14]. According to Heller [12], the trochophores of the genus *Phorcus* hatch down shore, within approximately one day and the veliger settles 4–5 days with about 1 mm. For *P. lineatus* in the United Kingdom, the recruits achieve 5–6 mm shell length by the first autumn and are detected on the bare rock between September and November and recognized, with 6–14 mm, through their first year [33].

The gap in size at settlement and size at first capture reported for topshells may be understood as a potential argument for the existence of nursery areas, underneath boulders or fissures, in which small juveniles are much commoner, but there appears to be no uniform pattern [14]. For instance, in Madeira archipelago, the juveniles of *P. sauciatus* are commonly found under boulders, with the smallest individuals having 2 mm in diameter (pers. obs.). These boulders may function as a nursery for topshell juveniles as they provide protection against abiotic factors, such as wave action and desiccation, and biotic factors, such as predation and substrate competition.

3. Anthropogenic impacts on the genus *Phorcus*

3.1. Harvesting

Intertidal and shallow-water grazers are extremely vulnerable organisms because of their limited habitat and their accessibility to human activity [50]. Hunter-gatherers have exploited intertidal grazers, since prehistoric times, and there are evidences that the densities and the

maximum sizes of several species were reduced by the exploitation [51, 52]. Studies performed in Northern Spain showed that topshells and limpets were collected, at subsistence exploitation levels, from intertidal areas of exposed shores, leading to the formation of huge shell middens [53]. In fact, intertidal resources have always been collected by humans as food supplement or used as a bargaining chip with other products worldwide [54, 55].

Several studies were carried out aiming to investigate the temporal patterns of worldwide topshell exploitation. A proven approach to study these temporal patterns of prehistoric shellfish exploitation is the analysis of the oxygen isotopic ratio (d18O) of the latest growth increment of mollusc shells [56, 57]. Variations in oxygen isotope ratios from shell carbonates are mostly dependent on sea surface temperature (SST), which enables the estimation of temperatures during periods of shell growth and helps to determine the season of the year when the mollusc died [58]. Colonese et al. [59] applied this approach to the topshell *P. turbinatus* from archaeological sites in Italy and concluded that Mesolithic *P. turbinatus* exploitation was carried out almost exclusively during the colder and intermediary seasons, with very sporadic harvesting during the warmer seasons.

The same approach has been followed by Gutiérrez-Zugasti et al. [58] that confirmed the potential of oxygen isotope analysis on *P. lineatus* for paleoclimate reconstruction and also showed that the aragonite of those shells grew under conditions of isotopic equilibrium, opening new avenues for future research. This species is commonly found in Holocene archaeological deposits of Atlantic Europe and is one of the most abundant subsistence resources utilized during the Mesolithic in northern Spain.

Continued exploitation of these species is likely to incur in shifts on size and shape over time. Colonese et al. [59] observed a significant change in shell shape of *P. turbinatus*, with slender Mesolithic shells being replaced by squatter forms in the Meso-Neolithic. These differences were explained with collection shifting from sheltered shores in the Mesolithic to exposed rocky shores in the Meso-Neolithic, thus confirming the potential effect of human collection on size and shape of this exploited species.

In recent times, however, the pattern of exploitation has changed both quantitatively and qualitatively, due to the expansion of human population, to the commercial value of several species and to the industrial development that facilitated shipping and flying products around the world [25]. Limpets, abalones, chitons, winkles, and topshells are common gastropods of intertidal rocky shores; however, some species are in serious decline mainly as a consequence of overexploitation [60]. The exploitation of these resources has plentiful direct and indirect effects on the trophic chains of marine ecosystems, with potential complex cascading effects [61].

The direct effects of exploitation are the decline of the exploited species′ abundance and a shift in size composition of their populations that results from the size-selective nature of harvest. Ramírez et al. [42] assessed the effects of human impacts over the abundance and size patterns of topshells (*P. atratus* and *P. sauciatus*) and limpets (*Patella aspera* Röding, 1798, *Patella candei* d'Orbigny, 1840, and *Patella rustica* Linnaeus, 1758), usually collected in the Canaries archipelago. The authors observed significant differences in size structure of these

species among islands, according to the level of human influence and verified that not only all large-sized individuals disappeared from the most populated island, but also that there was a decrease in numbers for the majority of size ranges, concluding that the observed differences among islands were mainly a consequence of the human activities. In fact, in exploited populations of broadcast spawners such as topshells and limpets, the decrease of larger individuals will reduce the reproductive success leading to a decrease in population abundance and, in extremes cases, conduct to the disappearance of the species [62, 63].

Also, differences on spatial distribution of the abundance and biomass of *P. articulatus* were observed by Cheour et al. [64] along the coast of Tunisia. The authors concluded that these differences were related to several anthropogenic and environmental factors.

Even though species of the genus *Phorcus* have been exploited by humans since prehistoric times, information regarding the status of exploited stocks is scarce and exploitation is generally unregulated. Recently, some efforts have been undertaken in the Canaries archipelago, aiming to contribute to the recovery of the stock of *P. sauciatus* and *P. atratus* in a short and medium term, namely by implementing a minimum capture size of 15 mm of shell longitude for both species [65].

Overexploitation of marine organisms prompts the implementation of management policies in order to protect the exploited populations and mitigate human impacts. Currently, protection of *Phorcus* species is mostly guaranteed by the implemented marine protected areas (MPAs); however, further measures should be equated especially in regions where exploitation of these species is more intensive. Management measures and regulation aiming at a sustainable exploitation of these species are therefore warranted, as is the improvement in enforcement of existing legislation and involvement of all interested stakeholders; otherwise, protection of topshells will remain ineffective.

3.3.1. Harvest of Phorcus sauciatus in the Madeira archipelago: an historical perspective

P. sauciatus is the most abundant of the two species of the genus *Phorcus* described for the Madeira archipelago and has a wide geographical distribution, occurring in all islands including Madeira, Porto Santo, Desertas, and Selvagens. *P. atratus* is also present in this archipelago as the endemic subspecies *Phorcus atratus selvagensis* restricted to the Selvagens Islands. However, according to Donald et al. [5], the classification of this subspecies needs additional clarification.

P. sauciatus is harvested in the Madeira archipelago since early colonization times, remounting back to the beginning of the fifteenth century. According to Silva and Menezes [66], *P. sauciatus*, formerly identified as *Trochus colubrinus* Gould, 1849, occurred in the intertidal zone of all the islands and was consumed salted or pickled, being imported from the Selvagens Islands in a relatively large quantity. This species was also used as bait for fisheries.

Nowadays, *P. sauciatus* continues to be caught in Madeira and Porto Santo, except in marine protected areas, without harvest regulation or auction obligation. The harvest of this species in the Madeira archipelago became more intensive due to the development of their commercial

exploitation supported by technological advances in methods of collection, processing, storage, and transportation, but also due to the increase in human population density and the accessibility to the coastal zones. As such, shifts in abundance and/or size structure of this species occurred mainly in the south coast of Madeira Island, resulting in a reduction in abundance levels and sizes of the caught specimens, due to the existence of more favourable environmental conditions, higher population density, and easier accessibility.

Given the current scenario, it has become vital to know the biological and ecological traits of *P. sauciatus* in Madeira archipelago and its population dynamics. As such, the Fisheries Research Service from the Regional Directorate of Fisheries of the Autonomous Region of Madeira presently develops a full study on this species aiming to establish proper conservation strategies, in order to preserve this important keystone resource of the intertidal zone, that would contribute towards the reduction of the risks of overexploitation and promotion of a sustainable harvest of *P. sauciatus* in the Madeira archipelago, through the implementation of suitable regulation and management measures considering the biological and ecological specificities of this species in this region. At a first glance, the implementation of regulation concerning harvest techniques, maximum allowable catch weight, and minimum catch size should be considered and also mandatory landings. Depending on the results obtained in the study, other measures might have to be pursued in order to provide an adequate management for a sustainable exploitation of this resource such as the establishment of a closed season to ensure optimal reproductive success.

3.2. Pollution: topshells as bioindicators of habitat health

The ecological effects of increasing levels of heavy metal concentrations in the environment are of great concern due to their high bioaccumulative nature, persistent behaviour, and high toxicity [67].

The increase of human population and anthropogenic activities, such as the development of industry on the coastline, are the major responsible factors for pollution hot spots that occur predominantly close to major ports, industrial areas, and cities [68]. Maritime traffic also acts as a source of pollution due to the antifouling paints of boats [69]. Marine and especially coastal ecosystems are increasingly endangered by the large amounts of metal pollutants, arriving to this environment mainly by superficial runoff of rain, by direct atmospheric deposition, and by discharges from sewage effluents, spillage, and industrial establishments [70, 71]. Biological and physiological alterations in benthic communities may occur due to the toxic effects of metals and due to the sedentary lifestyle of these species [72]. Aquatic organisms can accumulate petrogenic and anthropogenic compounds such as n-alkanes, polycyclic aromatic hydrocarbons (PAHs), and polychlorinated biphenyls (PCBs) from the environment into their lipid tissues, some of which can be carcinogenic and/or highly toxic for living organisms [71]. Most of the comparative studies between taxonomic groups indicate that bioaccumulation of pollutants in molluscs is, in general, much superior than in fish [73]. Mollusc shell and tissues reflect the higher degree of environmental pollution by heavy metals and are the most useful bioindicator tools. The metal body burden in molluscs may reflect the concentrations and availability of heavy metals in the surrounding water and sediment and may thus be an

indication of the quality of the surrounding environment [74]. These organisms accumulate comparatively higher concentrations of metals, both from water and sediment, because of their sedentary nature [67].

The worldwide increase of pollution levels on coastal zones has led to the awareness of the need to perform ecotoxicological research and to define sensitive bioindicators that allow the evaluation of contamination degrees, aiming to recommend the appropriate measures to conserve the ecology of the coastal areas. The species of the genus *Phorcus* act as sentinel species due to their particular ecobiological characteristics, as abundance and wide geographical distribution, long life span, suitable dimensions, easy identification, and collection, becoming a useful biomonitoring system of pollutants in the marine environment and making these organisms suitable to measure for hazard and risk assessment. These molluscs are also sturdy enough to survive in laboratory and field studies and tolerant to environmental alterations and various contaminants [67].

P. turbinatus is generally considered as a bioindicator of metal pollution in coastal areas [69, 75], because of their ability to tolerate temperature and salinity fluctuations and survive even in hypoxia [75, 76]. Boulajfene et al. [77] evaluated the degree of metal contamination in *P. turbinatus* and monitored the impact of metals on metallothioneins functioning in the Northeastern and the Eastern coasts of Tunisia and found that sedimentological metallic contents of copper, zinc, and cadmium vary according to the area where these species live. It seems that this species has an ability to accumulate these metals and that the metal effect on protein induction may be linked to physical factors such as temperature, oxygen, and copper contents in sediment. Boucetta et al. [78] assessed the health status of *P. turbinatus* on the Algerian East coast through the analyses of the concentrations of trace metals in this species tissue and of biomarkers such as the activity of acetylcholinesterase (AChE) and glutathione-S-transferase (GST) and verified that the alteration of the activity of AChE with the induction of GST was mainly due to the presence of high concentrations of trace metals and abiotic factors including salinity and pH.

In fact, several environmental factors such as water current, water flow, renewal of water, pH, and salinity affect the distribution of heavy metals in molluscs as reported by Grupta and Singh [67]. Survival is significantly affected by salt concentration and by temperature, as well as by the interaction between them, so that the toxicities of salts are generally enhanced at higher temperatures.

Other studies support the efficacy of topshells as bioindicators, such as Bordbar et al. [79] who investigated the impact of a ferronickel smelting plant on the coastal zone of Northern Greece through the study of metal bioconcentration in *Patella caerulea* and *P. turbinatus*, concluding that the ferronickel smelting plant had heavily impacted the coastal zone. Another study on the southeast coast of Tenerife reports the use of *P. atratus* in the evaluation of the concentrations of *n*alkanes and PAHs in the visceral mass and demonstrated that this species is contaminated with a chronic background of aliphatic and hydrocarbons strongly retained in their lipid tissues and suggested this species as a bioindicator of petrogenic contamination [71]. Cabral-Oliveira et al. [80] presented further evidence by comparing the accumulation of trace elements in edible molluscs *Mytilus galloprovincialis* Lamarck, 1819, *Patella ulyssiponensis* Gmelin, 1791, and *P. lineatus*, between one sewage-impacted area and two reference areas in

central western coast of Portugal and suggested that the concentrations of trace elements in the soft tissues of these molluscs can be affected by the presence of sewage discharges.

Bioaccumulation of pollutants in molluscs is, in general, much superior than in fish due to their sedentary nature. Thus, their shell and tissues reflect the levels of environmental pollution and are the most useful bioindicators regarding the quality of the surrounding environment. As such, there is a growing interest in the use of these marine gastropods as bioindicators, due to their ecobiological features, both in a scientific and ecosystem management perspective. This approach will contribute to the establishment of conservation measures targeting marine coastal environments. Also, all species of the genus *Phorcus* are a food source for other species; therefore, if these species are contaminated there is a potential for biomagnification in the food chain that can carry serious risks both to wildlife and human health.

3.3. Climate change effects on intertidal communities: impacts on topshells of the genus *Phorcus*

The history of earth is riddled with events that have shaped different ages, each with specific conditions that characterized them. One of these characteristics is global temperature that has oscillated numerous times over the course of earths' long history and thus shaped biodiversity throughout the ages. For instance, the change in mean temperature between the late Pleistocene (colder conditions) and the early Holocene (warmer conditions) lead to a taxa alteration between these two periods. The more abundant species adapted to cold water, such as the periwinkle *Littorina littorea* (Linnaeus, 1758) and the limpet *Patella vulgata* (Linnaeus, 1758) in the late Pleistocene, were replaced by species better suited to warmer conditions such as *P. lineatus*, *Patella depressa* Pennant, 1777, and *P. ulyssiponensis* in the Holocene [81, 82]. A similar pattern is visible today in the Cantabrian coast, with a predominance of warmer species such as *P. lineatus* and the absence of *L. littorea* [53].

Nowadays, however, global climate change is recognized as a reality, driven mostly as a direct consequence of human activity [83, 84], namely, through the cumulative postindustrial carbon emissions to the Earth's atmosphere [85]. Known consequences of climatic change in the marine environment are the increasing global temperature, perturbed regional weather patterns with increasing wind velocity and storm frequency, rising sea levels, ocean acidification, changed nutrient loads, and altered ocean circulation [86]. These and other physical consequences are affecting marine biological processes from genes to ecosystems, over scales from rock pools to ocean basins, impacting ecosystem services and threatening human food security [85]. The rates of physical change are unprecedented in some cases and biological changes are also likely to occur at a quick rate, although the resilience of organisms and ecosystems is highly variable. Biological changes founded in physiological response manifest as species range changes, invasions and extinctions, and ecosystem regime shifts [85].

Coastal ecosystems are among the most vulnerable to climate change, especially the intertidal areas, which have shown faster biogeographic changes [87, 88] than those found in terrestrial environments [89]. Long-term monitoring studies have shown that the distribution limits of the intertidal biota of hard substrates have progressed towards the poles at a rate of over 50 km per decade [88, 90, 91].

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Invertebrates and seaweeds, inhabiting the intertidal, may be particularly vulnerable to fluctuating temperatures, since individuals must adapt to the extreme temperatures of both the terrestrial and marine environments [92]. Even in small spatial scales in the intertidal zone, a broad range of thermal conditions is found that may exceed the range of large latitudinal bands. Therefore, intertidal organisms are believed to be at the limit of their physiological tolerance since these organisms are sorted by zonation in which the upper limit of one species is set by physiological stress, and species replace one another moving up the shore [88, 93]. The species most tolerant to heat and desiccation live at the top of these zones [94]. Since these organisms are thought to live at the utmost extremes of their physiological tolerance limits, any changes in abiotic parameters such as temperature and air exposure time could lead to death or local extinction [95, 96].

On the other hand, these changes can also lead to the expansion of the range and distribution area of some species. Thus, intertidal ecosystems are thought to be among the first to show responses to increases in global temperatures [95, 97] and are potential environments to assess the effects of climate change [98].

Rising temperatures can result in increased thermal stress and desiccation at low tide and in latitudinal changes in species abundance and distribution. However, changes in temperature affect the rocky intertidal; for instance, rising sea levels can result in altered zonation of intertidal biota and compression on vertical engineered defences. Also, increased storm frequency can result effectively in higher levels of wave exposure, resulting in shifts in community structure, due to a replacement of grazers by filter feeders, and shifts in direction of trophic control [85].

Intertidal organisms are subject to other factors that can lead to significant physiological stress and mortality such as shifts in salinity, increased levels of siltation, and prolonged oxygen or nutrient deprivation [99–102]. These factors play an important role in reproduction and survival of these organisms and are predicted to change in the coming decades as a result of global climate change. In fact, some of these changes are believed to have already occurred as ecological impacts on coastal ecosystems [103].

A species geographic limit reflects the interactions of organisms and their environment and is likely one of the first signals of the effect of climate change on the biota of the planet [89]. Geographical range limits impose environmental stresses, such as temperature, to populations that restrict adult survival or juvenile recruitment [88, 93]. This is related to the organisms' physiological tolerance to temperature. Exceeding these tolerance limits results in the organism's death and can lead to the local extinction of a population if temperatures are extreme enough [89]. Changing climatic conditions results in shifts of geographical limits in which populations can survive and reproduce thus acting as indicators of the processes of long-term climate change [88, 89, 93].

Species of the genus *Phorcus* like other intertidal organisms are considered good indicators of the effects of climate change in marine ecosystems. For instance, *P. lineatus* has been identified as an indicator species for monitoring climate changes around the coasts of Western Europe [104] due to its extensive biogeographic distribution, ranging from North Wales and Ireland

to Morocco [5, 14]. Crothers in 1994 [33] showed evidences that this species has extended its geographical distribution range in the Bristol Channel eastwards along the Somerset coast for atleast 20 km in the past 50 years and suggests that it may be still advancing. In fact, a decade after Mieszkowska et al. [105] reported that *P. lineatus* and *Gibbula umbilicalis* (da Costa 1778) have undergone North and Northeastern range extensions in Britain, with the increase in abundance of the populations and a decrease in adult size. According to Mieszkowska et al. [104], the range limits of *P. lineatus* in the British Isles have extended by up to 55 km, between the 1980s and the 2000s, even though the extremely cold winter of 1963 in the west and south of Britain [106] prompted a cold-induced mortality [107]. The recovery of these populations occurred in subsequent warmer years with breeding populations being found up to and beyond their limits before the cold spell [105]. These shifts have been synchronous throughout this geographic region, strongly suggesting that a large-scale factor such as climate is responsible for the observed changes.

The extension of northernmost geographic limits of *P. lineatus* in the Northeastern Atlantic is one among several evidences of range shifts that have been reported in recent years and is in accordance with Helmuth et al. [88] who reported that intertidal species range limits may be shifting by up to 50 km per decade.

Another possible example of geographic range extension due to climate change could be the colonization of Santa Maria Island in the Azores archipelago by *P. sauciatus* that occurred very recently, probably after 2009. The founder population has been able to recruit itself and is currently mostly constituted by specimens under 2 years of age. Presently, this species is restricted to the most occidental island of the Azores archipelago, the nearest island to the Portuguese mainland, and to the archipelagos of Madeira and Canaries where this species is well established since colonization times. According to the same authors, a successful colonization of the remaining islands of the Azores is predicted. Even though the driving forces that lead to the recent establishment of a population of this species in this island are unknown, there is a strong possibility that it is related to the increase of sea surface temperature (SST) in the Northeastern Atlantic (**Figure 3**) [108–110]. One of the determinants of successful reproduction and recruitment of *P. sauciatus*, a subtropical species, is sea surface temperature. According to Hutchins [110], subtropical species require warmers summers in order to guarantee reproductive success; as such, an increase in SST in Azorean waters could have played an important role in the successful establishment of *P. sauciatus* in Santa Maria Island.

Changes in the abundance and distribution of *P. sauciatus* could be directly or indirectly related to climate and oceanographic events that result in an increase of SST [18]. Historically, the geographic range of this species on the Northwest coast of the Iberian Peninsula is characterized by the existence of a distribution gap between southern Galicia and northern Portugal possibly related to upwelling events in the region. The first records of *P. sauciatus* having colonized, in the early years of the twenty-first century, at least partially its distribution gap were presented by Rubal et al. [18]. These authors suggested that colonization occurred from the east and north in westward and southward direction from South Galicia to North Portugal. The beginning of the expansion in the distribution of this species in South Galicia coincided with a warming in SST in the Northeastern Atlantic due to global warming [111, 112] by the end of the 1980s and early 1990s that was responsible by similar range expansion of warm

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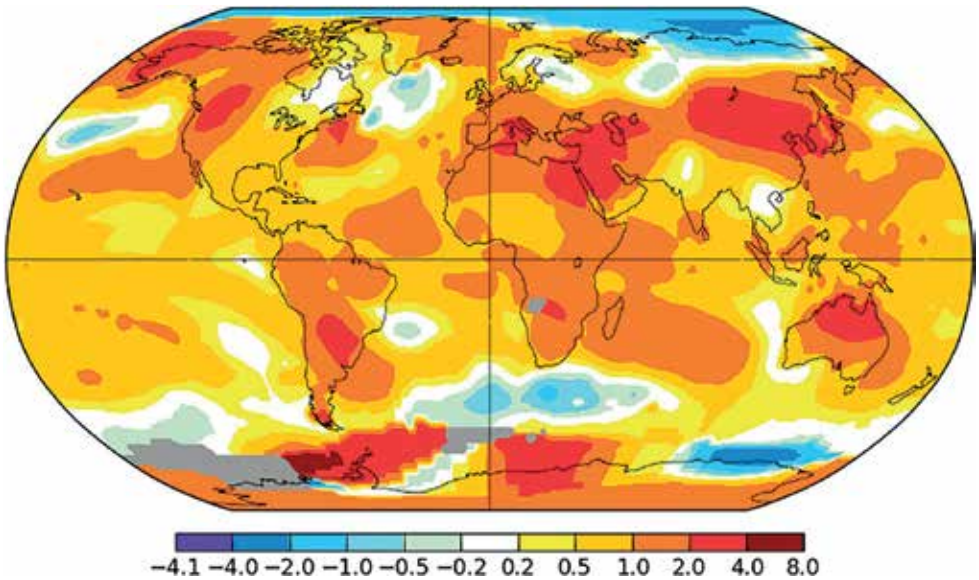

Figure 3. Map representing the spatial variability in surface warming. The temperature anomaly (°C) is represented for July 2017 compared to the mean surface temperature for the period of 1951–1980 [108].

water species in the English Channel [103, 113]. Rubal et al. [18] suggest that the weakening of the upwelling since the 1940s led to an increase in SST that could have been responsible for the recent colonization of these regions by *P. sauciatus*.

These changes in oceanographic conditions could result in shifts in the distribution and abundance of *P. sauciatus* along its northern boundary; such patterns have already been reported for other gastropods in this area [114] and other *Phorcus* species at northern latitudes [104, 105].

Climate change increases the level of environmental stress to which intertidal organisms are traditionally subjected to and these may severely affect the functioning of biological systems at different levels of organization. The reviewed works of several authors provide strong evidence of the suitability of *Phorcus* species as indicators of global climate change. This is particularly true for populations in the geographic boundaries of these species that can expand up to 50 km per decade, affecting ecological interactions and community structure of the intertidal ecosystems. Alteration of vertical zonation of these assemblages is another consequence of climate change that can be inferred using *Phorcus* as indicators, since these species occur at their physiological tolerance limits. Changes in temperature, climatic patterns, and oceanographic features directly affect biological processes, which can scale up to the assemblage level, thus affecting different levels of biological organization. For instance, reduction in body size and changes in reproductive cycles are recognized as universal responses of intertidal organisms to global warming. Further studies are required in order to provide information concerning biological parameters of *Phorcus* species and how they are affected by climate change, consolidating these species as indicators of large-scale ecological impacts of climate change.

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Chapter 8

Freshwater Crustaceans Decadpos: An Important Resource of Guatemala

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Additional information is available at the end of the chapter

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Abstract

Guatemala is a mega diversity country because it has several ecosystems and the physiography has a high diversity. However, the local population uses this biodiversity as a natural resource of food mainly. The country had three main drainage slopes for their rivers and aquatic reservoirs with several basins (the Gulf of Mexico, the Caribbean Sea, and the Pacific Sea). In these slopes, crayfish, freshwater prawns, and crabs compose the aquatic biological resources. Several fieldtrips were performed around these slopes in order to identify the species which were used as natural aquatic resources and verify if the diversity supports the food needs of the local population. Our findings were that the country has at least four crayfish species of genus *Procambarus* spp., those living in the high and middle altitude areas. Five freshwater prawn species with abbreviated larval development of genus *Macrobrachium*, that is, *Macrobrachium cemai* were also found. The bigger species of *Macrobrachium* was also identified on the three slopes as *Macrobrachium americanum*, *Macrobrachium tenellum*, *Macrobrachium occidentale*, and *Macrobrachium digueti* on the Pacific slope, while on the Gulf of Mexico and the Caribbean Sea, *Macrobrachium carcinus*, *Macrobrachium acanthurus*, *Macrobrachium heterochirus*, *Macrobrachium olfersii*, and *Macrobrachium hobbsi* were recorded, and therefore, the nonnative species *Macrobrachium rosenbergii*; with respect to other shrimps, *Palaemon pandaliformis*, *Palaemonetes octaviae*, and atyids as *Atya scabra* and *Potimirim glabra* were found. According to the freshwater crabs, the Pseudothelphusidae family is the best to represent in comparison with Trichodactylidae where only one population was recorded. Also, we register the uses of these species around the main markets in the country and we found two main ways: the first one is for the bigger species of freshwater prawns and crabs that are offered very expensive in kilogram and are almost offered in restaurants as exclusive dishes. The second one is more for the local consumption, and many families of fishery species that include crayfishes, freshwater prawns with abbreviated larval development, and smaller crabs, and so on, are sometimes found in the markets, with the prices being cheaper and can be bought only

by the local people. Our findings show that Guatemala has an enormous potential in the crustaceans decapods for use as natural aquatic resources as protein sources at low cost, especially for the families with low economical level.

Keywords: Guatemala, crustacean decapods, biological resources

1. Introduction

Currently, Guatemala has been included as a mega diversity country, because it represents a geographical area where convergence of a lot of different ecosystems involved a change in the physiography, climates, and biomes [1]. Especially, the climate variations in small territory have been playing an important role in the speciation process and specializations and adaptations in diverse populations of plants and animals.

Originally, Guatemala was cataloged as diversity, mainly for the data from terrestrial ecosystems, which has been studied with more emphasis [2]. The marine environment has been few studied; however, the geographical position of the country indicates that there is an important marine diversity in both coasts (Atlantic and Pacific) [2]. Recently, due the interest in the sea resources exploitation, the attention on these resources has increased. In contrast, the aquatic epicontinental resources have scarcity attention as study subject and natural resources, and their potential social and economic benefits are limited. The richness and importance of these small sources of life, important to the subsistence of human populations closers has not evaluated before 2000 year, due to this, it was not possible to give their real value when it has been planning strategies to management and conserve these resources.

In Guatemala, the natural epicontinental aquatic resources begin to acquire an important role in the priorities in the country, mainly due the latent threat of climate change and desertification [3]. The freshwater springs now are considered in the planning and land preservation strategies. As an example of this, Atitlán lake (located in the Sololá Department) worry internationally, due the massive cyanobacteria bloom, due the waste water from human closer to the basin [4]; this case induces that the scientific research activity on freshwater resources increase to get data from springs and streams as bigger basins as source to know the diversity and establish management plans and uses on this natural resources (fisheries, transport, and water sources to human use).

Due to this interest, the biological resource increases in importance, and one group that has been well represented in these environments are the crustaceans, mainly freshwater shrimps, crayfishes, and crabs, together several species of fishes and mollusk are dominant in these habitats [2].

In Guatemala, these resources have been exploited economically [5]. However, their importance in production is so low that there are not records and hard data. Several human communities used directly or indirectly the river resources from springs through the coastal connection. As happened with freshwater crabs from family Pseudothelphusidae that is possibly found in the majority of rivers or springs of country on east slope, even the Maya communities from highlands have a fishery and are used for self-consumption [6]. On another slope (Ocean Pacific), *Macrobrachium tenellum* and *Atya margaritacea* have important fisheries and commercialization among the coastal towns on the Pacific especially on south of country mainly in the estuaries of rivers María Linda and Los Esclavos [7]. The aim of this chapter is the potential

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in the freshwater aquatic natural resources in Guatemala, especially on those native species, and the analysis of native human populations that use these biological resources as protein sources.

2. Study area

Guatemala as country, has a great biological diversity on the subject of aquatic continental systems. Firstly, as the country is divided into three main slopes, two with drainage on Atlantic Ocean (Gulf of Mexico and Caribbean Sea), both very well defined by bigger basins that occupied all center to north of country [8]. The main river to drainage on Gulf of Mexico is the Usumacinta conformed by important rivers as La Pasión, Chixoy, Salinas e Icbolar, all these rivers with origins on highlands flow through lower lands, which permit to have different physical and chemical water conditions, and producing several habitats that bearing an important biological diversity [9].

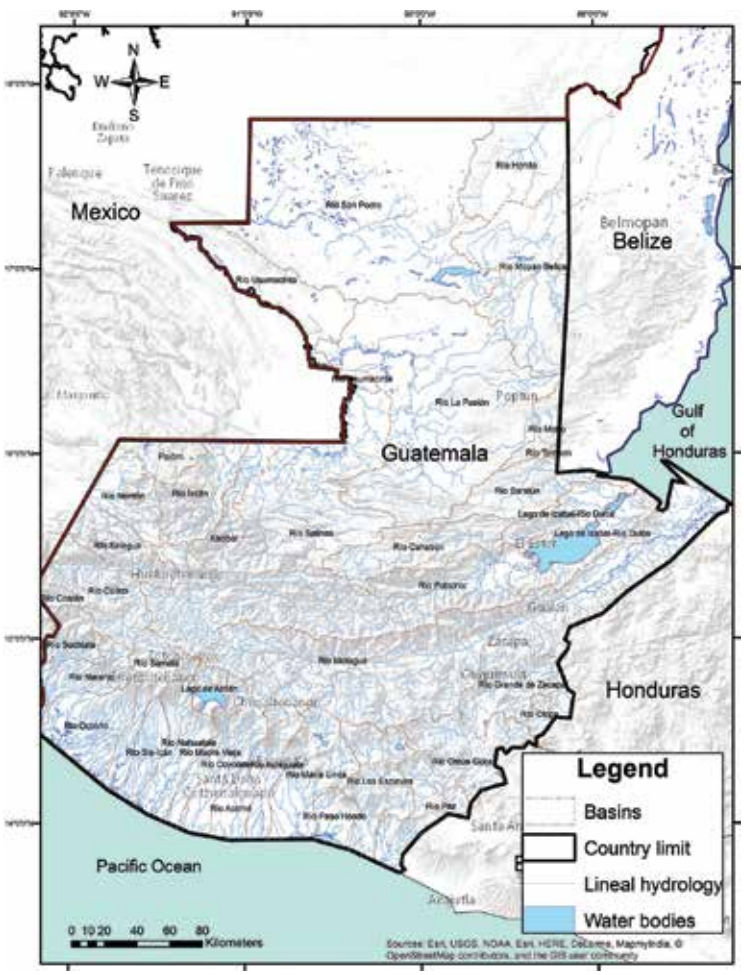

Figure 1. Location of aquatic reservoir in Guatemala (rivers and lakes).

On the Caribbean slope, Guatemala has other rivers with conditions completely different as Dulce, Motagua, and Sarstún Rivers, that with another they end on this slope. River Dulce is the main effluent of important aquifers on Sierra de las Minas and Cerro San Gil, as well as highlands from Alta Verapaz, all these rivers produce the Río Polochic that end on Izabal Lake with connection with the sea in the Amatique Bay [8].

The Motagua River is the longest of the country. However, due to their origin (closed to Guatemala City) and magnitude, induces an excessive carry of solid wastes mainly plastics and nondegradable material that reach the Caribbean Sea and produce marine pollution [10].

Finally on the Pacific Slope, some main rivers are María Linda, Los Esclavos, La Paz, Achiguate, Coyolate, and Naranjo are located and drainage on the Departments of Escuintla, Rethauleu, Santa Rosa, Jutiapa, Sichitepéquez, San Marcos Quetzaltenango y Sololá [7] (**Figure 1**).

3. Materials and methods

Two structured sampling trips were made on the Atlantic Slopes (Gulf of Mexico and Caribbean), and in Pacific Slope, different trips were made (**Figure 2**). In these trips, the crustaceans were collected using nets and hand. At that time, the GPS and physical and chemical water data were recorded using a GPS Garmin and YSI Oxygen Dissolved recording. In each place, the animals were preserved in ethanol to posterior lab identification. Also, in each

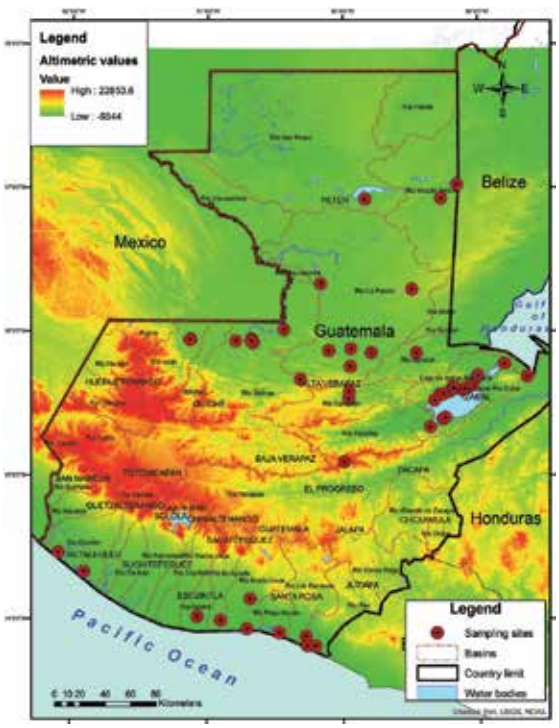

Figure 2. Location of sampling sites around the three main slopes Gulf of Mexico, Caribbean Sea and Pacific Ocean.

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place, the use and the value that local community has on these animals were recorded. In several markets around the country, make a survey to identify the different crustaceans species that are to sale and know if the local or exotic species have special preferences or major value.

4. Results

Guatemala has three main basins and several economical decapod species associated to these. The first basin analyzed was Caribbean where it was possible to find the following species *Macrobrachium carcinus* (**Figure 3A**), *Macrobrachium acanthurus* (**Figure 3B**), *Macrobrachium olfersii*, *Macrobrachium hobbsi*, *Macrobrachium heterochirus*, and one introduced species *Macrobrachium rosenbergii*; In general, all these species have a good acceptation in the market and in some cases their acceptation had reached high levels, for example *Macrobrachium carcinus* ("La pigua" in Spanish) in the Departments of Quiche, Alta Verapaz, Izabal, and Petén, the populations recently shown an important decrease in the fisheries, due to the over exploitation, pollution of rivers and reservoirs, and therefore, the barriers as dams in rivers that limited their migrations. In contrast, the exotic species *M. rosenbergii* is more frequently in the market and fisheries at least in the harbor fishery of Río Dulce, just now is evaluated if this last species have an impact on native species in several rivers of region. Also, in this region, there are several populations that have an abbreviated larval development and recently was described *Macrobrachium cemai* (**Figure 3C**), that is, used as food by the autochthonous community of Qek'chi in Cerro San Gil, Puerto Barrios, and Izabal. Therefore, there are another small decapods that lack economical value as *Palaemon pandaliformis*, *Palaemonetes octaviae*, and some atyids as *Atya scabra* and *Potimirim glabra* (**Figure 3D**).

In this basin, the crayfish species of *Procambarus* spp. (**Figure 3E**) was recorder and in some rivers there are freshwater crabs of family Trichodactylidae (**Figure 3F**) and Pseudothelphusidae (**Figure 3G**) and the local populations especially the indigenous communities as Qek' chi used by self-consumption and these species are hard to be found in the markets and fisheries, but the families use the children to search these species and by this way, they contribute with food to family.

The second basin is the Gulf of Mexico, the rivers and aquatic reservoirs drainage on the Usumacinta River and the main species are *Macrobrachium carcinus*, *Macrobrachium acanthurus*, and *Macrobrachium heterochirus*, but in this case due to few important markets to sale these products in general are to self-consumption. On this basin, we have recorded two populations with abbreviated larval development but in general few people know their existence and only indigenous populations used this biological resources together crayfish (*Procambarus* spp.) and freshwater crabs of family of Pseudothelphusidae.

In contrast on the Pacific slope in Guatemala, the biological aquatic resources are more diverse in small areas because the mountain chain is a barrier to limit their distribution. But in this area, the species of freshwater prawns are *Macrobrachium tenellum*, *Macrobrachium americanum*, *Macrobrachium occidentale*, and *Macrobrachium digueti*, all these with high commercial value and are easy found in the markets or the people just sale in their houses. The water pollution on rivers or dams are sometimes especially those sites close to cities where few control of waste water exists, the data of oxygen registered was lower in these sites, and the animals were absent, in contrast with those sites so far from human effects. The *Macrobrachium* species are a good indicator of the water quality because there are species as *M. heterochirus* where the oxygen requirements are higher in comparison with another species.

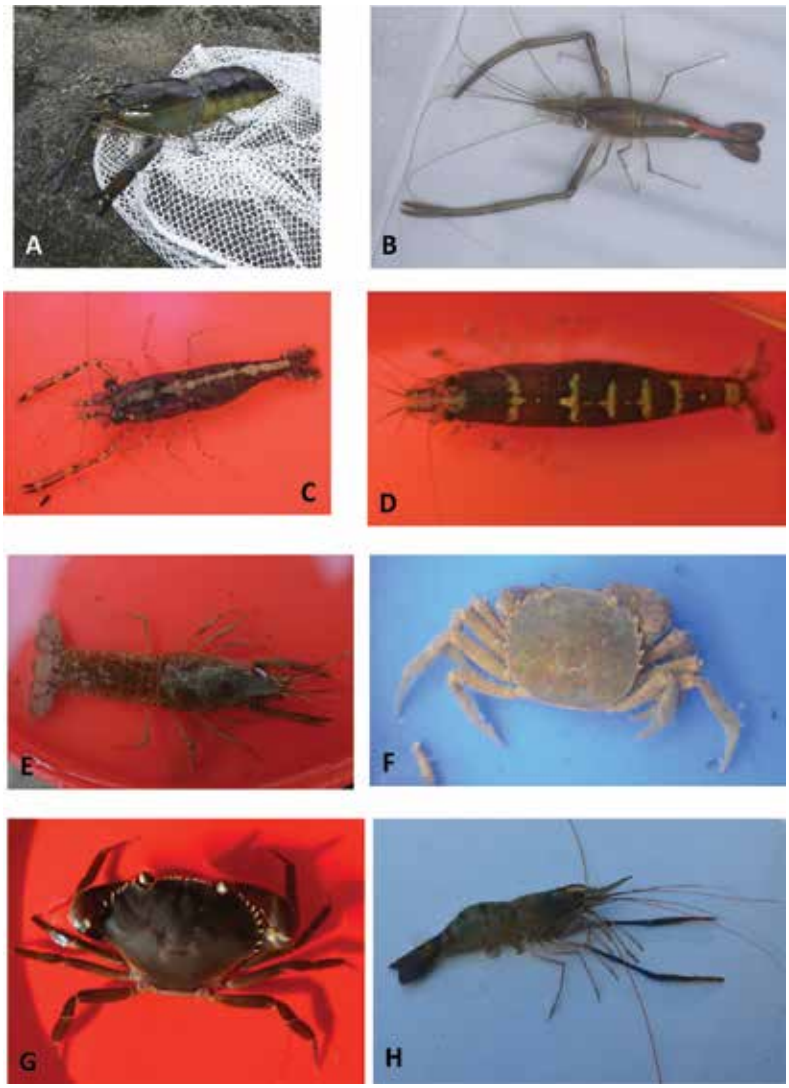

Figure 3. Freshwater decapods species in Guatemala. (A) *Macrobrachium carcinus*; (B) *Macrobrachium acanthurus*; (C) *Macrobrachium cemai*; (D) *Potimirim glabra*; (E) *Procambarus* spp.; (F) *Trichodactylidae*; (G) *Pseudothelphusidae*; (H) *Macrobrachium rosenbergii*.

5. Discussion

The aquatic biological resources in Guatemala are best represented with freshwater decapods species, but the economical values of these resources are lower in comparison with freshwater fishes and only few species have an important acceptance in their consumption of local population. The species of *Macrobrachium* that are the largest freshwater decapod have a big distribution on the three basins because the majority of sampling sites in this study were recorded (**Table 1**).

Site	Locality	Department	GPS X	GPS Y	Altitude	Taxa	Population richenss
Caribbean and Gulf of Mexico Slope							
Escobas Cerro San Gil	Las Escobas	Puerto Barrios	−88.6456667	15.6851667	116	*Macrobrachium cemai*	High >200
						Potimrim sp.	Medium >50
						Raddus sp	Low >10 org
El Boqueron	El Estor	Izabal	−89.2844722	15.5660278	9	*Trichodactylidae*	Low >10
						Pseudothelphusidae	Low >10
Río Zarco	El Estor	Izabal	−89.2951111	15.5571944	20	*Macrobrachium sp.*	High >200
Puente Pedernales	El Estor	Izabal	−89.0426944	15.6364722	9		
Puente la Máquina	El Estor	Izabal	−89.0753333	15.6157222	26		
Sumache	El Estor	Izabal	−89.0941667	15.6056389	19		
Puente Manaco	El Estor	Izabal	−89.1230278	15.5952778	16		
Balneario caliente	El Estor	Izabal	−89.2085556	15.5908611	35		
Afluente remanso	Sumache	Izabal	−89.1074444	15.6054444	65	*Procambarus sp.*	High >100
Aldea Manantiales, Esmeralda del Paraiso	Agua caliente	Izabal	−89.2206111	15.5833056	55		
Rio Zarquito	Río Oscuro	Izabal	−89.3595278	15.3377222	5	*Palaemon pandaliformis*	High >200
Rio Chapin	Chapin abajo	Izabal	−89.2665278	15.3924444	9		
Río Balandra	Quinel/Estor	Izabal	−89.2481944	15.4031111	14		
Puente Prieto	Sa Rosita/El Estor	Izabal	−89.2623889	15.5692222	13	*Pseudothelhusidae*	Low <50
El lago	El Estor	Izabal	−89.3294722	15.5239722	12	*Palemonetes octaviae*	High >200
Arroyo colorado	El Bongo/El Estor	Izabal	−89.1920833	15.6183333	212	*Procambarus sp.*	Medium >50
Río Bouro	El Bongo/el Estor	Izabal	−89.1861667	15.6092222	159	*Procambarus sp.*	Medium >50
Río Branche	Esmeralda	Livingston	−89.0110278	15.6929722	17	*Palaemon pandaliformis*	High
La Palmera	Esmeralda	Livignston	−89.0103056	15.6942500	29	*Procambarus sp.*	High >200

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Site	Locality	Department	GPS X	GPS Y	Altitude	Taxa	Population richenss
Cenote de Sarstum	Sarstum	Livingston	−89.9428056	15.8822500	15		
Siete Altares	Livingston	Livingston	−89.7918889	15.8542778	83	*Macrobrachium heterochirus*	Low <10
						Macrobrachium carcinus	Medium >50
						Macrobrachium ofersii	Medium >50
						Potimirim sp.	High >200
Cueva del tigre	Barra Lampara	Livingston	−88.8125278	15.7747500	74	*Pseudothephusidae*	Low <10
Las Conchas	Chasac	Alta Verapaz	−89.4616944	15.8533056	144	*Macrobrachium sp.*	High <100
Río Lachua	Santa Lucia, Reserva Lachua	Alta Verapaz	−90.6639722	15.9245833	171	*Pseudothelphusidae*	High>100
Puente la machaca	Santa Lucia, Reserva Lachua	Alta Verapaz	−90.6750833	15.9486389	195		
Arroyo El Caoba	Santa Lucia, Reserva Lachua	Alta Verapaz	−90.6758333	15.9406944	175	*Macrobrachium sp.*	Low <20
Arroyo las ranas	Santa Lucia, Reserva Lachua	Alta Verapaz	−90.6761944	15.9376389	180	*Procambarus sp.*	High >100
Hunal-Ye	Chisec	Coban	−90.3143333	15.6699722	403	*Macrobrachium sp.*	High>200
						Pseudothelphusidae	Medium>30
Semuc- Champey	Larkin	Alta Verapaz	−89.9595833	15.5336667	353	*Macrobrachium sp.*	High >200
Cueva las Marias	Semuc-Champey	Alta Verapaz	−89.9555556	15.5875000	357	*Macrobrachium sp.*	Medium>50
Las Mesas	Rio Hondo Zacapa		−89.5932000	15.0545000		*Macrobrachium heterochirus*	Low <10
Pacific Slope							
Las Pozas	Buena Vista	Santa Rosa	−90.16264"	13.52519	14	*Macrobrachium americanum*	Medium >50
Manchon Guamuchal	Manchon Guamuchal	Rethauleu	−92.05112	14.27499	17	*Macrobrachium occidentale*	Medium >50

Site	Locality	Department	GPS X	GPS Y	Altitude	Taxa	Population richenss
La verde	Champerico	Rethauleu	−91.5408	14.195107	13	*Macrobrachium tenellum*	High >200
Málaga	Málaga	Escuintla	−91.0416.5	14.01048	19		
Otacingo	Otacingo	Escuintla	−90.53562	13.59368	24	*Macrobrachium digueti*	Low >10
Brito	Brito	Escuintla	−90.40555	14.08333	44		
El Paraíso	Santa Rosa	Santa Rosa	−90.12144	13.485168	5	*Macrobrachium tenellum*	High >200
Las Lisas	Las Lisas	Santa Rosa	−90.15489	13.48516	6	*Macrobrachium tenellum*	High >200
Iztapa	Iztapa	Escuintla	−90.42251	13.55576	7	*Macrobrachium tenellum*	High >200
						Macrobrachium americanum	Medum >50
La Avellana	Monterrico	Santa Rosa	−90.28119	13.54231	6		
Peten Zone							
La campana	La campana	Peten	−91.07213	15.56554	231		
Trinitaria	Trinitaria	Peten	−90.47287	15.56126	168	*Macrobrachium sp.*	Medium>50
Tres Rios	Tres Rios	Peten	−90.26084	16.00559	138		
Las Pozas	Las Pozas	Peten	−90.09585	16.20044	168	*Pseudothephusidae*	Low >10
Flores	Flores	Peten	−89.50106	16.55238	131	*Macobrachium sp.*	Medium >50
Melchor de Mencos	Melchor de Mencos	Peten	−89.09262	17.012404	106	*Macrobrachium sp.*	Medium >50
Salpet	Salpet	Peten	−89.16325	16.55458	146		
Poptun	Poptun	Peten	−89.293103	16.17421	420	*Procambarus sp.*	High >100
Chabilchoch	Chabilchoch	Peten	−89.56472	15.45272	190		
San Antonio Las Cuevas	San Antonio Las Cuevas	Peten	−90.061916	15.52079	244	*Macrobrachium sp.*	Medium >50
La Campana	La Campana	Peten	−91.07391	15.564503	244	*Procambarus sp.*	High >100

Table 1. Relation of sites explored, GPS, species, and population richness data.

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But only two or three species are possibly found in the market in different places around the country. In the Pacific slope, the acceptance of these resources are major, but it is due to the cultural aspects on the indigenous people. In the Gulf of Mexico and Caribbean Sea slopes, the Qek' chi people use these resources for self-consumption and only in the local markets, sometimes by seasons, this aquatic resource is possibly found. Also, there are another species smaller or with less economical value as crayfishes and freshwater prawns, that in general the people that fishing their animals are children as part of their contribution to food in the family. These problems on the acceptance and sale of resource is only in some areas because for special species (largest), the over exploitation of *Macrobrachium carcinus* in the recent years has produced an important decrease in the fishery on this species as was reported in another countries [11].

Therefore, less important problem is the increase in the pollution on rivers due the chemical products used in the sugar and palm farmers and their respective industries reported not only in Guatemala because it is a normal practice in Central America and Mexico.

In general, the commercial species have migrating behaviors, the constructions of dams, and the water use to agricultural activities also decreased the native populations producing that exotic species occupied the niches empty [12].

However, just now Guatemala has an important opportunity to make plans to development according the basin and their resources. To protect those species over exploited and increase their potential of those species that only are using by indigenous people could be establish farmers because there are the technologies to producing by tons, and could be one mechanism to conserve the biological diversity and have management plans on aquatic biological resources.

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Chapter 9

Assessment of Proximate and Bioactive Lipid Composition of Black Sea Mussels (*M. galloprovincialis*) from Bulgaria

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Abstract

Farmed marine mussels from genera *Mytilus* are important for the human diet by providing high levels of proteins, omega-3 polyunsaturated fatty acids (PUFAs), fat soluble vitamins and carbohydrates. Recently, black mussels are commercially important species from the Bulgarian Black Sea. The aim of this study was to assess the seasonal changes in proximate composition and to focus on the lipid bioactive components such as fatty acids, cholesterol, fat-soluble vitamins (A, E and D_3), and carotenoids (astaxanthin, beta-carotene) in farmed mussels (*M. galloprovincialis*) from the northern part of the Bulgarian Black Sea coast. All analyzed samples presented high protein and low lipid content. The fatty acids (FA) profile was characterized by the highest amount of PUFA, as 22:6 omega-3 (n-3) dominated, regardless of the seasons. In all seasons, the content of n-3 was significantly higher than the omega-6 PUFA. The amounts of cholesterol were in the range 62.3 (summer) to 78 (autumn) mg 100^{-1} g ww. The highest amounts of vitamin D_3 (3.1 μg 100^{-1} g ww), vitamin E (2525 μg 100^{-1} g ww), astaxanthin (0.470 mg 100^{-1} g ww), and beta-carotene (0.445 mg 100^{-1} g ww) were found in the summer season. The studied mussel aquaculture from Bulgaria presented a high beneficial potential in all seasons in terms of human health protection.

Keywords: *M. galloprovincialis*, astaxanthin, cholesterol, fat soluble vitamins, seasonal changes, omega-3PUFA

1. Introduction

The phylum Mollusca represents one of the most diverse groups of marine animals. The Bivalves comprise some of the best-known invertebrates including the mussel species, represented in all

marine environments. Nowadays, mussels are harvested commercially and are of considerable significance for aquaculture worldwide. Farmed marine mussels from the Mytilidae family, especially genera *Mytilus*, are important for the human diet in the provision of high levels of proteins, omega-3 polyunsaturated fatty acids (PUFAs), fat-soluble vitamins, and carbohydrates. In recent years, the functional properties of mussel lipids have been investigated and few dietary supplements, based on lipid extracts of mussels, have been presented at the market [1, 2]. Due to these facts, the importance of marine mussels as a source for bioactive substances with anti-inflammatory, antimicrobial, and lowering cholesterol level agents, is increasing rapidly. In addition, mussels have recently become one of the most commercially important species from the Bulgarian Black Sea [3]. The assessment of the proximate composition and the lipid qualities may facilitate consumer acceptance and predict the market feasibility of aquaculture mussels in our region. However, the information about the nutritional qualities of mussels from the Bulgarian Black Sea waters, based on their chemical composition, fat-soluble pigments, cholesterol and PUFA contents is very limited. In this article, we studied the seasonal changes of mussel primary metabolites as proteins, lipids, and carbohydrates with a focus on lipid bioactive components such as fatty acids, cholesterol, fat-soluble vitamins (A, E and D_3) and pigments (astaxanthin, beta-carotene) in farmed mussels (*Mytilus galloprovincialis*) from the Northern part of the Bulgarian Black Sea coast.

2. Materials and methods

2.1. Sample collection

All mollusk samples were purchased from two mussel farms in spring (March 2015), summer (July 2015), and autumn (October 2015). The mussel farms are located in one of the most ecologically non-polluted areas along the Northern part of the Bulgarian Black Sea coast (Kavarna). The samples were immediately frozen at −20°C and stored in a fridge. The biometric characteristics as mean weight (g) and mean length (cm) were determined (**Table 1**).

Average 40 specimens of mussels (from each season and each farm) were used for a proximate, fatty acid and fat-soluble vitamins, cholesterol and pigments analysis. All shucked mussels were cut into small pieces and homogenized at 800 rpm for 5 min, using a Moulinex blender.

	Spring (n = 45)	**Summer (n = 43)**	**Autumn (n = 46)**
Mean weight	11.0 ± 0.5	12.0 ± 0.5	13.0 ± 0.5
Mean length	4.5 ± 0.3	5.5 ± 0.5	6.0 ± 0.5
Habitat		Demersal	
Food habits		Herbivorous	

n, number of specimens; SD, standard deviation.

Table 1. Biometric characteristics of mussel samples (mean ± SD).

2.2. Standards and reagents

Fatty Acid Methyl Esters (FAME) Mix standard (SUPELCO FAME, Mix C4-C24), and nonadecanoic acid and methyl ester nonadecanoic acid standards were purchased from Sigma–Aldrich™. Pure solid substances of all-trans-retinol, cholecalciferol, alpha-tocopherol, astaxanthin, beta-carotene, and total-cholesterol are HPLC-grade reagents, purchased from Sigma-Aldrich™. All used chemicals were of analytical, HPLC, and GC grade (Scharlau, Scharlau Sourcing Group, Spain).

2.3. Proximate composition analysis

The homogenized mussel tissues (2.000 ± 0.005 g) were dried at 105 ± 2°C in an air oven for 16–18 hours to a constant weight [4]. The moisture was calculated as weight loss. The crude protein content was determined by the Kjeldahl method [5]. The total lipids (TLs) were estimated according to Bligh and Dyer procedure [6] and the results were presented as g per 100 g wet weight (g 100 g^{-1} ww). The carbohydrates were determined according to [7]. The method was based on the treatment of the mussels' tissue with a methanolic KOH solution, followed by acid hydrolysis of starch to glucose. The glucose quantity was determined through the oxidation with a bivalent copper from a copper reagent and was then converted into starch. The energy values were calculated by multiplying fat, protein, and carbohydrate with appropriate coefficients (4.0 kcal/g for proteins and carbohydrates and 9.0 kcal/g for lipids) [8].

2.4. Fatty acid analysis

Fatty acid composition of total lipids at edible mussel tissue was determined by GC of the corresponding methyl esters. The residual lipid fraction was methylated by base-catalyzed transmethylation, using 2 M methanolic KOH and n-hexane according to [9]. To determine the analytical recoveries, the samples of homogenized tissue were spiked with a methanolic solution containing C19:0 (1 mg/ml). Gas chromatography analysis was performed by a FOCUS GC, autosampler A 3000, Polaris Q MS detector (Thermo Scientific, USA). The capillary column was a TR-5 MS (Thermo Scientific, USA), 30 m, 0.25 mm i.d. Helium was used as a carrier gas at flow rate 1 ml/min. Chromatographic separation of fatty acids methyl esters was performed under the following temperature regime: 40°C initial temperature for 4 min, followed by 10°C increase per minute until 235°C were reaches, temperature increase up to 280°C with a stay at this level for 5 min. The sample volume was 1 μl. The injector was a split/splitless injector operated in the split mode (1:10). Peak identification was measured by: retention time (RT) based on fatty acid methyl esters (FAME) mix standard (SUPELCO F.A.M.E. Mix C4-C24), and mass spectra (ratio *m*/*z*) compared to the internal Data Base (Thermo Sciences Mass Library; Thermo Corporation, Waltham, USA). FAMEs were quantified by the method of external standard. The FA content was expressed as a percentage of total FAs content [10].

2.5. Extraction of fat soluble vitamins, cholesterol, pigments, and HPLC analysis

The edible tissue of the mussels from the three different farms was used to evaluate its astaxanthin, beta-carotene, and cholesterol content. The extraction and quantity analysis

was performed by the method of Dobreva et al. [11]. An aliquot of the homogenized sample (1.000 ± 0.005 g) was weighed into a glass tube with a screw cap, 1% of methanolic L-ascorbic acid and 0.3 M methanolic KOH were added. Six parallel samples of edible tissue from each mussel farm were prepared and subjected to saponification at 50°C for 30 min. The fat-soluble components of interest were extracted with two portions of n-hexane: dichloromethane = 2:1 solution. The combined extracts were evaporated under a nitrogen flow and the dry residue was dissolved in methanol: dichloromethane and injected (20 μl) into the HPLC/UV/FL system. All fat-soluble compounds were analyzed simultaneously by an HPLC system, equipped with an RP analytical column (Synergi Hydro-RP (80 Å, 250 × 4, 6 mm, 4 μm)). Astaxanthin, beta-carotene, and cholesterol were identified by UV detection. The mobile phase composition was ACN:MeOH:iPrOH = 75:20:5 v/v, with the flow rate being 1 mL/min. The qualitative analysis was performed by comparing the retention times of pure substances: at λ_{max} = 208 nm for cholesterol, at λ_{max} = 450 nm for beta-carotene, and λ_{max} = 474 nm for astaxanthin. The quantitation was performed by external calibration, comparing the chromatographic peak areas of the corresponding standards (Astaxanthin, Supelco; Cholecalciferol, Supelco, and Beta-carotene, Supelco). The results were expressed as μg per 100 g wet weight (μg·100 g^{-1}ww).

2.6. Nutritional quality indices

Nutritional qualities were estimated by several indices and ratios of fatty acid composition: the indices of atherogenicity (AI), thrombogenicity (TI), cholesterolemic index (h/H), n-6/n-3 and PUFA/SFA ratios, according to Simopoulos [12]. Ulbricht and Southgate [13] suggest two indices, AI and TI, which might better describe the atherogenic and thrombogenic potential of different unsaturated FA. AI indicates the relationship between the sum of the main saturates and that of the main unsaturated, the former being considered pro-atherogenic (favoring the adhesion of lipids to cells of the immunological and circulatory systems), and the latter being anti-atherogenic (inhibiting the aggregation of plaque and diminishing the levels of esterified FA, cholesterol and phospholipids, thus preventing the appearance of micro- and macrocoronary diseases). TI shows the tendency to form clots in the blood vessels and is defined as the relationship between the pro-thrombogenic (saturated) and the anti-thrombogenic FAs (MUFA, n-6 PUFA, and n-3PUFA). The cholesterolemic index (h/H) presents the functional effects of different PUFAs of the cholesterol metabolism (hypo- and hyper-cholesterolemic effect) and is calculated in accordance with the method, described elsewhere [14]. In addition, the hyperlipidemic and atherogenic potential of mussel lipids, related to cholesterol, SFA, and unsaturated FA content, were determined. To assess the dietary effect of the mussel lipid consumption on serum cholesterol levels, two indices were calculated: cholesterol/SFA index (CSI) and cholesterol index (CI) [15, 16].

2.7. Statistical analysis

All analytical estimations were performed in triplicate. The results were expressed as a mean and standard deviation (mean ± SD). The obtained data were analyzed using Graph Pad Prism 5.0 software. An unpaired t-test statistical analysis was performed to estimate the differences between the analyzed samples. Thus, the comparison was made for proximate compositions,

individual FA, FA groups, fat-soluble vitamins, cholesterol and carotenoids, and nutritional quality indices. The differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Proximate composition

The proximate composition of edible mussel tissue varied with the season (**Table 2**). The assessment of the nutritional quality based on the macronutrients content in black mussel was conducted in accordance with Commission Regulation (EC) No. 116/2010 [17]. As water is the main component of mussel tissue, the levels of moisture are ranged between 73.35 and 77.15%, being higher in the summer samples in comparison to other seasons.

Seafood products are considered "low fat" when containing below 3 g of lipids per 100 g wet weight (ww). In the present study, the range of the total lipids (TLs) content is between 1.40 and 2.89 g 100^{-1} g ww. The highest TL was found in spring mussels, whereas summer specimens presented twice lower values ($P < 0.001$). TLs that amount below 3 g 100^{-1} g ww were found in all analyzed seasons; therefore, Black Sea mussels can be classified as "low fat" food.

The lack of considerable variation in the protein content during the seasons is well illustrated in the results (see **Table 2**). According to [8], the seafood protein content below 15% is considered low. In this study, a significant decrease of the protein content was found in the autumn period as compared to the spring sample ($P < 0.001$). However, protein levels were significantly above 15% in all samples, and the analyzed mussels can be classified as protein-rich regardless of the season.

The observed seasonal pattern in the carbohydrate amounts showed the highest levels in the autumn season and the lowest in the summer period. The accumulated carbohydrates could be utilized under unfavorable conditions and the observed variation in the mussel tissue indicates that the level of mobilized carbohydrate reserves may vary widely and rapidly in response to fluctuation in environmental conditions [18, 19]. It was observed that in warmer seasons, the carbohydrate contents were higher than TL contents in the mussel

	Lipid	Protein	Carbohydrate	Moisture	Energy value
Spring	2.89 ± 0.10	19.92 ± 0.80	2.25 ± 0.08	73.35 ± 1.55	115.00 ± 5.50
Summer	1.40 ± 0.08[a]	18.30 ± 0.50	2.00 ± 0.06	77.15 ± 1.60[a]	94.50 ± 4.50[a]
Autumn	2.51 ± 0.12[c]	17.40 ± 0.55[b]	2.73 ± 0.10[b,c]	76.20 ± 1.40[b]	103.20 ± 5.20[b]

[a]$P < 0.001$ (spring vs. summer).
[b]$P < 0.001$ (spring vs. autumn).
[c]$P < 0.001$ (summer vs. autumn).

Table 2. Proximate composition in molluscs tissue, in g 100 g^{-1} ww and kCal 100^{-1} g ww (mean ± SD).

tissue. This may be explained by the higher mussels' metabolic activities during the summer-autumn period. Some studies suppose that the carbohydrate reserve may not be fully depleted during the mussel growth and remains relatively high throughout the spawning season [20, 21].

Further, on this study, it was demonstrated that seasonal changes of chemical composition largely depend on the mussel reproductive cycle. The moisture content is often used as an indication of the spawning time, hence the highest water content (July) correlated with the spawning period for the Black Sea mussels. The observed seasonal variation especially in TL and carbohydrates may be explained by the biochemical balance in relation to the mussels' reproductive activity. Mussels usually accumulate lipid reserves prior to gametogenesis. During the period of gametogenic development in spring (March and April) and autumn (September and October), the mussels showed two peaks of spawning after an important gonad ripeness. In this study, the accumulation of lipids and carbohydrates followed the main reproductive cycle in March and April (highest TL levels). The intensive spawning period between May and August was well correlated by the lowest TL and carbohydrates content found in the summer mussel samples. In addition, some authors [22] assume that the protein maximum and minimum levels also correspond to the mussel development phases (spawning and resting cycles). Similar seasonal variation in TL and protein and carbohydrate amounts of farmed and wild populations of *M. galloprovincialis* from Sinop (South Black Sea) and Romanian coast (North-west Black Sea) are reported by [21, 23]. The protein content in Bulgarian mussels was significantly higher than the values presented for mussel populations from the other parts of Black Sea (7–12%). For black mussel species from different regions such as the Adriatic Sea and Mar Grande of Taranto, different TL contents are reported in 2008 and 2010 [24, 25]. These patterns of temporal variability of TL in bivalve mollusks in previous studies result from several environmental factors acting simultaneously such as temperature, food availability, plankton composition, and physiological factors. Additionally, the differences mentioned above may be attributed to the longer reproductive periods in a warmer climate.

3.2. Fatty acid composition

The changes of fatty acid (FA) profile, main FA groups, n-3 and n-6 PUFA, EPA and DHA (as a percentage of the total FA and g 100 g^{-1} ww, mean ± standard deviation), FA ratios, and indices for the study period are shown in **Table 3**. Saturated fatty acids (SFAs), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFAs) underwent statistically significant changes during the observed period ($P < 0.05$). SFA ranged from 25.60% (July) to 31% (October), MUFA showed minor changes and ranged between 16.0% (October) and 17.95% (July), while PUFA was the dominant group and ranged from 53.0% (October) to 56.5% (July). The bivalves are considered herbivores with phytoplankton as the main component of their diet and FA profile, respectively. However, several studies show that bivalves can use other food sources such as detritus, bacteria, micro zooplankton and meso-zooplankton [18, 20]. Orban et al. [20] and Zlatanos [26] presented a relative pattern PUFA > SFA > MUFA in the black mussel from the Adriatic coast and the local Mediterranean mussel farms. Our results

Fatty acid	Spring	Summer	Autumn
C 8:0	0.26 ± 0.01	0.20 ± 0.01	0.11 ± 0.01
C 10:0	0.18 ± 0.01	0.35 ± 0.02	0.26 ± 0.01
C 12:0	0.36 ± 0.01	0.70 ± 0.02	0.88 ± 0.03[b]
C 13:0	Nd	Nd	Nd
C 14:0	2.93 ± 0.06	2.60 ± 0.08	2.40 ± 0.10[c]
C 16:0	19.69 ± 0.23	17.80 ± 0.35[a]	21.33 ± 0.85[b,c]
C 17:0	0.06 ± 0.01	Nd	1.06 ± 0.05
C 18:0	3.62 ± 0.08	3.24 ± 0.20	4.45 ± 0.15[b,c]
C 20:0	0.25 ± 0.01	0.23 ± 0.01	0.08 ± 0.01
C 21:0	0.04 ± 0.01	0.05 ± 0.01	0.02 ± 0.01
C 22:0	0.17 ± 0.01	0.18 ± 0.01	0.17 ± 0.01
C 24:0	0.56 ± 0.02	0.25 ± 0.02	0.24 ± 0.01
∑SFA	28.12 ± 0.55	25.60 ± 0.30	31.00 ± 0.85
C14:1n5	0.83 ± 0.03	0.62 ± 0.03	0.14 ± 0.01
C16:1n7	7.77 ± 0.15	8.20 ± 0.20[a]	6.00 ± 0.21[b,c]
C17:1n8	0.06 ± 0.01	Nd	0.18 ± 0.01[b]
C18:1n9	5.48 ± 0.10	7.10 ± 0.30[a]	5.22 ± 0.35[c]
C20:1n9	2.80 ± 0.06	1.60 ± 0.08[a]	4.00 ± 0.40[b,c]
C22:1 n9	0.10 ± 0.01	0.17 ± 0.01	0.12 ± 0.01
C24:1n9	0.39 ± 0.01	0.22 ± 0.01	0.34 ± 0.01
∑MUFA	17.43 ± 0.25	17.91 ± 0.18	16.00 ± 0.20
C18:3 n6	0.60 ± 0.02	0.80 ± 0.03	0.30 ± 0.01[c]
C18:2 n6	1.16 ± 0.07	2.00 ± 0.12[a]	1.05 ± 0.15[b,c]
C18:3 n3	1.05 ± 0.04	1.40 ± 0.09	0.65 ± 0.02[c]
C20:5 n3	6.90 ± 0.20	9.80 ± 0.24[a]	7.70 ± 0.85
C20:4 n6	10.55 ± 0.45	12.00 ± 0.30[a]	13.60 ± 0.69[c]
C 20:3 n6	1.15 ± 0.16	0.80 ± 0.03	2.00 ± 0.25
C 20:2 n6	0.41 ± 0.02	0.50 ± 0.02	0.60 ± 0.03
C 20:3 n3	1.72 ± 0.10	1.25 ± 0.11	0.90 ± 0.04
C 22:6 n3	30.39 ± 1.80	27.30 ± 0.54[a]	26.00 ± 0.88[b, c]
C 22:2 n6	0.53 ± 0.03	0.65 ± 0.01	0.20 ± 0.01

Fatty acid	Spring	Summer	Autumn
∑PUFA	54.45 ± 2.20	56.50 ± 2.10	53.00 ± 2.06
∑n 3	40.06 ± 1.70	39.75 ± 1.56	35.25 ± 1.30[b]
∑n 6	14.39 ± 0.85	16.75 ± 0.90	17.75 ± 1.05[b]
n6/n3	0.35 ± 0.02	0.42 ± 0.01	0.49 ± 0.02
PUFA/SFA	1.94 ± 0.12	2.20 ± 0.18	1.71 ± 0.10
DHA/EPA	4.40 ± 0.80	2.78 ± 0.25	3.38 ± 0.42
C16:1n7/C16:0	0.39 ± 0.02	0.46 ± 0.03	0.28 ± 0.02
CSI*	3.85 ± 0.50	4.04 ± 0.50	4.79 ± 0.38
CI*	3.73 ± 0.80	3.72 ± 0.43	4.06 ± 0.41
AI*	0.44 ± 0.01	0.39 ± 0.01	0.46 ± 0.01
TI*	0.19 ± 0.01	0.17 ± 0.01	0.22 ± 0.01
h/H*	2.45 ± 0.10	2.92 ± 0.15	2.28 ± 0.10
Fatty acid, g 100 g^{-1} ww			
∑SFA	0.731 ± 0.050	0.320 ± 0.030	0.722 ± 0.040
∑MUFA	0.453 ± 0.030	0.224 ± 0.025	0.373 ± 0.015
∑PUFA	1.416 ± 0.065	0.706 ± 0.040	1.235 ± 0.080
∑n3	1.042 ± 0.050	0.496 ± 0.020	0.821 ± 0.060
∑n6	0.374 ± 0.020	0.210 ± 0.015	0.414 ± 0.020
EPA	0.180 ± 0.012	0.123 ± 0.010[a]	0.179 ± 0.014
DHA	0.790 ± 0.045	0.341 ± 0.018[a]	0.606 ± 0.035
EPA + DHA	0.970 ± 0.048	0.464 ± 0.020	0.785 ± 0.050

[a]P < 0.001 (spring vs. summer).

[b]P < 0.001 (spring vs. autumn).

[c]P < 0.001 (summer vs. autumn).

*CSI = (1.01×SFA g 100^{-1} g ww) + (0.05×cholesterol mg 100^{-1} g ww); CI = 1.01(SFAg 100^{-1} g ww – 0.5PUFA g 100^{-1} g ww) + (0.06 cholesterol mg 100^{-1} g ww); AI = [(C12:0 + (4 × C14:0) + C16:0)]/(n6PUFA + n3PUFA+ MUFA); TI = (C14:0 + C16:0 + C18:0)/[(0.5MUFA) + (0.5n6PUFA) + (3n3PUFA) + (n3PUFA/n6PUFA)]; h/H = (C18:1n9 + C18:2n6 + C18:3n3+ C20:4n6 + C20:5n3 + C22:6n3)/(C14:0) + C16:0).

Table 3. Comparison of FA profiles (FA, % of total FA), ratios, indices, EPA, and DHA contents of the edible tissue of black mussels (mean ± SD) during seasons.

are in agreement with the above mentioned authors. A deflection of this pattern (SFA > PUFA >MUFA) was presented by Badiu et al. [27] for wild mussel samples from the Baia Mamaia Zone-Park, Constanza (the Black Sea), and Cape Galata (the Bulgarian Black Sea) [28]. It is known that temperature and food availability are two of the most important factors regulating the growth of the marine bivalve mollusks.

The major SFA was palmitic acid (C16:0), followed by stearic acid (C18:0), which demonstrated highest levels in autumn (21.7 and 4.45%) and lowest in the summer season. Generally, three saturated FAs, which presented the next distribution C16:0 > C18:0 > C14 > 0 in all seasons, accounted for 90.0–93.0% of the total SFA. A possible explanation of the significant lowering of saturated FAs levels of energetic character in the summer season is that these acids were probably catabolized for the acquisition of the energy required for diverse metabolic functions as spawning, etc. In addition, some authors [29] pointed that elevated amounts of short-chain SFA such as C12:0, C14:0, and C16:0 SFAs displayed omnivorous feeding.

The main MUFAs were palmitoleic acid (C16:1n7), oleic acid (C18:1n9), and gondoic acid (C20:1n9). These FAs values are between 92% (spring) and 95% (autumn) of the total MUFAs and show C16:1n7 > C18:1n9 > C20:1n9 alignment in all seasons. According to Ref. [30], C16:1n7 and EPA (C20:5n3) are used as an indicator of diatom-based mussel diets. Another FA food behavior marker C16:1n7/C16:0 has been used to differentiate between diatoms versus phytoflagellate feeding. The phytoflagellate contained high levels of C16:0. Significant variation in C16:1n7/C16:0 ratio, from 0.28 (autumn) to 0.46 (summer), was observed and we could assume that in the summer season, diatoms prevail in mussels' food. Additionally, there was a significant increase of long-chain C20:1n9 ($P > 0.001$) in the autumn season. Some investigations reported that gondoic acid may be used as an indicative marker for zooplankton in the mussels' diet [31, 32]. Although mussels are herbivores species, several studies have demonstrated that species of micro- and mesozooplankton have been ingested by suspension form marine bivalves [25, 30]. A possible explanation of the raised levels of gondoic acid (two and a half times, $P < 0.001$) is that zooplankton may comprise the bigger part of the mussels' food in the autumn season. It is known that high levels of C 18:1 n9 are characteristic for deep-sea organisms as an adaptive response to high water pressure [32]. The lower amount of oleic acid in the Black Sea mussels is specific for specimens from shallow (especially warm) waters. The analyzed farmed mussels live at 12–18 m depths, which could explain the reported low levels of C18:1 n9 regardless of the season.

Among PUFA of black mussels, the docosahexaenoic acid (C22:6n3, DHA) was the predominant FA, followed by arachidonic acid (C20:4n6, AA) and eicosapentaenoic acid (C20:5n3, EPA) in all seasons. These FAs accounted for 87–89% of the total PUFA during the year. Significant seasonal variations were observed for DHA ($P < 0.001$) and EPA ($P < 0.001$) levels. Long-chain n3 PUFAs (LCPUFAs) showed lower levels in the autumn season, whereas AA presented highest values in the same season. These LCPUFAs are synthesized from the main mussels' food such as phytoplankton and microalgae in high quantities. Some authors [18] suppose that the water temperature may strongly affect the EPA and DHA levels. This statement has been confirmed by the fact that the Black Sea mussels lower their tissue DHA levels in warmer seasons (with 16% in autumn, $P < 0.001$) compared to the spring period. Due to the high biological activities of these n3 LCPUFAs, the DHA/EPA ratio characterized the deceleration of the mussel metabolism activities in the autumn period, which could be related to the higher water temperature and the reproductive cycle.

On the other hand, the DHA/EPA ratio is used to determine the degree of carnivory food, ingested by the mussels. As dinoflagellate contain greater amount of DHA and EPA is a specific marker for diatoms, the ratio could be used to assess the relative proportions of dinoflagellate and diatom contents in the mussels' food. Dinoflagellates prevail in the mussels' diet when this ratio is greater than 1 and diatoms are predominant when it is less than 1 [31–33]. In this study, we supposed that dinoflagellates are dominant in the analyzed spring-autumn period in the Black Sea mussels' food as DHA/EPA > 1. Other essential PUFAs as C18:3n3 and C18:2n6 were obtained in small amounts in the analyzed periods and ranged between 1.35 and 3.4%. The sum of both C18:3n3 + C18:2n6 was used as a terrestrial marker; therefore, the levels above 2.5% indicated a significant input of terrestrial material in the mussels' food [31]. Values above the cut-off levels (3.4%) were determined only for the summer period as for the other seasons, the sum of C18:3n3 + C18:2n6 was under 2.5% and it could be assumed that terrestrial matter was present in low levels in mussels' food from this Black Sea region.

The beneficial lipid quality of the mussel tissue was well displayed by the high levels of n3 PUFA. During the year, n3 FAs showed 35–40% of the total FAs, whereas n6 PUFA presented significantly lower values from 14 to 17.75%. The n6/n3 and PUFA/SFA ratios were used as indicators when comparing the relative nutritional values of sea food. The observed seasonal changes in these ratios are discussed in Section 3.4.

The fatty acid content in absolute amounts in g/100 g wet weight provides more useful and accurate information to assess the quality of mussels as food and to raise the consumers' interest. The European Food Safety Authority [34] recommends a daily intake of 0.500 g EPA + DHA n3 PUFA. Taking into account the above, the percentage values of this LCPUFA were recalculated to g/100 g of raw mussel tissue according to Ref. [35]. In the present study, the highest PUFA, n3 and EPA + DHA amounts were found in spring mussels compared to other seasons.

The present results are in accordance with some previous investigations [23, 26, 28] of farmed Mediterranean and Black Sea mussels. Some authors [25] report significantly lower PUFA and n3 PUFA values for mussels from Mar Grande of Taranto (7.55–11.16%) in comparison to our findings. Orban et al. [20] and Badiu et al. [27] present higher EPA than DHA levels in wild black mussels from the Adriatic, the Tyrrhenian, and the Romanian Black Sea coasts. The observed discrepancy and variations of n3 LCPUFA contents could be related to the type of food available and ingested by the mollusks and the lipid metabolism of EPA to DHA.

3.3. Fat soluble vitamins, cholesterol, beta-carotene, and astaxanthin content

Fat soluble vitamins, cholesterol, and carotenes contents are expressed as an average and standard deviation (mean ± SD). The results are shown as microgram per 100 grams wet weight (μg 100^{-1} g ww) for fat soluble vitamins (A, D_3, and E) and as milligram per 100 grams wet weight (mg·100 g-1 ww) for cholesterol, astaxanthin, and beta-carotene (**Table 4**).

Fat soluble components	Spring	Summer	Autumn
Vit A (μg/100 g)	36.4 ± 4.0	50.2 ± 5.0[a]	47.0 ± 4.5
Vit D_3(μg/100 g)	2.7 ± 0.3	3.10 ± 0.5	2.5 ± 0.3
Vit E (μg/100 g)	2315.7 ± 50.0	2525.0 ± 55.0	1975.5 ± 45.0[b,c]
Cholesterol, mg	62.3 ± 0.50	68.0 ± 0.60[a]	75.00 ± 0.40[b]
beta-carotene, mg	0.409 ± 0.035	0.445 ± 0.030	0.228 ± 0.018[b,c]
Astaxanthin, mg	0.428 ± 0.040	0.470 ± 0.038	0.142 ± 0.025[b,c]
Percentage of the daily recommended intake of fat soluble vitamins			
Vit A	4.9%	6.7%	6.3%
Vit D_3	54%	62%	50%
Vit E	15.4%	16.8%	13.2%
Vit E/PUFA	1.63	3.60	1.60

[a]$P < 0.001$ (spring vs. summer).
[b]$P < 0.001$ (spring vs. autumn).
[c]$P < 0.001$ (summer vs. autumn).

Table 4. Seasonal variations in fat soluble vitamins, cholesterol, carotenoids, and RDI in edible mussel's tissue (mean ± SD).

In this study, significant seasonal changes in all fat soluble biologically active compounds were observed. The analyzed fat-soluble components with high antioxidant activity are vitamin E (alpha-tocopherol) and carotenoids (beta-carotene and astaxanthin). Vitamin E was found in highest levels in edible mussel tissue, followed by vitamin A and vitamin D_3, regardless of the season. Our previous investigation of wild and aquaculture *M. galloprovincialis* from the Bulgarian Black Sea coast shows a similar distribution of fat-soluble vitamins: vitamin E > vitamin A > vitamin D_3 and their contents are similar to the autumn mussel sample levels [36]. One possible reason for the observed high vitamin E content is that its amount reflects a higher degree of antioxidant protection, necessary for the n3PUFA-rich organisms [19]. Our study illustrated a strong correlation for the black mussel tissue: highest PUFA contents—highest vitamin E content (summer) and lowest PUFA—lowest amount of vitamin E (autumn). Discrepancies with our results are reported for fat soluble vitamin (A, D, E) contents in wild common clam *Donax cuneatus* from the Southeast coast of India [37]. Authors find that vitamin A (105.6 mg/g) and vitamin D (38.2 mg/g) dominate in clam tissue, whereas vitamin E content is drastically low (just 3.64 mg/g). The authors do not describe the sampling details, hence possible reasons for the observed differences could be sampling season, available food, environmental conditions, etc. Earlier investigations report significantly lower levels of vitamin E (790 μg/100 g) and similar values for vitamin A (38.7 μg/100 g) in aquaculture green-shell mussels from New Zealand [38]. Shulman and Soldatov [39] found the highest levels of alpha-tocopherol (vitamin E) and carotenoids for *M. galloprovincialis* from Northern Part of Black Sea (Sevastopol) in a warmer period, whereas in the cold months, their levels decrease. Our results showed a similar trend for all fat-soluble vitamins and carotenoids contents (except cholesterol) in mussel tissue.

The cholesterol content in the mussel edible tissue was found in significantly low levels regardless of the season (see **Table 4**). The cholesterol amounts increased slowly in the sequence: spring > summer > autumn. Earlier investigations report lower cholesterol levels for *M. galloprovincialis*—from 20 mg/100 g [40] from the Southern part of the Black Sea (Sinop) to 53 mg/100 g ww [28, 36] from the Bulgarian part of the Black Sea. Li et al. [41] reported similar seasonal changes of cholesterol content for cultured *Perna viridis* from Guangdong, China. The authors present a slow increase of cholesterol levels from 26% (spring) to 37% (autumn) of total sterols. A possible explanation is that the main diet of the mussels is plankton, which contains cholesterol precursors. The mussels can synthesize cholesterol from specific precursors, but cholesterol metabolism depends on different factors such as the reproductive cycle, sex, etc. [41]. According to the Bulgarian dietary standards [42], consumption of less than 300 mg per day of cholesterol may help maintain normal blood cholesterol levels and prevent cardiovascular disease. All analyzed mussel samples were characterized with low cholesterol content (RDA <300 mg/day, [42]) regardless of the season.

Interest in the study of marine carotenoids increased after the discovery of the antitumor activity of beta-carotene and vitamin A [39]. Further on, astaxanthin has a beneficial effect on the human health due to its high antioxidant activity—10 times more than beta-carotene and 100 times more than alpha-tocopherol. Until recently, the effect of carotenoids on human health was focused mainly on beta-carotene. Information and clinical trials on the effect of the various carotenoids on the human endocrine and immune system, metabolism, etc., are scarce. De Carvalho and Caramujo [43] suppose that at present, natural beta-carotene accounts for 20% of the world demand, and a similar interest in natural astaxanthin is now emerging in the nutraceutical market. Thus, the information about the marine indigenous sources of these compounds is important for consumers. The main source of carotenoids in marine ecosystems is plankton microalgae. Pigments as beta-carotene and astaxanthin are synthesized in their cells and then distributed, and subjected to metabolic transformation in trophic chains. Most microalgae contain species-specific carotenoids. However, the composition of the main groups of these compounds remains unchanged within the microalgae classes. Shulman and Soldatov [39] report that the domination of diatoms in the composition of phytoplankton in the Black Sea ecosystem leads to an increase in pigments (such as beta-carotene, fucoxanthin, zeaxanthin, etc.), whereas astaxanthin is species-specific for dinoflagellates. The bivalves, which are filter-feeding mollusks, can accumulate carotenoids directly via dietary microalgae or after modification through metabolic reactions [43]. In this study, the astaxanthin content of the analyzed samples was lower in comparison to the beta-carotene amounts only in the autumn sample mussels, whereas in the spring and summer periods, it presented higher values (see **Table 4**). Therefore, we classified cultured Black Sea mussels as good sources of astaxanthin. In accordance with our previous conclusion based on DHA/EPA ratio, the observed higher astaxanthin levels in comparison with the beta-carotene content confirmed that dinoflagellates are dominant in the Black Sea mussels' food for the spring-summer period. No comparative study for seasonal changes of Black Sea mussel fat-soluble vitamins and carotenoids composition is available in the literature. Posleslova and Nehoroshev [44] reported the 0.5 mg·100 g^{-1} ww beta-carotene for wild Black mussels from the Northern part of the Black Sea (Sevastopol, The Ukraine). This result is consistent with our findings for the summer mussel samples (**Table 4**). Desnica et al. [45]

determined the average concentration of astaxanthin (1.5 mg·100 g^{-1} ww) in blue mussel *M. edulis* samples from Iceland. This result is higher than ours. The most likely reason for this difference is an algal availability in the region—a key factor for the astaxanthin and beta-carotene production in herbivorous mollusks [39, 45]. Earlier studies on edible mussel *M. edulis* found that beta-carotene can be converted to astaxanthin by oxidative (and reductive) metabolic processes. Other interesting properties of carotenoids are reported for bivalves from the littoral region of the Northern Black Sea coast. The authors present a correlation between carotenoid contents and pollution in the region. They suggest that mollusks with high carotenoid content in their tissue show higher resistance to environmental pollution and therefore, carotenoids may be an important part of oxygen metabolism in bivalves [46].

3.4. Assessment of the nutritional quality of the mussel lipids

The analyzed mussels have been assessed from a nutritional quality perspective. The evaluations of the lipid functional properties were based on fatty acids ratios, indices, contents of long-chain n3 PUFAs (EPA + DHA), recommended daily intake (RDI) for fat-soluble vitamins, cholesterol, and carotenoids contents. In addition, the seasonal changes of lipid quality indices, FA ratios, fat-soluble RDI, cholesterol, and carotenoid content were studied and discussed. There are two FA ratios—n6/n3 and PUFA/SFA, which are traditionally used in the estimation of the lipid quality. In this study, these ratios are significantly varied between seasons. An increase in the human dietary n6/n3 PUFA ratio is essential for preventing coronary heart disease by reducing plasma lipids and the risk of cancer [12]. In the present study, this ratio ranged from 0.35 to 0.49 in agreement with earlier published results for the Black Sea mussels [23.27, 36]. According to Ref. [12], the beneficial n6/n3 ratio for the human health is below 1 and the findings confirmed the high quality of mussel lipids. In addition, PUFA/SFA ratio described the FA balance in mussel lipids well. The Department of Health [47] recommends values of PUFA/SFA ratio which should be higher than 0.45. In this study, the PUFA/SFA ratio ranged from 1.7 to 2.2 (see **Table 3**) and we may conclude that all culture mussels have a well-balanced and beneficial FA profile regardless of the season.

As mentioned above, EFSA [34] recommends a daily intake of 0.500 g EPA + DHA n3 PUFA. A 100 g of mussel edible tissue contains from 0.464 to 0.970 g of EPA + DHA n3 PUFA and provides from 93% (summer) up to 194% (spring) of the recommended daily intake (**Table 3**). Thus, in accordance with [48], the analyzed farmed Black Sea mussels can be classified as high in omega-3 fatty acids regardless of the season. In our previous study [28], the reported results for EPA + DHA contents of Black Sea mussels are lower (0.45 g/100 g). Comparable information in literature for seasonal changes in long-chain n3 PUFAs contents in Black Sea mussel edible tissues was not found. The functional properties of mussel lipids were assessed by the following indices (**Table 3**): indices of atherogenicity (AI), thrombogenicity (TI), and cholesterolemic index (h/H). The mentioned indices were in the respective range: for AI—from 0.39 to 0.46; for TI—from 0.17 to 0.22; and for h/H—from 2.28 to 2.92. AI and TI seasonal variations showed opposite trends compared to h/H index. In the summer season, their values were the lowest, whereas h/H index presented the highest amounts in the same season. In contrast, a new investigation of wild black mussels, harvested in the Bulgarian part of Black Sea (Varna Bay), shows different levels for these indices [49]. The authors find

twice higher AI (up to 0.94) and higher TI (up to 0.37) values, but significantly lower h/H levels (up to 1.92) in comparison with our results. Moreover, the authors report different seasonal changes in FA distribution, especially between individual SFA and PUFA. The possible reasons for the observed discrepancies are different mussel populations (subtidal mussels) and locations, available food, sex, etc.

The hypercholesterolaemic-atherogenic potential of mussel lipids is related to their cholesterol content and FA profile. In the present chapter, cholesterol/SFA index (CSI) and cholesterol index (CI) were determined for the assessment of this potential. CSI was used to compare different types of food, whereas CI predicted the possible variation in an average individual serum cholesterol, which could be affected by individual portions of food. Seasonal variation was found for CSI (3.85–4.79) and CI (3.7–4.06). The calculated CSI and CI values are comparable with those calculated for red and pink shrimps from the Ionian Sea [50]. The low values of both indices found for the Black Sea mussel lipids indicated their high functional properties and protective role against the risk of cardiovascular disease [51].

The amount of fat-soluble vitamins provided by 100 g raw mussel tissue was calculated as a percentage of the average daily allowance (ADA) and was compared with the RDI, accepted in Bulgaria [42]. Bulgarian dietary standards for ADA are close to those accepted in the European Union [52] with the exception of the RDI for vitamin D3 (5 μg for adults in our country, while the recommendation of the European Union is 10 μg). According to the Dietary Standards in Bulgaria, the analyzed mussels could supply a low percentage of RDI of vitamin A (4.9–6.7%) and of vitamin E (13.2–15.4%). Substantial amounts of vitamin D3 were found in farmed mussels, where 100 g raw mussel tissue could provide between 50 and 62% of the average daily intake. Minor seasonal changes were found for the recommended daily intake values of vitamins A and E, whereas a more significant fluctuation was observed for vitamin D3. The highest RDI levels for all three fat-soluble vitamins were found in the summer season. An earlier investigation of farmed and wild mussels from Bulgaria [36] presents similar low amounts of aquaculture mussel vitamin A and E levels of RDI and lower values of vitamin D3 RDI in comparison with the present study.

The relationship between vitamin E and PUFA intake (for adults), presented as >0.5 for mg vitamin E/g total PUFA ratio, could also be used as a criterion for evaluation of the functional qualities of the mussel lipids (see **Table 4**). This ratio is based on the minimum requirement for vitamin E content, allowing for cellular synthesis and PUFA cellular membrane retention; and vitamin E amounts, required to protect and metabolize dietary PUFA [53, 54]. Based on the calculated ratio, which ranged between 1.6 (autumn) to 3.57 (summer), we can conclude that mussel lipids contained compounds with high biological activity and well-balanced Vit E/PUFA ratio.

4. Conclusion

The study investigated seasonal changes in the quantities of macro-components and fat-soluble biologically active compounds in the edible tissue of the aquaculture mussels from

the Bulgarian Black Sea coast. Regardless of the observed variations in the chemical composition, the mussels are rich in proteins (average 18.5%) and contain low levels of total lipids and carbohydrates, and a low energy value (average 104 kcal/100 g). A well-expressed tendency of decreasing levels of total lipids and carbohydrates during the summer season (July) was established, which correlates well with the reproductive cycle of the Black Sea shellfish. Additionally, significant variations were found for bioactive lipid components such as fatty acids, fat-soluble vitamins, cholesterol, and carotenoids. High levels of n3 PUFAs (average 0.786 g/100 g) were found during the whole study period. Mussels are a rich source of EPA + DHA n3LCPUFAs (average 0.74 g/100 g), supplying 148% of RDI for these FAs. An interesting trend was determined for the summer season: the highest levels of all three fat-soluble vitamins and carotenoids were observed at the lowest total lipid values. Within the study period of catching and distribution of mussels to the Bulgarian markets, the levels of cholesterol that were subject to control were low (average 68.4 mg/100 g). The functional properties of the lipids were estimated by FA ratios, FA indices, and interactions between cholesterol and SFA (low CSI levels), and vitamin E and PUFA (high alpha-tocopherol/PUFA levels). The results demonstrated very good hypocholesterolemic (high h/H values), anti-atherogenic (low levels of AI), and anti-thrombogenic (low levels of TI) potential of the lipids. Valuable new information on changes in beta-carotene and astaxanthin contents in the intestinal tissue was provided. Beneficial carotenoid contents confirm the very good antioxidant potential of the mussel lipids. Although proximate and FA composition, fat-soluble vitamins, cholesterol, and carotenoids contents of mussel tissue are multifarious and strongly dependent on biotic and abiotic environmental factors, we can summarize that the present results illustrate well the high potential of mussels as healthy food. Moreover, mussel consumption could promote dietary recommendations for the consumption of low-fat and cholesterol, rich in n3 PUFA, vitamin D3, and astaxanthin foods. In addition to the study of the bioactive lipid composition of aquaculture black mussels, more detailed investigations devoted to the seasonal changes of different lipid classes as phospholipids, sterols, waxes, carotenoids, etc., are needed. The assessment of the proximate composition and the lipid quality of the black mussel edible tissue may promote their consumption. The findings concerning aquaculture mussels in Bulgaria may support consumers' dietary regimes and help them make healthy food choices.

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Chapter 10

Biological and Medicinal Importance of Sponge

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Additional information is available at the end of the chapter

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Abstract

Sponges are multicellular, heterotrophic parazoan organisms, characterized by the possession of unique feeding system among the animals. They are the most primitive types of animals in existence, featuring a cell-based organization where different cells have different tasks, but do not form tissues. Sponges (Porifera) are a predominantly marine phylum living from the intertidal to the abyssal (deepest ocean) zone. There are approximately 8500 described species of sponges worldwide with a prominent role in many reef coral communities. Several ecological studies reported have shown that secondary metabolites isolated from sponges often serve defensive purposes to protect them from threats such as predator attacks, biofouling, microbial infections, and overgrowth by other sessile organisms. In the recent years, interest in marine sponges has risen considerably due to presence of high number of interesting biologically active natural products. More than 5300 different natural products are known from sponges and their associated microorganisms, and every year hundreds of new substances are discovered. In addition to the unusual nucleosides, other classes of substances such as bioactive terpenes, sterols, fatty acids, alkaloids, cyclic peptides, peroxides, and amino acid derivatives (which are frequently halogenated) have been described from sponges or from their associated microorganisms. Many of these natural products from sponges have shown a wide range of pharmacological activities such as anticancer, antifungal, antiviral, anthelmintic, antiprotozoal, anti-inflammatory, immunosuppressive, neurosuppressive, and antifouling activities. This chapter covers extensive work published regarding new compounds isolated from marine sponges and biological activities associated with them.

Keywords: sponges, anticancer, antibacterial, chemical constituents

1. Introduction

Sponges are the ancient, efficient designed multicellular parazoan organisms and show relatively little differentiation and tissue coordination. A sponge is a sessile, sedentary, filter-feeding

primitive aquatic invertebrate animal which attaches itself to solid surfaces from intertidal zone to depths of 29,000 ft (85000m) or more, where they can get sufficient food to grow [1, 2]. Sponges feed on microscopic organisms (protozoa, bacteria and other small organisms in water) and organic particles [3]. There are about 10,000 known species inhabit a wide variety of marine and fresh water habitats and are found throughout deep ocean depths to rock pools, warm tropical seas to frozen arctic seas, rivers and streams [3, 4]. They are very diverse and occur in various colors, sizes and shapes such as tubular (tube-like), globular (ball-shaped), caliculate (cup-shaped), arboresecent (plant-shaped), flabellate (fan-shaped) and amorphous (shapeless). The scientific term for sponges is Porifera meaning "pore-bearing" and has bodies full of pores and channels allowing water to circulate through them, consisting of jelly-like mesohyl sandwiched between two layers of cells [5]. The shapes of their bodies are adapted for maximal efficiency of water flow through the central cavity, where it deposits the nutrients, and leaves through a hole called the osculum. Several sponges have spicules of silicon dioxide or calcium carbonate and a mesh of proteins called spongin as an internal skeleton. One of the remarkable properties of sponges is their ability to suffer damage and regenerative capacity [6–8]. Marine sponges have attracted growing attention as a source of overwhelming structurally diverse secondary metabolites with potential biological activities and were placed at the top with respect to discovery of biologically active chemical constituents [9, 10]. Although thousands of chemical compounds have been reported in the literature from these sponges, only few of them are clinically described. Many studies revealed that sponge-derived metabolites are used directly in therapy or as a prototype of bioactive leads to develop more active and less toxic analogs [11, 12]. Sponges are most primitive type of aquatic animals in existence which are dominating many benthic habitats, featuring a cell-based organization where different cells conduct all forms of bodily function, but do not form tissues [13]. They consume food and excrete waste products within cells without a body cavity [14]. Several ecological studies reported that high quantity of bioactive constituents produced by sponges often serve defensive against environmental threats such as predation, microbial infection, competition for space or overgrowth by fouling organisms [15, 16]. For this reason marine sponges are the subject of attraction for chemists due to the sheer number of metabolites produced, the novelty of structure encountered, and the therapeutic potential of these compounds in the treatment of human diseases. Scientists working in the field of natural product chemistry and research suggest that these sponges have promising potential to provide future drugs which can serve various diseases. In this chapter, we describe main isolated chemical entities from sponges and their pharmacological application.

2. Anticancer agents

In the recent years, marine natural products bioprospecting has yielded a considerable number of drug candidates, most still being in preclinical or early clinical development, with only a limited number already in the market [17]. A typical example of marine anticancer drugs is eribulinmesylate, a derivative of halichondrin B isolated from the marine sponge. *Halichondria okadai* has achieved success in phase III clinical trials. Literature studies have shown sponge-derived discodermolides antitumor compounds can play remarkable role in future to treat cancer. Plethora of secondary metabolites is produced by marine sponges and their symbionts. The spongothymidine and spongouridine nucleosides were the first successful sponge-derived

pharmaceutical drugs isolated from *Tectitethya crypta* [18]. Ara-C (cytarabineor1-beta-D-Arabinofuranosylcytosine) recently used for the cure of leukemia [19, 20] and its combination with Daunoribicin and other anticancer drugs, is screened in clinical trials for the treatment of acute myeloid neoplasms [21] During the last few years several marine derived natural compounds are in the pipeline for evaluation in Phase I–III clinical trials for various cancers treatment [22]. A review in 2003 listed the most important anticancer candidate from marine natural compounds undergoing preclinical and clinical (I, II, III) trials and following compounds were from sponge origin: Isohomohalichondrin B, Halichondrin B, Laulimalide/Fijianolide,5-methoxyamphimedine(alkaloid)Discodermolide, Hemiasterlins A and B, Fascaphysins (alkaloid), modified halichondrin B, KRN-70000, Alipkinidine (alkaloid), and Variolin (alkaloid) [23]. Moreover marine sponges are the important source for vital diverse bioactive constituents including alkaloids, terpenoids, sterols and macrolides. Renieramycins, members of tetrahydroiso-quinoline family were isolated from marine sponges from genus Reniera with promising anticancer potential. The preclinical results reported that Renieramycin M, a natural constituent from sponge induced lung cancer cells apoptosis through p53-dependent pathway and may inhibit progression and metastasis of lung cancer cells [24]. A novel polycyclic guanidine alkaloid monanchocidin isolated from *Monanchora pulchra* marine sponge reported to induce cell death in human cervical cancer (HeLa),human monocytic leukemia (THP-1) and mouse epidermal (JB6 Cl41) cells [25]. In the early 1987, as esquiterpene aminoquinone, Smenospongine extracted from *Smenospongia* sp. reported to induces cytotoxic, antiproliferetive, antiangiogenic, and antimicrobial activities [26]. Spongistatin a macrocyclic lactone polyether isolated from *Spongia* sp. marine sponge in 1993 was shown to inhibit microtubule assembly, mitosis, and the binding of tubulin to vinblastine thereby inducing cytotoxic cell death in numerous cancer cell lines [27, 28]. Recently a very important compound named lectin has been isolated from *Cinachyrella apion* marine sponge was evaluated for antiproliferative, hemolytic, and cytotoxic properties, besides the ability to induce cell death in tumor cells. Results showed that the lectin induces cell death by apoptosis activation by pro-apoptotic protein Bax, promoting permeabilization of mitochondrial membrane, S phase cell cycle arrest and acting as both dependent and/or independent of caspases pathway. These results indicate the potential of lectin for treating cancer [29]. Another marine sponge component, heteronemin a sesterterpene isolated from *Hyrtios sp*. has attracted the interest of researchers as an antimour agent especially for its pharmacological effects on chronic myelogenous leukemia cells. Results revealed that heteronemin affected the various cellular processes such as cell cycle, nitrogen-activated protein kinases pathways, apoptosis, and nuclear factor kappa B signaling cascade. Thus the compound has shown anti-inflammatory as well as anticancer agent [30]. A collaborative program between experimental therapeutics laboratory of Henry Ford Hospital in Detroit and University of California Santa Cruz initiated in 1990 focused on the development and discovery of anticancer drugs from sponge extracts. About 2036 extracts from 683 individual sponges were examined by using novel *in vitro* assay led to the identification pure bioactive compounds from many sponges for treating solid tumors. The collaborative efforts and analogs led to the isolation of number of constituents with of anticancer potential [31].

Thus the possibility of development of new anticancer drugs for curing or reducing cancer is promising. Until now, *in vitro* antitumor activity studies of sponge-derived compounds were tested. Thus, the detailed pharmaceutical studies to investigate the mechanism of action and clinical trials are needed. Moreover, the extensive ongoing research on sponges and development

of new advanced techniques have made it possible to access deep sea, new anticancer marine isolates with unprecedented carbon skeleton and inhibitory activities of human cancer cell continued to be discovered and developed, which will offer in future the new candidate for cancer therapy. The chemical constituents so far reported for anticancer activity include (**Table 1**).

Categories	Species	Active agents	Antitumor tested	References
Alkaloids	*Papua*	Hyrtiocarboline	H522-T1, MDA-MB- 435, U937 tumor cell lines	[31]
	Penares sp.		HL-60, HeLa	[32]
	Aaptos suberitoides	Aaptamine	L5178Y	[33]
	Monanchora arbuscula	Norbatzelladine		
		Dinorbatzelladine	MDA-MB-231 breast cancer	[34]
		Dinordehy-drobatzelladine		
		Dinorbatzelladine		
		Dihomodehy-drobatzelladine	MDA-MB-231 breast cancer	[34]
	Clathria calla	Norbatzelladine		
		Clathriadic acid		
	Xestospongia sp.	Renieramycin T	HCT116, QC56, AsPC1	[35]
			T47D tumor cell lines	
	Smenospongia sp.	6′-Iodoaureol	MOLT-3, HepG2 cells	[36]
	Hyrtios sp.	Hyrtimomine A	Human epidermoid carcinoma KB, murine leukemia L1210	[37]
	Pseudoceratina verrucosa	Aplysamine	HeLa, NFF cells	[38]
	Amphimedon sp.	Pyrinodemin G	P388 murine leukemia cells	
		Pyrinodemin H		[39]
	Oceanapia sp.	Sagitol C	PC12, L5178Y, HeLa cells	[40]
	Monanchora pulchra	Monanchocidins B		
		Monanchocidins C	HL-60 human leukemia cells	[41]
		Monanchocidins D		
		Monanchocidins E		
	Agelas sp.	Hexazosceptrin		
		Agelestes A-B	U937, PC9 human	
		(9S, 10R, 90S, 100R)-nakamuric acid	Cancer cell lines	[42]
Sterols	*Ianthella sp.*	Petrosterol-3,6-dione	A549 (lung), HT-29 (colon),	
		5α,6α-epoxy-petrosterol	SK-OV-3 (ovary), MCF-7 (breast) HL-60 and U937	[43]
	Lissodendryx fibrosa	Manadosterol A-B	Ubc13–Uev1A complex	[44]
Terpenoids	*Carteriospongia* sp.	Homoscalarane sesterterpenes	A2780, H522-T1, A2058	[45]
	Monanchora sp.	9Sesterterpenoids	A498, ACHN (renal cancer)	[46]
			MIA-paca, and PANC-1 (pancreatic cancer)	[47]
	Psammocinia sp.	Scalarane sesterterpenes	A498, ACHN MIA-paca,PANC-1	[48]
	Pseudoaxinella flava	Diterpene isonitrile	PC3(prostate cancer cell line)	[49]
	Agelas axifera	Three axistatins (pyrimidine diterpenes)	P338, BXPC-3 MCF-7, SF-268 NCI-H460, KML20L2, and DU-145 cell lines growth	[50]

Categories	Species	Active agents	Antitumor tested	References
	Thorectare ticulate	Metachromins U Metachromins V	SF-268, H460,MCF-7, HT-29, and CHOK1 (mammalian cell line)	[51]
	Dactylospongia elegans	Nakijinol B and CHO-K1	SF-268, H460, MCF-7, HT-29	[51]
	Coscinoderma sp.	Sesterterpenes coscinolactams C	K562 and A549 (human cancer cells)	[52]
		Coscinolactams D,		
		Coscinolactams E		
		Coscinolactams F		
		Coscinolactams G		[53]
Macrolide	*Cinachyrella enigmatica*	Enigmazole A	NCI 60 human tumor cells	[54]
	Jaspis splendans	Jaspamide M	MCF-7and HT-29	[55]
		Jaspamide N	(antimicrofilament)	
		Jaspamide O		
		Jaspamide P		
	Mycale hentscheli	Peloruside A	P388 HL-60 cells	[56]
		Peloruside B		
	Pipestela candelabra	Pipestelide A	KB cell lines	[57]
		Pipestelide B		
Polyketone	Plakortis simplex	Simplextone C	HeLa, K562, A-549 cell lines	
		Plakortoxide A		[58]
	Plakortis halichondrioides	Epiplakinidioic acid	DU-145, A2058	[59]
		Plakortoxide A	tumor cell lines	
	Lithoplocamialithistoides	Polyketides PM050489	HT-29, A549, MDA-MB-231	
		Polyketides PM060184	Human tumor cell lines	[60]
Peptides	*Homophymia* sp.	Homophymines B	KB, MCF7, MCF7R, HCT116	
		Homophymines E	HCT15, HT29, OVCAR 8, OV3,	
		Homophymines A1-E1	PC3, Vero, MRC5, HL60, HL60R, K562, PaCa, SF268, A549, MDA231, MDA435, HepG2, and EPC human tumor cells	[61]
	Neamphius huxleyi	Neamphamide B	A549,HeLa, LNCaP,	
		Neamphamide C	PC3, NFF human tumor	
		Neamphamide D	cell lines	[62]
	Eurypon laughlini	Rolloamide A	LNCap, PC3MM2, PC3, DU145 (Prostrate), MDA361, MCF7, MDA231 (breast), OVCAR3, SKOV3, U87MG (Glioma), (ovarian), A498 (renal)	[63]
	Stylissa caribica	Stylissamide H	HCT-116.	[64]
	Homophymia lamellose	Pipecolidepsin A	A549, HT-29 MDA-MB-231	
		Pipecolidepsin B	Human tumor cells	[65]
Glycosides	*Pandaros acanthifolium*	Acanthifoliosides A–E	L6 cell lines	[66]
	Rhabdastrella globostellata	Rhabdastin E-G	HL-60	[67]
Quinones	*Dysidea avara*	Dysidavarone A	HeLa, A549, MDA231, QGY7703	
		Dysidavarone D	HeLa tumor cells	[68]
	Dactylospongia metachromia	5 Sesquiterpene aminoquinones	L5178Y mouse cancer cell lines	[69]

Categories	Species	Active agents	Antitumor tested	References
	Dactylospongia avara	3 Dysideanones A–C	HeLa HepG2 cancer cell lines	[70]
Miscellaneous	*Petrosia* sp.	3(−) Petrosynoic acids A–D	A2058, H522-T1,	
			H460 human tumor cell line	
			IMR-90 human fibroblast cells	[71]
	Subereamollis	Subereaphenol D	HeLa cell lines	[72]
	Mixture of *Smenospongia aurea*	(E)-10-benzyl-5,7-dimethylun-1 deca,5,10-trien-4-ol	HL-60 human leukemia	
	Smenospongia cerebriformis			
	Verongula rigida			[73]
	Myrmekioderma dendyi	Myrmekioside E-2	NSCLC-N6 and A549 tumor cell lines	[74]
	Genus *Suberea*.	Four novel Psammaplysin analogs	Cytotoxicity	[75]

Table 1. Marine sponge-derived anticancer compounds and their effects.

3. Antibacterial active agents

Marine sponges are among the richest sources of interesting chemicals produced by marine organisms. Exploitation of bioactive metabolites by natural product chemist from marine sources by using antimicrobial or cytotoxic assays started back in 1970s. Later, various reputed pharmaceutical companies joined hands for this effort using more advance assay systems, including enzyme inhibition assays. As a result several new promising bioactive candidates have been discovered from marine sponges [76]. Bioactive constituents are claimed for potent *in vivo* or *in vitro* activity against infectious and parasitic diseases, such as bacterial, fungal, viral and protozoan infections. Studies revealed that the crude extracts of marine sponge have shown high incidences of antibacterial activity against terrestrial pathogenic bacteria, but very low incidences of antibacterial activity against marine bacteria [77, 78]. Very few cases of sponge infection by exogenous microorganisms are known, presumably due to the accumulation/or product by the marine sponges of substances which have antimicrobial activity [1]. A number of new metabolites with antibiotic applications are discovered every year, but in marine sponges their ubiquity is remarkable. Antibacterial screening of marine sponges led to identification and characterization of wide range of active chemical constituents, including some with promising therapeutic leads [79, 80]. Around 850 antibiotic constituents are reported from marine sponges [81]. Various antibacterial substances were identified from marine sponges by continuous efforts of marine natural product community. Despite of discovery of huge number of natural product from marine sponges, none of them has yet led to antibacterial product, but currently several are under investigation. Examples of some isolated substances from marine sponges with antibacterial activity are shown in **Table 2**. The first discovered antibiotic from a marine sponge was manoalide, a seterterpenoid isolated from *Luffariella variabilis* [82]. The most promising constituents with antibacterial properties reported from marine sponges include: agelasine D, cribrostatin 3 and 6, petrosamine B, psammaplin A and alkylpyridines (haliclonacyclamine E, arenosclerins) and among these constituents, manzamine A and psammaplin A are in preclinical trials. Many of these have excellent potential for drug development, but no commercial medication has been originated from them so far.

Categories	Species	Active agents	Antibacterial tested	References
Alkaloids	*Axinella* sp.	Axinellamines B-D	*H. pylori* Gram-(-ve)	[83]
	Acanthostrongylophora sp.	12,34-Oxamanzamine E,	*M. tuberculosis*	[84]
		8-Hydroxymanzamine J		
		6-Hydroxymanzamine E		
	Arenosclera brasiliensis	Haliclonacyclamine E,	*S. aureus, P. aeruginosa*	
		Arenosclerins A-C		[85]
	Spongosorites sp.	Deoxytopsentin, bromotopsentin	*S. aureus* (MRSA strain)	
		4,5-Dihydro-6″-deoxybromotopsentin, bis(indole)		[86]
	Cribrochalina sp.	Cribrostatin 3	*N. gonorrheae*	[87]
	Cribrochalina sp.	Cribrostatin 6	*S. pneumonia*	[88]
	Spongosorites sp.	Hamacanthin A	*S. aureus* (MRSA strain)	[86]
	Oceanapia sp.	Petrosamine B	*H. pylori*	[89]
	Latrunculia sp.	Discorhabdin R	*S. aureus, M. luteus*	[90]
			S. marcescens, E. coli	
	Hamacantha sp.	Hamacanthin A 1	*C. albicans*	
		Hamacanthin B 2	*C. neoformans*	[91]
Nitrogenous	*Pachychalina* sp.	Cyclostellettamines A-I,	*S. aureus* (MRSA strain),	[92]
		Cyclostel K-L	*P. aeruginosa* (antibiotic-resistant strain), *M. tuberculosis*	[93]
	Pachychalina sp.	Ingenamine G	*S. aureus* (MRSA strain)	
			E. coli, M. tuberculosis	[92]
	M. sarassinorum	Melophlin C	*B. subtilis, S. aureus*	[47]
	Agelas sp.	Agelasine D	*M. tuberculosis Gram (+ve, -ve)*	[94]
Terpenoids	*Cacospongia* sp.	Isojaspic acid, cacospongin D, jaspaquinol	*S. epidermidis*	[95]
	Myrmekiodermastyx	(S)-(+)-curcuphenol	*M. tuberculosis*	[96]
Miscellaneous	*Oceanapia* sp.	C14 acetylenic acid	*E. coli, P. aeruginosa, B. subtilis, S. aureus*	[97]
	C. sphaeroconia	Caminosides A-D	*E. coli*	[98]
	A. coralliphaga	Corallidictyals A-D	*S. aureus*	[99]
	C. varians	CvL	*B. subtilis, S. aureus*	[100]
	N. magnifica	Latrunculins	*S. aureus* and *B. cereus*	[101]
	Discodermia sp.	Polydiscamide A	*B. subtilis*	[93]
	Psammaplysilla	Psammaplin A	*S. aureus* (MRSA strain)	[102]

Table 2. Marine sponge-derived antibacterial compounds and their effects.

4. Antiviral compounds and their efficacy

The search for new antiviral substances from marine sources led to the isolation of several promising therapeutic leads which are presented in **Table 3**. The literature presents a good number of reports about different biological activities of marine sponges. Several papers

Categories	Species	Active agents	Antiviral tests	References
Alkaloid	*Aaptosa aptos*	4-Methylaaptamine	HSV-1	[110]
	Halicortex sp.	Dragmacidin F	HSV-1	[111]
	Indo-Pacific	Manzamine A, 8-hydroxymanzamine A, 6-deoxymanzamine X neokauluamine	HIV-1	[112]
Nucleosides	*Mycale* sp.	Mycalamide A-B	A59 coronavirus, HSV-1	[113]
	Hamacantha sp.	Coscinamides 60-62,		
		Chondriamides 63-65	Anti-HIV	[91]
Cyclic depsipeptides	*Theonella* sp.	Papuamides A-D	HIV-1	[114]
	S. microspinosa	Microspinosamide	HIV-1	[115]
Sterols	Haplosclerid sponges	Haplosamates A	HV-1	
		Haplosamates B		[116]
Terpenoids	*D. avara*	Avarol 6′-hydroxy avarol, 3′-hydroxy avarone	HV-1	[117]
Nucleoside	*Cryptotethya crypta*	Ara-A	HSV-1, HSV-2, VZV	[105]
	Mycale sp.	Mycalamide A-B	A59 coronavirus, HSV-1	[118]
Miscellaneous	*Dysidea avara*	Callyspongymic acid	HIV, hepatitis B virus	[119]
		2′-5′ Oligoadenylates	Viral replication	[120]
	H. tarangaensis	Hamigeran B	Herpes, polio viruses	[121]
	Petrosia weinbergi	Weinbersterols A-B	Leukemia virus, mouse influenza virus, mouse corona virus	[122]

Table 3. Antiviral compounds from marine sponges and their effects.

reports the screening results of marine organisms for antiviral activity, and a diverse range of active constituents have been isolated and characterized from them [80, 103, 104]. For some of these isolated substances important antiviral activities were reported. Perhaps the most important antiviral lead of marine origin reported thus far is the nucleoside ara-A (vidarabine) isolated from the sponge *Cryptotethya crypta*. Ara-A is a semisynthetic compound, based on the arabinosyl nucleosides, that inhibits viral DNA synthesis [105]. Once it was realized that biological systems would recognize the nucleoside base after modifications of the sugar moiety, chemists began to substitute the typical pentoses with acyclic entities or with substituted sugars, leading to the drug azidothymidine (zidovudine). Ara-A, ara-C (1-β-Darabinosyl cytosine, cytarabine), acyclovir, and azidothymidine are in clinical use and are all examples of products of semisynthetic modifications of the arabinosyl nucleosides [106]. Several of these substances have a great potential for drug development. Ara-A has been used for the treatment of herpes virus infections, but it is less efficient and more toxic than acyclovir [107, 108]. However, ara-A is capable of inhibiting a cyclovir-resistant HSV and VZV (varicella-zoster virus) [109]. The most promising antiviral substances from sponges appear to be 4-methylaaptamine, avarol, manzamines, mycalamide A and B. Among these substances, preclinical assessments were started for avarol and manzamine A. In general, antiviral molecules from sponges do not give protection against viruses, but they may result in drugs to treat already infected individuals. In addition, broad-based antiviral agents such as 2-5A and α-glucosidase inhibitors may be useful in cases of sudden outbreaks of (less familiar) viruses such as SARS and Ebola [80].

5. Antifungal compounds

Marine sponges have been considered a gold mine for the discovery of marine natural products during the past 50 years. The need of new antifungals in clinical medicine due to various kinds of mycoses, in particular invasive mycoses have become serious health problems as their incidences has increased dramatically during last few years in relation to AIDS, transplant recipients, hematological malignancies, transplant recipients and other immunosuppressed individuals. One of the major causes of death in patients suffering from malignant disease is fungal infections and emerging resistance is also an important problem. Immunocompromised patients are mainly infected by *Aspergillus*, *Cryptococcus*, *Candida*, and other opportunistic fungi. *Candida albicans* is most often associated with serious invasive fungal infections, but other *Candida* species and yeast-like organisms (*Blastoschizomyces*, *Trichosporon* and *Malassezia*) have emerged as etiological agents of severe mycoses problem [123–126]. Fungicides which are presently being used are less diverse than antimicrobials, and the usage of many of them is restricted because of their toxic effects to animals, plants and humans. Moreover the progress in this area is slow as comparison to antibacterial agents [126]. Antifungal compounds isolated from marine sponges are listed in **Table 4**.

Categories	species	Active agents	Antifungal tests	References
Alkaloids	*A. brasiliensis*	Arenosclerins A-C	*C. albicans*	
		Haliclonacyclamine E		[127]
	Acanthostrongylophora sp.	Manzamine A	*C. neoformans*	[112]
	Leucetta cf.	Naamine D	*Chagosensis C. neoformans*	[128]
	Pseudoceratina sp.	Ceratinadins A-C	*C. albicans*	[129]
	A. citrina	(−)-Agelasidine F,	*C. albicans*	
		(−)-Agelasidine C		[130]
	M. arbuscular	Batzelladine L	*A. flavus*	[131]
Terpenoids	*L. variabilis*	Secomanoalide	*C. glabrata, C. krusei*	
			C. albicans	[132]
	M. herdmani	Microsclerodermins A-B	*A. fumigatus*	[133]
	Hyrtios sp.	Puupehenonol	*C. neoformans*, *C. krusei*	[134]
Sterols	*Euryspongia* sp.	Eurysterols A-B	*C. albicans*	[135]
	Topsentia sp	Geodisterol-3-O-sulfite, 29-demethylgeodisterol-3-OCl-sulfite	*S. cerevisiae, C. albicans*	
			C. albicans	[136]
Peptides	*Discodermia* sp.	Discobahamin A-B	*C. albicans*	[137]
	Jaspis sp.	Jasplakinolide or jaspamide	*C. albicans*	[138]
	Latrunculia sp.	Callipeltins F-I	*C. albicans*	[139]
	Latrunculia sp.	Callipeltin J-K	*C. albicans*	[42]
	T. swinhoei	Theonellamide G	*C. albicans*	[140]
	Theonella sp.	Theonellamide TNM-F	*Candida* spp, *Trichophyton* spp, *Aspergillus* sp.	[141]
Purine derivatives	*Agelas* sp.	Agelasines, agelasimines	*C. krusei*	[142]

Categories	species	Active agents	Antifungal tests	References
Miscellaneous	*P. reticulate*	Crambescin A2 392	*C. albicans*	
		Crambescin A2 406	*C. neoformans var. gattii,*	
		Crambescin A2 420	*C. glabrata, C. krusei*	
		Sch 575948		[143]
	Sponge	Theonellamides	Antifungal	[144]
	Melophlus sp.	Aurantoside K	*C. albicans* (wild-type)	[145]
	P. halichondrioides	Plakortide F	*C. albicans*, *C. neoformans*, *A. fumigatus*	[146]
	H. viscosa	Haliscosamine	*C. neoformans*, *C. albicans*	[147]
	D. herbacea	3,5-Dibromo-2-(3,5-dibromo-2-methoxyphenoxy) phenol	*Aspergillus*	[148]
	P. onkodes	Two α and β1,2-dioxolane peroxide acids	*C. albicans*	[149]
	T. laevispirulifer	Nematocide, onnamide F	*S. cerevisiae*	[150]
	T. swinhoei	Swinhoeiamide A	*C. albicans*, *A. fumigates*	[151]
	Family *Neopeltidae*	Neopeltolide	*C. albicans*	[152]
	Plakinastrella	Epiplakinic acid F	*C. albicans*	[153]
	H. communis	(−)-Untenospongin B	*C. albicans, C. tropicalis, F. oxysporum*	[154]
	H. lachne	Hippolachnin A	*C. neoformans, T. rubrum, M. gypseum*	[155]

Table 4. Antifungal compounds from marine sponges and their effects.

6. Anti-inflammatory compounds

Marine organisms and microorganisms have provided a large proportion of the anti-inflammatory and natural antioxidants products over the last years. Reports suggest that marine invertebrates represent new marine resources for the isolation of novel agents which are active on inflammatory conditions have also been found in the literature. Herencia and coworkers [156] studied the effects of dichloromethane and methanol extracts from some Mediterranean marine invertebrates on carrageenan-induced paw edema in mice. Extracts partially decreased elastase activity and PGE2 levels measured in homogenates from inflamed paws, without affecting the levels of this prostanoid present in stomach homogenates. Within the framework of the European MAST III Project, extracts of different polarity from sponges, ascidians and cnidarians have been screened for immunomodulating activities [157]. It was demonstrated that endotoxin-free samples of marine origin possess effects on certain components of the immune system. As a result of all these investigations, bioassay-directed separation of active extracts identified many structurally diverse compounds as future leads. Anti-inflammatory compounds found in the marine environment include terpenes and steroids, alkaloids, peptides and proteins, polysaccharides and others. Examples of anti-inflammatory compounds marine sponge origin are presented in **Table 5**. Also includes diterpenes of (8*E*, 13*Z*, 20*Z*)-strobilinin and (7*E*, 13*Z*, 20*Z*)-felixinin from a marine sponge *Psammocinia* sp. [158], and novel anti-inflammatory spongian diterpenes from the New Zealand marine sponge *Chelonaplysill aviolacea* [159].

Categories	species	Active agents	Anti-inflammatory tests	References
Terpenoids	*F. cavernosa*	Cavernolide	TNF-α, NO and PGE2 production	[160]
	Axinella spp.	6-Cycloamphilectenes	NO, PGE2 and TNF-α production	[161]
		2-Cycloamphilectenes	Inhibit NF-KB pathway	[161]
	Psammocinia spp.	Chromarols A-E	Inhibition of 15-LOX	[162]
	Psammocinia spp.	(8*E*, 13*Z*, 20*Z*)-strobilinin	Anti-inflammatory	
		(7*E*, 13*Z*, 20*Z*)-felixinin	Anti-inflammatory	[158]
	C. violacea	Spongian	Anti-inflammatory	[163]
	D. avara	Avarol, avarone,	Inhibition of eicosanoid release	[164]
		Spongiaquinone, ilimaquinone	and depression of superoxide generation	[165]
	Dysidea spp.	Dysidotronic acid	Inhibited production of TNF-α, IL-1 PGE2, and LTB4	[166]
	Plakortis spp.	Plakolide A	Inhibit iNOS	[167]
	D. elegans	Cymopol	DNA binding of NF-KB	[168]
	L. variabilis	Manoalide, scalaradial	Inhibited IL-1 and TNF-α	[169]
	F. cavernosa	Cacospongiolide B	Inhibited PLA2	[170]
	Dysidea spp	Dysidenones A-B	Inhibited human synovial PLA2	[171]
	L. variabilis	Cladocorans A-B	Inhibition of secretory PLA2	[172]
	P. nigra	Petrosa spongiolides	Inhibitor of PLA2	[173]
	P. nigra	Petrosa spongiolide M	Inhibited LTB4 levels	[174]
	Cacospongia spp.	Scalaradial	Inactivate the enzyme PLA2	[175]
	G. sedna	Homoscalarane	Moderate activity to inhibit mammalian PLA2	[176]
	Hyrtios sp.	Puupehenone, hyrtenone	A high potency against 12-human, 15-human and 15-soybean LOX	[177]
	C. linteiformis	Cyclolinteinone	iNOS and COX-2 protein expression in LPS-stimulated J774 macrophages	[178]
	Callyspongia spp.	Akaterpin	Inhibitor of phosphatidylinositol-specific Phospholipase C	[179]
Steroids	*C. lissosdera*	Clathriol	*In vitro* anti-inflammatory activity against human neutrophil and rat mast cells	[180]
	Euryspongia spp.	Petrosterol, 3β-hydroxy-26-*nor*-campest-5-en-25 oic acid	Against 6-keto-PGF1α release in a human keratinocyte cell line HaCaT	[181]
Alkaloids	*X. testudinaria*	Hymenialdisine	Inhibitor of NF-KB and ILs production	[182]
	Agelas spp.	Nagelamides A-H	NF-KB in inflammatory diseases	[183]
	S. flabellate	Stylissadines A-B	Antiinflammatory activity	[184]

Table 5. Anti-inflammatory compounds from marine sponges and their effects.

7. Marine sponge-derived compounds with enzyme inhibitory activity

Derivatives of halenaquinone and xestoquinone showed various enzyme inhibitory activities besides the phosphatidylinositol 3-kinase and topoisomerase I and II inhibitory activities mentioned above. Compound xestoquinone inhibited both Ca^{2+} and K^{+}-ATPase of skeletal muscle myosin [185]. SAR Investigations showed that halenaquinone and three synthetic analogs with a quinone structure significantly inhibited Ca^{2+} ATPase activity. In contrast, four xestoquinone

Categories	Species	Active agents	Enzyme-inhibitory	References
Quinones	*X. exigua*	Halenaquinone	Ca^{2+} ATPase activity	[191]
	X. exigua	Xestoquinone	Ca^{2+} and K^{+}-ATPase activity	[192]
	X. sapra	Halenaquinol	Protein tyrosine kinase activity	[193]
	X. cf. carbonaria	14-Methoxyhalenaquinone	Protein tyrosine kinase activity	[187]
	Xestospongia sp.	Adociaquinone B	Protein tyrosine kinase activity	[194]
	Xestospongia sp.	3-Ketoadociaquinone B	Cdc25B phosphatase activity	[195]
	Xestospongia sp.	Adociaquinone A	Cdc25B phosphatase	[194]
	Xestospongia sp.	3-Ketoadociaquinone	Cdc25B phosphatase	[195]
Cyclostellettamines	*Xestospongia* sp.	Cyclostellettamine	A histone deacetylase derived inhibition	
		Cyclostellettamine G		
		Dehydrocyclostellettamine D		
		Dehydrocyclostellettamine E		[189]
Fatty acids	*X. testudinaria*	Xestospongic acid ethyl ester	inhibit the Na+/K+ ATPase	[190]

Table 6. Marine sponge-derived compounds showing enzyme-inhibitory activities.

analogs in which the quinine structure was converted to quinol dimethyl ether did not inhibit the Ca^{2+} ATPase activity [186]. The protein tyrosine kinase (PTK) inhibitory activities of halenaquinone, halenaquinol, and 14-methoxyhalenaquinone were the most remarkable with IC_{50} values <10 mm. The other analogs was either less potent or inactive, and a rationalization for this SAR pattern was also reported [187]. Xestoquinone also showed significant protein kinase inhibitory activity toward Pfnek-1, a serine/threonine malarial kinase, with an IC_{50} value of ca. 1 mm, and moderate activity toward PfPK5, a member of the cyclin-dependent kinase (CDK) family [188]. Adociaquinone B and 3-ketoadociaquinone B were the most potent inhibitors of the Cdc25 B phosphatase inhibitory activities, and the dihydro-benzothiazine dioxide in compounds Adociaquinone A, Adociaquinone B, 3-Ketoadociaquinone A, and 3-Ketoadociaquinone B appeared to be an important structural feature for this enhanced activity. Four cyclostellettamines, cyclostellettamine A, cyclostellettamine G, dehydrocyclostellettamine D and dehydrocyclostellettamine E inhibited histone deacetylase derived from K562 human leukemia cells with IC_{50} values ranging from 17 to 80 mm [189]. Xestospongic acid ethyl ester (207) was found to inhibit the Na+/K+ ATPase [190]. Compounds are listed in **Table 6**.

8. Sponge-derived immunosuppressive compounds and their efficacy

Recently natural constituents isolated from marine sponges were tested for immunosuppressive activities and in the end of 1980s, deep water marine sponges resulted in isolation of pure compounds with immunosuppressive properties. Two important compounds: 4a-merhyl-5a-cholest-8-en-3~-ol and 4,5-dibromo-2-pyrrolic acid discovered by American scientist from deep water sponge *Agelasfla bellrform is* showed significant immunosuppressive activity. Both compounds were found significantly active in suppression of the response of murine splenocytes in the two-way mixed lymphocyte reaction (MLR) with little to no demonstrable cytotoxicity at low doses [196]. Constituents isolated from the Aurora globostellata marine sponge showed

immunomodulatory potential. The immunomodulatory potential was evaluated by oral administration of ethyl acetate extract of marine sponge (200 mg/kg) to Wistar rats and the results obtained showed that extracts exhibited immunosuppressant activity and can further be studied [197]. A recent investigation on an Indian marine sponge aimed to isolate and characterize bacteria with immunomodulatory and antimicrobial activity. *Callyspongia difusa* (Gulf of Mannar province) a marine sponge resulted in isolation of 10 marine bacterial strains which exhibited remarkable antagonistic activity against clinical bacterial pathogens. These findings suggested that the sponge associated bacterial strain *Virgibacillus* sp. can contribute the search for novel antibiotics to overcome infections and also for the production of potential immunomodulators [109].

9. Hypocholesterolemic compounds

In the last decade studies reported that marine sponges could have been a source of hypocholesterolemic compounds. For example, lysophosphatidylcholines and lyso-PAF analogs derived from *Spirastrella abata* are reported as successful inhibitors of cholesterol biosynthesis in vitro study [198, 199]. Zhao et al. [200] extracted novel lysophosphatidylcholines from marine sponges with hypocholesterolemic properties and thereby aroused an interest of compounds from marine sponge due to short lifespan of conventional lysophosphatidylcholines *in vivo*.

10. Sponge–derived antibiotics

Also, over the years marine sponges are considered as a rich source of natural products and metabolites for antibiotics possessing strong inhibitory against bacteria, fungi and microbes. Several studies revealed that many natural bioactive components isolated from various marine sponges can be useful for the production of new antibiotics and antimicrobial drugs. In the recent years many scientific studies provided evidences for marine sponge metabolites with efficient antibiotic, antibacterials and antimicrobial properties. Purpuroines A-J, halogenated alkaloids isolated from *Lotrochota purpurea* marine sponge showed promising inhibitory activities against bacteria and fungi related diseases [201]. *Haliclona* sp. sponge from Korea resulted in isolation of novel cyclic bis-1,3-dialkylpyridiniums and cyclostellettamines, which showed moderate cytotoxic and antibacterial activities against A549 cell-line and Gram-positive strains, respectively [202]. A number of new alkaloids were isolated from the marine sponge *Agelas mauritiana*: (+)-2-oxo-agela-sidine C, (−)-8′-oxo-agelasine D,4-bromo-N-(butoxymethyl)-1H-pyrrole-2-carboxamide, ageloxime B, and (−)-ageloxime D and some of these isolated components exhibited antifungal activity against *Cryptococcus neoformans*, antileishmanial activity in vitro and antibacterial activity against *S. aureus* and methicillin-resistant *S. aureus in vitro* [203]. Extracts prepared from the sponge's species *Petromica citrina*, *Haliclona* sp. and *Cinachyrella* sp. exhibited antibacterial activity against 61% of the coagulase-negative staphylococci (CNS) strains, including strains resistant to conventional antibiotics. *P. citrina* extracts showed the largest spectrum of inhibitory activity. This current study according scientist shows potential of marine sponges to become new sources of antibiotics and disinfectants for the control of CNS involved in bovine mastitis in future [204]. Isolation of isonitriles ditepene from *Cymbastela hooperi*, tropical marine sponge and the axisonitrile-3 sesquiterpene isolated *Acanthella kletra*, from

the tropical marine sponge were tested for series of bioassays antibacterial, antiphotosynthetic, antifouling, antialgal, antifouling, antialgal, antiphotosynthetic, antifungal, and antitubercular. The results showed majority of the tested compounds were active against at least two of the applied test systems [152]. Recently, sponge-derived actinomycetes and sediments isolated from marine sponge were tested for bioactive constituents with antifungal and antimicrobial activity. Out of 15 prepared active extract nine were found active against *Enterococcus fascism* (vancomycin-resistant) and *Candida albicans* multidrug-resistant [132], including strains resistant to conventional antibiotics. Thus the bacterial actinomycetes from marine sponges and other marine organisms have been proved prolific producers of pharmacologically active compounds. Literature studies revealed that 70% of naturally derived antibiotics which are currently in clinical use have been derived from actinomycetes. In the recent study, *Streptomyces* sp. strains from Mediterranean sponges and secondary metabolite namely, cyclic depsipeptide valinomycin, indolocarbazole alkaloid staurosporine and butenolide, were screened for anti-infective activities. All the isolated compounds along with *Streptomyces* sp. exhibited antiparasitic activities. Researchers also claim the anti-infective potential of marine actinomycetes is very promising.

11. Marine sponges-derived antifouling and antibiofilm compounds

Bacterial biofilms are surface-attached microorganism's communities that are protected by an extracellular matrix of biomolecules. Continuous use of chemical antifoulants resulted in increased tributyltin concentration and created extensive pollution problems in marine organisms. Natural antifouling molecules from marine have been recently reviewed and researches hope that will provide more specific and less toxic antifouling activity in future. Antifouling compounds derived from sponges were found to be very effective, environmentally friendly biocides and less toxic [205]. In the last few years several studies were directed to find the most promising alternative technologies to antifouling in marine organisms, especially from sponges. In a recent study structurally different compounds containing 3-alkylpyridine moiety were evaluated for antifouling potential. The compounds, namely haminols, saraine and 3-alkylpyridinium salts extracted from *Reniera sarai, Haliclona* sp. and the mollusk *Haminoea fusar* is obtained by synthesis, showed very good antifouling potential larvae of the barnacle *Amphibalanus amphitrite*. Bromopyrrole or diterpene alkaloids derivatives isolated from *Agelas linnaei* and *Ageles nakamurai* Indonesian marine sponges exhibited cytotoxic activity. Moreover, agelasine derivatives inhibited settling of larvae of *Balanus improvisus* in an antifouling bioassay as well as the growth of planktonic forms of biofilm forming bacteria *S. epidermidis* [206].

12. Conclusion

Marine invertebrates (Porifera, Cnidaria, Mollusca, Arthropoda, Echinodermata, etc.) are considered as one of the major groups of biological organisms which gave huge number of natural products and secondary metabolites with interesting pharmacological properties and led in the formation of novel drugs. Among marine invertebrates, marine sponges (phylum: Porifera) is the most dominant responsible group for discovering significant number of natural components, which has been used as template to develop therapeutic drugs. These natural products

possesses vast range of therapeutic application, including antimicrobial, antihypertensive, antioxidant, anticancer, anticoagulant, anti-inflammatory, immune modulator, and wound healing and other medicinal effects. Therefore, marine sponges are considered a rich source of chemical diversity and health benefits for developing drug candidates, nutritional supplements, cosmetics, and molecular probes that can be supported to increase the healthy life span of humans. In this chapter we included the most important and biologically active marine sponge-derived compounds and presented selected studies of most important bioactive and promising natural products and secondary metabolites from marine sponges.

Conflict of interest

The authors declare that they have no conflict of interest.

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Chapter 11

Chemical Ecology of Biocompounds in Molluscs

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Additional information is available at the end of the chapter

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Abstract

Among aquatic creatures, molluscs are one of the main phylums of marine organisms because of their vast biodiversity, nutrition advantages and their natural compounds. Chemical ecology discusses study of natural compounds in organisms, analyzes their structures and tracing their production and variation in response to environmental parameters. There are about 600 natural compounds identified, in which their metabolism are studied more in four classes of molluscs: polyplacophora, gastropoda, cephalopoda and bivalves. The main identified compounds are amino acids, lipids and fatty acids, terpens and steroids. Fatty acids and lipids are the main pre-structures for biological membranes, and their physical characteristics create membrane structure and its activities. Therefore, many cell functions depend on membrane activity and chemical composition of membrane lipids and environmental parameters as follows. Normally, the omega 6 to omega 3 ratio has moderate amounts in natural food sources. The more the ratio closer to 1, the more body metabolism could be sensitive for absorption. Environmental parameters, such as temperature, salinity, pH and food type source, could change the amount and structure of natural compounds. Biological factors such as sex type, reproduction and breeding cycles, different tissues and their activities could receive different types of natural compounds.

Keywords: mollusc, chemical ecology, biocompounds, environmental parameters

1. Introduction

The name mollusc (mollusk) was derived from Latin word mollus meaning soft. This term was first used by Cuvier in 1798 to describe squids, cuttlefish and animals whose shells is reduced. After the arthropods the molluscs are the most successful of the animal phyla in terms of numbers of species. Considering the vast species of molluscs and the large number of fossil species, they are the largest marine phylum, comprising about 23% of all the named marine organisms [1].

Molluscs live in very different habitats and are highly diverse especially in their ecological behaviors. The phylum consist 10 taxonomic classes, which two are entirely extinct. Among the existing classes, Cephalopods such as squid, cuttlefish and octopus, are among the most neurologically advanced of all invertebrates, and gastropods (snails and slugs) are the most numerous classes in this phylum [2].

Molluscs are highly successful animal group in terms of ecology and adaptation and they are found in all habitats ranging from deepest ocean to intertidal zone, freshwater and terrestrial lands where they occupy a wide range of habitats, but the highest diversity could be found in the sea in comparison with freshwater and terrestrial habitats. Between all classes in the phylum Mollusca, the most important class is gastropoda comprising more than 80% of all living Mollusca species. The species belonging to this class occurs in marine, freshwater and terrestrial habitats. Whereas bivalves occurs both in freshwater and marine environments, but there is not any species in terrestrial habitats. In the all classes of molluscs, 6 classes are exclusively marine species [3, 4].

Molluscs are consumed as a food source for humans, birds, fish, mammals and other invertebrates, and also play a key role in the recycling of nutrients, soil-generation and water filtration. They are good bio-indicators too, for environmental quality in all types of aquatic habitats [4, 5].

Molluscs have very different forms among the other animal phylums. Snails, slugs and other gastropods; clams, oysters, scallops and other bivalves; squid, cuttlefish, octopus and other cephalopods; and also lesser known subgroups have interesting diversities in structure, color and size [6]. The giant squid, which had not been observed alive in its adult form recently, is one of the largest invertebrates, with 10 m (33 ft.) long and 500 kg (1100 lb.) weighed [7].

Molluscs are an important food source for humans as mentioned earlier, but there is a risk about poisoning from toxins which can accumulate in certain molluscs under specific conditions. Besides, they are a good source of many luxury goods, such as pearls, mother of pearl, Tyrian purple dye, and sea silk. Also, in ancient periods, their shells have also been used as money [4].

Mollusca are very abundant and form an important trophic level in the aquatic food chain. A large number are filter feeders and hence, are important in nutrient recycling along with the other soil invertebrates. Numerous molluscs are important food source for humans such as Clams and Snails. Some gastropods are pest and damage crops or others hosts for some disease causing parasites such as lung worm which causes schistosomiasis and liver worm for fascioliasis in humans [3, 5].

In addition to the wide usages of molluscs in food industry, shell decorations, dyes and medicines; determination, identification and extraction of their bioactive compounds and secondary metabolites have been an important scientific field of research recently. For instance, isolated natural products from molluscs and their structural analogues are particularly well represented in the anticancer compounds in clinical trials. These compounds and their different chemical structures could be change in each species [8–10].

The marine environment is highly competitive and being able to produce fundamental compounds which have both industrial and medical applications. Based on the species number, molluscs are the second largest phylum in the marine environment. Their morphological and physiological features attract many investigators [11]. Among molluscs, gastropods have a particular role in commercial shell craft industry. A wide variety of species exists on land, fresh water and the sea. Marine gastropods form only a minor component of marine fishery resources. Many species are exported for the purposes of manufacturing ornaments, curious and various other artifacts of commercial value. Women and children collect this gastropods and bivalves from shallow estuaries for nutritional food. Shells and shell crafts of gastropods are the major economy for the local peoples in marine coasts. Marine bivalves and gastropods are also rich source of many biologically active compounds. Owing to their medicinal and industrial properties, several species are traditionally fished for food and shell [2].

Mollusc species could be hazardous or pests. For example, blue-ringed octopus which is often fatal, and Octopus apollyon causes inflammations which can last for over a month. Toxic cone shells could kill or cause inflammations, while some times their venoms could become important tools in neurological research. Also, some snails and slugs are serious agricultural pests or dangerous vectors for transition parasitic diseases [10, 11].

2. Concept of chemical ecology

The different species from molluscs probably utilizes the neutral and total lipids during cold seasons in order to survive and stores them for hot seasons. The importance of stored lipids is for reproductive purposes. However, they have also been shown to provide energy during winter, when carbohydrate reserves are depleted. This indicates that the fatty acid compositions of animals, neutral lipids in particular, are dictated by their metabolic activities and components of their dietary lipids [12].

The feeding habitats and diet composition are important factors that cause changes in the levels or type of the fatty acids in the different groups of molluscs. There are different feeding habits (such as filter feeder and detritus feeder) in the different groups of this phylum. Most of the lipids and considerable amounts of C20:5ω3 and C22:6ω3 acids are provided by diatoms and dinoflagellates, respectively. For example, diet composition of bivalves which are filter feeders, consist of dinoflagellates, bacteria and particulate organic material. It is found that diatoms have high levels of C20:5ω3 acid and low C22:6ω3 acid, but dinoflagellates have high concentrations of C22:6ω3 acid. Some species of molluscs are detritus feeder, and amounts of lipids, SFAs and MUFAs of 14–18 carbons are provided by detritus. Therefore, diet composition has the important role in the variation in the level and type of the fatty acids between different groups during four seasons of the year [13–15].

The different metabolic processes play an important role in the changes of levels and type of the fatty acids, because there are significant differences in amount of consumed energy between different metabolic processes. Between all metabolic processes, reproductive cycles is the main process for consume of energy, and this process need high levels of energy (fatty

acid). Therefore, there is probably an apparent relation between reproduction cycles and fatty acid profiles. In between all fatty acids same as C20:4ω6, is mostly associated with the reproductive enzymes and highest levels of this compound is consumed in spawning times and reproductive processes.

Growth is one of the processes which needs high levels of energy too, and the energy levels (fatty acid) change in the different stages of the growth. The growth ratio is not similar for different organs and species, and different types of organs need different fatty acids level for growth. Among different organs, sexual organs such as gonads need high levels of fatty acids for growth, and the highest levels of energy are consumed for gonad growth. Therefore, metabolic ratio and followed energy level are varied in different processes, and it could be found that metabolic ratios are key roles in fatty acid amounts and their profiles [4, 5, 14].

The decrease in the ∑PUFA level of neutral lipids of mollusks may probably due to transport of fatty acids to the reproductive organs responsible for gonad maturation. In the different species of mollusks, which the winter is reproductive time, the level of fatty acids in the winter is low, in comparison with other seasons [6, 7].

Many studies indicate that there are a positive correlation between fatty acids and temperature in the tissues of mollusks. Accumulation and increase in the amount of lipids especially PUFAs during summer and decrease in winter may be related to the adaptable regulation of the melting point of cellular lipids. Therefore, many researches are indicated that the amount of lipids in summer is higher than the winter, which returns to; (1) consume of lipid in the reproductive organs for gonad maturation and (2) the adaptable regulation of the melting point of cellular lipids. Finally, variations in the lipid levels in their tissues are related to environmental parameters (such as temperature, light and salinity), seasonal variations, feeding habitats, spawning time and reproductive processes, sexual development and growth metabolisms of molluscs [15–17].

In the total body lipids analyses of molluscs, fatty acids, phospholipids and neutral lipid fractions identified from different tissues. These fatty acids are mostly common in marine and freshwater mollusks. Also, odd-numbered fatty acids, such as C13:0, C15:0, C17:0, and C20 polyunsaturated fatty acids in body lipids of different species, were identified. As mentioned, temperature, food availability, metabolic and physiological activities can affect the lipid and fatty acids compositions of molluscs [6, 8].

3. Chemical ecology of natural compounds in molluscs

Marine molluscs have become the focus of many chemical studies aimed at isolating and identifying novel natural products and secondary metabolites. As scant information is available on the chemistry of terrestrial and freshwater species, this review focuses on marines. Considering the chemical redundancy between species, at least 977 distinct compounds have been isolated from about 251 species in the annual reviews of marine natural products [6], which indicates different chemical diversities and related compounds derived from their biochemical pathways. These compounds could be isolated from a single species merely, or from the same family or genus [9].

Distribution histogram of species diversity reveals multiple metabolites, with a median number of two and a maximum of 58 compounds isolated from a single species [18, 19].

Search results typically show small groups of structurally related compounds (analogues), regarding that the compounds vary in different habitats for the same species. For example, 25 compounds such as terpenes, nitrogenous aliphatic compounds, macrolides and fatty acid derivatives have been isolated from the sea hare *Aplysia kurodai*. Eight novel metabolites were isolated from this species in new environment, further [20].

Its close related species *Aplysia dactylomela*, had 58 compounds which were primarily terpenes derived probably from algal diets of these cosmopolitan grazing sea hares. The *Patinopectin yessoensis* bivalve contained second highest number of sterols and algal toxins. Hence, it could be found that dietary sources contribute significantly to the chemical diversities in molluscs. Evidence for the biogenesis of secondary metabolites mostly stems from feeding experiments, which demonstrate the incorporation of radio-labeled precursors in certain groups of heterobranch molluscs. The secondary metabolites isolated, fall into a wide range of structural classes, with some compounds being more dominant in certain taxa [21, 22].

Clearly, all the secondary metabolite types are present in both gastropods and bivalves. Terpenes are dominated In Gastropods, while only three terpenes were identified in bivalves. Terpenes have been an important field of research in soft-bodied grazing gastropods, which they might gain these compounds from their diet for their own defense [5]. Sterols are dominated in bivalves partly because of their role in reproduction cycles, while they are rare in gastropods, taking into account that the large number of researches in bivalves is probably due to their importance in fisheries and aquaculture. Polyproprionates and alkaloids have been isolated from both classes, whereas aliphatic nitrogenous compounds are relatively uncommon in both [8].

There are an extraordinary series of unusual compounds in marine invertebrates, many of them cause interesting biological properties. For instance, opisthobranchs and pulmonates, particularly are important due to their secondary metabolites, and the ecological role and biosynthesis of these compounds could be related to their diet such as microalgae and diatoms. Opisthobranchs which are unprotected with reduced or completely absent shells, have defensive strategies using different chemicals [12]. The selected sampling stations were along different ecosystems such as Indian, Chinese, Mediterranean, Australian and Atlantic coasts of Spain, and strongly indicate that the metabolism of the opisthobranchs is influenced by geographical location, ecosystem type and habitats. The feeding ecology and habitats of all molluscs species are very selective, so feeding metabolites possessed by related species are more similar, while those de novo biosynthesized are most identical in species belonging to the same family but with different geographically habitat. Also, some recent biosynthetic experiments possessed had been discussed [23, 24].

Natural products research aimed at the isolation and identification of novel secondary metabolites, has only been undertaken on a small proportion of molluscan species to date. The bioactivity of many molluscan traditional medicines is yet to be substantiated, but preliminary data available from bivalves, cephalpods and caenogastropods suggests that there is likely to be some chemical basis to their medical applications.

All compounds which are produced by molluscs are varied because of environmental factors such as temperature, salinity and seasonally variations. Therefore, changes in environmental factors could cause variations in the chemical components. Therefore differences in chemical components need different conditions for production, for example fatty acids and amino acids are related to specific temperature and salinity. In conclusion, environmental factor changes in different seasons could be caused in decrease or increase level of compounds. Also, other biological factors such as food availability, metabolic and physiological activities can affect the compounds such as lipid and fatty acids composition of molluscs [13–15].

4. Different types of natural compounds in molluscs

Amino acids are classified into essential amino acids (EAA) (cannot synthesized by humans) and non-essential amino acids (NEAA). In addition to oils and other hydrocarbon derivatives in the marine environment, the hydrocarbons synthesized by organisms occur normally in this environment. Aliphatic hydrocarbons are the principal group, and can occur in several species of marine as well as terrestrial plants and animals.

There are different type of fatty acids such as ∑SFA, ∑MUFA, and ∑PUFA in the whole body of molluscs. There could be changes or variations in their levels of different groups in the different seasons. These differences might be based on temperature, feeding habitats, or metabolic demands [24–26].

The triacylglycerol compounds store SFAs for energy purposes in different processes in body and they also may be interim PUFAs reservoir, which could be transferred to the structural lipids or directed to specific metabolic routes for function of different organs. In contrast, phospholipid compounds fractions of mollusks show considerably less seasonal variations to maintain structural exactness of the cell as compared to the store of saturated fatty acids to be used as a source of energy and store of PUFAs required for phospholipid synthesis to multiple membrane structures or to be integrated in several metabolic processes [25, 26].

Molluscs are sources of many important and different natural compounds such as amino acids, fatty acids, lipids, terpenes and steroids. Different types of fatty acids such as lipids, ∑SFA, ∑MUFA, and ∑PUFA, omega 6 to omega 3 and other compounds are produced by different classes of molluscs specially polyplacophora, gastropoda, cephalopoda and bivalves. Four classes are important, that they could produce about 600 natural compounds. The level of natural compounds between different species, organ and sexes are different, and many of biotic and abiotic factors can cause variations in those levels. Also, the process and metabolism are different for all compounds. Finally, amino acids, lipids and fatty acids, terpenes and steroids are important natural compounds that they could be produced [27–29].

5. The role and importance of fatty acids in molluscs

Lipids are major sources of metabolic energy and of essential materials for the formation of cell and tissue membranes, and they are important in the processes of egg productions.

They are very important in the physiology and reproductive processes of marine animals and reflect the special biochemical and ecological conditions of the marine environment. Lipids also provide energy for growth during conditions of limited food supply, when carbohydrate levels (the main energetic reserve in molluscs) are low.

The lipid composition can be affected by external (exogenous) factors, such as fluctuations in the environmental conditions and qualitative and/or quantitative changes in food availability, or by internal (endogenous) factors such as sexual maturation [28–30].

Accumulation and depletion of stored reserves in molluscs depends mainly on the stage of gonad development, environmental factors affecting metabolic activities and on the quantity and nutritional value of the food supply. Usually, the glycogen compound is the major energy source in species, while lipids are considered as the nutritive store source of the gonad organs. A high correlation between the gonad lipid content and the phase of the reproductive process cycle has been established in different species of bivalves and also prosobranch species.

Seasonal variations in lipid and fatty acid compositions have been reported for several marine molluscs and are generally related to the growth process and the maturation cycle: in the summer season and in the high temperature when the growth process takes place, receptacles of lipid compounds are build up and stored, and these are later consume for gametogenesis in the maturation cycle (often autumn or winter), normally are decreased during spawning process. However, the majority of these publications have focused on the class bivalve class, probably because of their major commercial importance and influence on the public health of people. Studies about biochemical compounds and their chemical structures, particularly fatty acid compounds in prosobranch gastropods, are strait [30].

Limpets are herbivore grazers which remove large quantities of unicellular microbes, algal germ lings and detritus, apparently unselectively, during feeding excursions around the home scar. As a consequence, there are considerable variations in their diets. There is a large amount of literature detailing about fatty acid compositions of a large number of species of marine algae. Availability and quality of algal lipids are very important in the nutrition, growth and development of aquatic animals such as marine fish larvae, shrimps and molluscs [28, 31, 32].

Molluscs phylum are of important aquatic invertebrates that the levels of the chemical compounds such as fatty acid components are higher in their tissues in comparison with other animals. They exhibit a range of lipid and fatty acid components in both freshwater and marine species and therefore fatty acid contents in mollusks are studied in many habitats, because of their importance in human's life. Among the marine invertebrates, the molluscs are the potential source of bioactive substances. The bioactive compounds isolated from the gastropods are considered to have a role in the chemical defense of the animals against their predators. Molluscs in the oceans are common sight and are virtually untapped resource for the discovery of novel compounds [27, 29].

Marine molluscs are excellent sources of nutritionally important compounds, such as fatty acids, amino acids and sterols. Fatty acids are essential for life, due to their key role as a good source of energy, membrane constituents, as well as metabolic and signaling mediators. In recent years, poly unsaturated fatty acids (PUFAs) have been recognized as a good remedy

for cardiovascular diseases. Marine organisms consume diets rich in n-3 PUFAs and the lipids of the animals can contain up to 50% unsaturated fatty acids, with five or six double bonds, including 22:6 n-3 and 20:5 n-3 [18, 19].

The term sterol refers to a compound with a fused cyclopentano phenanthrene ring with a 3-hydroxyl moiety. Early studies of gastropod sterols indicated cholesterol as the principal sterol of all species. Most species only one or two types of sterols present. Amino acids are the building blocks of protein molecules. They cause metabolites in the homeostasis of an organisms, due to their role as the regulation of several cellular processes and also as precursors of other molecules, such as hormones and nitrogenous bases. Lipid compositions and storage strategy in molluscs, particularly of bivalves and gastropods, have been studied since lipids constitute a major fraction of molluscan tissues. Almost all the data included in their lipid studies, concern the entire organism and only a few reports on the tissue distribution of fatty acids are available [24, 26, 28, 29].

The lipid in the gill tissue in the marine molluscs has important role for regulate of ions such as Na. In the marine animals, the primary site of Na uptake is gills. In addition to being the initially site of an ion transport, gills also captive food, have roles in gas exchanges and act as a brooding chamber for the larval glochidia in females species. Thus, gills activate in many different functions, regarding that their related importance may vary during the year. From the lipids, C20:4ω6 acid is an active substrate for prostaglandin productions involved in regulating Na uptake and it has relatively high contents in gill lipids. Therefore, high level of C20:4ω6 acid in the gill is probably related to prostaglandin synthesizing in the gills to regulate Na uptake. Finally the accumulation of C20:4ω6 acid in the gills was related to physiological activities in the organs [22, 30–33].

Fatty acids are organic compounds consist of hydrocarbon chains with terminal carboxyl groups. The fatty acid chains in sea foods differ from vegetables in length. In the presence of Omega-3 fatty acids, prostaglandins actions on epinephrine is diminished and thus constriction or narrowing of blood vessels is prevented. Therefore, marine Mollusca have been regarded as a good source of lipid compounds, and lipids are proper energy sources for animals and nutritive foodstuff for human diets [34].

6. Omega 6 to omega 3 ratio and its related effects

Normally, the omega 6 to omega 3 ratio has moderate amounts in natural food sources, especially in marine foods. In aquatic creatures, omega 6 to omega 3 ratio in the tissues of molluscs is significantly higher in comparison with others. Also, the omega 6 to omega 3 ratio vary between different organs and different species, as well as marine and freshwater species. There are significant differences in the omega 6 to omega 3 ratio in the gills, foot, mantle and whole body tissues of molluscs species, respectively [12, 14, 35].

Different species of the marine molluscs are generally rich in fatty acid compounds of ω3 (especially C18:3ω3, C20:5ω3 and C22:6ω3). The mussels species in freshwater, however, include a greater proportion of fatty acids compounds of ω6 (especially C18:2ω6 and C20:4ω6). The Σω6/Σω3 ratios is 2:4 in freshwater mussels, but the marine species have ratios of 0.1:1.0 [12].

Obesity disease which is a complex condition along with organs dysregulations and molecular pathways, such as adipose organ, liver, gastrointestinal tract, pancreas, central nervous system (CNS), and genetics. The role of the CNS in this disease needs more attention as obesity rates rise and relating treatments might fail. Since hypothalamus system has long been recognized in the regulation of appetite and food intake, the role of the CNS systems were examined as well as environmental impacts on energy balance. Furthermore, the omega-3 fatty acids have an important role in this disease and in the prevention and management of obesity [3, 4, 6].

The omega-6 and omega-3 polyunsaturated fatty acids (PUFAs) compounds are very important and essential fatty acids that must be derived from the diet compositions. Since omega-6 and omega-3 polyunsaturated fatty acids (PUFAs) compounds need endogenous enzymes for omega desaturation and there are no endogenous enzymes for omega desaturation in human and other mammals, these compounds cannot be made by man or other mammals and could be made particularly by Mollusca species. Modern agricultural western diets contain excessive concentrations of omega-6 PUFAs but very low concentrations of omega-3 PUFAs, leading to an unhealthy omega-6/omega-3 ratio of 20:1, instead of 1:1 proper for evolution process in the humans [9, 10].

Thus, an unbalanced omega-6/omega-3 ratio in favor of omega-6 PUFAs is highly prothrombotic and proinflammatory, which contributes to the prevalence of atherosclerosis, obesity, and diabetes. In fact, regular and balance of the omega-6/omega-3 ratio have positive effects for of these diseases and is the important factor for improve of these diseases (obesity, diabetes, atherosclerosis and cancer) [23, 24, 26, 30].

As mentioned earlier, omega-6 to omega-3 fatty acids compounds cannot be made and convert in humans and other mammalian cells, therefore, they cannot made enzyme for omega-3 desaturase and so they lack converting enzyme, omega-3 desaturase. Omega-6 and omega-3 fatty acids compounds are not interconvertible, and they are metabolically compounds and functionally distinct. Also they have important opposing physiological influences, therefore, omega-6 to omega-3 fatty acids balance in the diet is very important for better function and body protection [6, 7]. When fish consume by humans or predators, the EPA and DHA from the diet composition partially replace the omega-6 fatty acids, especially AA, in the skin and membranes of almost all body cells, but specifically in the membranes of platelets, erythrocytes, neutrophils, monocytes, and liver cells. The parent compounds for eicosanoid formation, are AA and EPA fatty acids. Because of high levels of omega-6 in the diet, the eicosanoid metabolic products from AA, especially prostaglandins, thromboxane, leukotriene, hydroxyl fatty acids, and lipoids, are formed in larger amounts than those derived from omega-3 fatty acids, especially EPA [32]. The eicosanoids from AA are biologically active in very small concentrations and, if they are formed in high levels, they contribute to the formation of thrombus and atheroma; allergic and inflammatory disorders, particularly in susceptible people; and proliferation of cells. Thus, a diet composition rich in omega-6 fatty acids shifts the physiological state to prothrombotic, proinflammatory, and proaggregatory effects with increases in blood viscosity, vasospasm, and vasoconstriction and cell proliferation. Omega-6 and omega-3 fatty acids balance is a physiological state that is less inflammatory in terms of prostaglandin, gene expression and leukotriene metabolism activity, and interleukin-1 (IL-1) production [28–31].

Novel agricultural technologies, by changing animal feeds for better and short term productions, have decreased the omega-3 fatty acid contents in many foods such as meats, eggs, and even fish. Foods from edible wild plants contain a good balance of omega-6 and omega-3 fatty acids. For instance, *Purslane*, a wild plant, in comparison to *Spinach*, red leaf lettuce, butter crunch lettuce and mustard greens, has eight times more ALA than the cultivated plants [30]. New aquaculture technologies produce fish with less omega-3 fatty acids than naturally grown fish in the ocean or freshwaters. The fatty acid composition in egg yolk from free-ranging chicken has an omega-6: omega-3 ratio of 1.3 whereas egg production supervising by the United States Department of Agriculture (USDA) conclude ratio of 19.9. By enriching the chicken feed with fishmeal or flaxseed, the ratio of omega-6: omega-3 decreased to 6.6 and 1.6 respectively [33].

High omega-6/omega-3 ratios cause some disorders such as increasing in the end cannabinoid signaling and related mediators, which could lead to change inflammatory state, energy homeostasis, and mood. In animal experiments a high omega-6 acid intake leads to decreased insulin sensitivity in muscle and promotes fat accumulation in adipose tissues. Nutritional approaches with dietary omega-3 fatty acids reverse the dysregulation of this system, improve insulin sensitivity and control body fat [5, 7].

End cannabinoids are lipids, derived from the omega-6 AA. Their concentrations are regulated by (1) dietary intake of omega-6 and omega-3 fatty acids; and (2) by the activity of biosynthetic and catabolic enzymes involved in the end cannabinoid pathway, which is an important parameter in regulation of appetite and metabolism. The end cannabinoid system is involved in preservation of energy balance and sustained hyperactivity of the end cannabinoid system which result obesity. Finally, omega 6 to omega 3 ratio is important factor in regulation metabolism and enzyme activities, and is important factor in control and improve of the nervous system diseases and genetics [9, 10, 13, 14].

7. Environmental parameters and nutrition effects on biocompounds variations

Environmental and biological parameters could change the amount and structure of natural compounds (fatty acids, amino acids and steroids). The environmental and biological factors could change in the different seasons, therefore, seasonal changes have the main role in the variations in the amount and structure of natural compounds. Studies of seasonal variations in biochemical contents of organisms explain how environment, biology, ecology and physiology can affect the compositions. As such, seasonal variations in the biochemistry of phylum Mollusca are known to be related to the complex interaction of both biological parameters (reproduction, growth, food type, food bioavailability, sex, tissue variance), and environmental parameters (temperature, salinity of water and pH) [21, 23, 24].

Observations the close correlations between temperature in the aquatic environment and different compounds in the tissues of Mollusca, could be explained by varying the level of metabolisms in different temperatures, which could change in the amount of

biocompounds in the tissues of animals. Also, salinity of water and pH has effect on the variations in the compounds such as fatty acids. The accumulation of fatty acids in the different tissues of organisms vary in different salinity and pH. Also, the accumulation of fatty acids in the different level of salinity and pH are not similar for different organs, and fatty acid profiles and their amounts in gill tissue for example, has more variations in the different salinities [4, 6].

Levels of proteins, lipids and carbohydrates (glycogen) have been shown to fluctuate with food availability. Food abundance allows for the accumulation of proteins and lipids in the tissues of the different species such as bivalves and gastropods. There are correlations between food type source and biocompounds structure, which increase in the food availability in the aquatic environment could result increasing the amount of the biocompounds in the tissues of the different species of Mollusca. When food availability levels are high in the environment the level of biocompounds are higher in comparison with other situations [17, 20]. The reproductive cycle and time spawning have the key role in the variation of chemistry compounds especially fatty acids, because of the high levels of energy needs for spawning processes and the high level of fatty acids consumed in this process [31, 32].

Lipids generally increase during the course of gametogenesis and decrease upon release of gametes. For proteins, diverging trends have been observed throughout gametogenesis and spawning. During gametogenesis, protein content was found to increase, decrease or even remain stable. During spawning, levels of protein were found to increase or decrease. Differences in food availability and water temperature conditions may partially explain the observed discrepancies since these factors are known to influence protein accumulation [1, 2].

Focusing on proteins and lipids, compounds involved in most biochemical and physiological processes of any organism is therefore useful for the recognition of ecological and physiological changes. Indeed, differences in seasonal trends have been observed among both AAs and FAs. More commonly reported, is the different behavior exhibited among free AAs in relation to salinity and that exhibited among FAs in relation to temperature. The biochemical composition of an organism is determined by endogenous (e.g., gametogenesis, maturation, spawning) and exogenous (e.g., food availability, salinity, temperature) processes. The temporal tests in the field of biochemical compounds permit intercrossing along with chronological and other variables allowing researchers to gain knowledge about ecology and physiology of an organism and also understanding how the surrounding ecosystem may affect [8, 9, 12].

There are significant differences between tissues and their activities for accumulation of amount and structure of natural compounds, and different tissues based on their activities can be accumulated fatty acids, amino acids and other compounds. Therefore, the level of compounds in the tissues are related to their activities. Some tissues such as gonad have highest level of biocompounds in comparison with the other tissues, due to this fact that gonad must have high level of energy for reproductive and spawning process. Since, gonad consume high amount of energy for this process, reproductive and spawning processes need high levels of energy. Also, gills need high energy levels for their metabolism, and so the high levels of fatty acids can be accumulate in this tissue [15, 16].

Sex types in the mollusca could affect variations in the concentrations and structures of natural compounds, because the biological factors are different between male and female animals and therefore changes in biological factors could cause variations in the compounds. One of the important factors in female animals is reproductive or spawning process, which could result variations during consuming of compounds. Since this process needs high energy, almost more energy levels are consumed in the reproductive cycles. Therefore, decreasing in energy levels of female species are observed. Also, other factors such as metabolism ratio vary between different sexes, therefore, level of compounds change between sex types [17].

Finally, according to many studies conclusions biotic and abiotic factors have effective results on variations of natural compounds. Throughout abiotic and environmental factors; temperature, salinity and pH, and in biotic factors; growth, reproduction cycle, food availability, sex type, tissue variances and functions, have the most important effects on the variations of natural compounds concentration and structure of lipids, fatty acids, amino acids and steroids.

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Section 2

Conservation and Sustainable Management

Chapter 12

Genetic Applications in the Conservation of Neotropical Freshwater Fish

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Additional information is available at the end of the chapter

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Abstract

Neotropical fish correspond to approximately 30% of all fish species worldwide. The diversity of fish species found in Neotropical basins reflects variations in life-history strategies and exhibition of particular morphological, physiological and ecological attributes. These attributes are mainly related to different forms of feeding, life maintenance and reproduction. Today, fish populations are being threatened by anthropogenic actions that are having a visible impact on the natural state of continental aquatic ecosystems. The main causes are overfishing, non-native species introduction, reservoir-dam systems, mining, pollution and deforestation. The biology and population dynamics of the species are still unclear due to lack of research. Genetic tools can be useful resources for the conservation of Neotropical fish species in several ways. Molecular genetic markers are considered powerful tools to identify cryptic and hybrid fish and also allow the evaluation of the genetic variability and structure of populations of Neotropical ichthyofauna. Several analyses of molecular markers have been performed on Neotropical fish, including allozyme analysis, restriction fragment length polymorphisms in regions of DNA (RFLP), randomly amplified polymorphic DNA (AFLP), randomly amplified polymorphic DNA (RAPD), microsatellites, single nucleotide polymorphisms (SNPs) and mitochondrial DNA (mtDNA) markers. In order to analyse a high number of markers, next generation sequencing has allowed researchers to generate a large amount of genomic information that can be applied to the conservation of Neotropical fish.

Keywords: molecular markers, genetic conservation, Neotropical ichthyofauna, overfishing, dam

1. Rivers of the Neotropical region

The distribution of freshwater fish around the world was mediated by historical climatic and geological events at different time points. Today, each global region has distinct patterns of distribution due to physical barriers obstructing species dispersion, representing different tolerances to environmental variables [1]. The tropics of the American continent are well known for their high biodiversity. This is due to habitat heterogeneity and a complex geological history. The Neotropical region is a biogeographic region that comprises Central America (including the southern part of Mexico and the peninsula of Baja California), the south of Florida, the Caribbean and South America. The origin and evolution of the Neotropical region arose through a process of synergism between its fauna that experienced local rainfall variations and gradual climate change resulting in a mosaic of habitats controlled by river migrations, sea-level fluctuations, local dryness and local uplifts [2, 3].

Regional geographical formations can affect the local hydrography and species distribution by forming distinct biogeographic barriers and allowing speciation of some isolated populations. Consequently, large basins separated by physical barriers with heterogeneous distribution across thousands of river systems, tend to have distinct species, with behaviours relating to environmental characteristics [4, 5]. The main hydrographic basins covering the Neotropical region are concentrated in South America, including the Amazon Basin, which covers the Colombian and Brazilian hydrographic regions, the Upper Paraná River Basin, the Paraguay-Paraná Basin, the São Francisco River Basin and the Uruguay River Basin [6].

The Amazon drainage basin covers 7.05 million km^2 occupying approximately 39% of the South American land mass. Around 72% of the basin is concentrated in Brazil, but it covers almost the whole continent from the Andes Mountains in the west to the Atlantic Ocean in the east. The mean water temperature in the basin is 27–29°C and reaches up to 34°C. Rainfall is the main source of water for the Amazon Basin, with about 50% of water originating from precipitation, being 6% of the basin area continuously flooded by large and medium rivers [7]. Several fish species of economical relevance in the Amazon River are migratory, such as catfishes of the Siluriformes order that can migrate for thousands of kilometres, and seed dispersers, such as *Brycon* spp., *Colossoma macropomum* and *Piaractus brachypomus* [8, 9]. The major habitats used by migratory fish are large channels and floodplains where they migrate and spawn in lotic areas [10].

The Upper Paraná-River Basin is formed by the junction of the Grande and Parnaiba rivers in the south-central region of Brazil. It is one of the longest rivers in the world at 4695 km with a 2.8×10^6 km^2 drainage area. It comprises 10.5% of the total area of Brazil and flows by the region that has the greatest population density of the country, subject to dam construction and agricultural, industrial and urban pollution. The Upper Paraná region has a tropical and subtropical climate with an average temperature of 22°C and 140 cm of precipitation per year [11]. There is a large floodplain located between the Porto Primavera and Itaipu dams, with a 230 km dam-free stretch, and is a region considered important for the conservation of local fish fauna. There are large migrators such as *Piaractus mesopotamicus* and *Pseudoplatystoma corruscans*, and a wide variety of short-distance migratory species and sedentary species, such as *Astyanax altiparanae* and *Serrasalmus* spp., present in the basin [12].

The Paraná-Paraguay Basin covers most of the southeastern region of Brazil and other countries such as Paraguay, eastern Bolivia and northern Argentina. Together with the Uruguay River, it covers most of central South America. The hydrographic basin covers 2.8 million km^2 and is considered the second biggest Brazilian basin. In contrast to the Amazon Basin, the climate in the Paraná-Paraguay Basin is drier, and the basin oscillates between harsh dryness and shallowness, with rains from October to March. The annual precipitation rate is 800–1200 mm leading to the formation of significant seasonal floodplains [13]. One of the biggest and most important wetlands of the world is the Pantanal located in the Upper Paraguay Basin. The complex hydrological cycle of the Pantanal wetland creates selective pressures on the adaptive and diversified traits of fish species. The Pantanal wetland consists of 5% of all existing Neotropical species [14, 15], but surprisingly few studies into diversity, structure and the population dynamics of fish populations have been carried out. Most of the fish in the Paraguay-Paraná River are economically important and are migratory, such as *Salminus brasiliensis*, *P. mesopotamicus*, *Brycon orbignyanus* and large catfish such as *P. corruscans*, *Pseudoplatystoma fasciatum* and *Paulicea luetkeni* [16].

The Uruguay River Basin system is located in the temperate latitudes near the southern coast of Brazil with altitudes reaching 1800 m. It runs along the border between the Santa Catarina and Rio Grande do Sul states of Brazil until the Paraná River where it forms the estuary of the Plata River in Argentina. As a result of its sloping profile and abundance of rapids, the Uruguay River is hard to navigate compared to other rivers. With faster water, there are a considerable number of hydropowered dams in the basin that can affect the reproduction of migratory species and their eggs and larvae drift [17].

The São Francisco River covers 7.4% of Brazil and represents a large number of reservoirs, representing the second highest source of hydropower in the country [18]. The headwaters rise in the southern region of Minas Gerais state, and run through Bahia, Pernambuco, Sergipe and Alagoas states to then empty into the Atlantic Ocean. The altitude reaches 1600 m above sea level, and there are diverse climate conditions raging from humid tropical to semi-arid, with temperatures from 18 to 27°C and high evaporation rates (2300–3000 mm/year). The São Francisco River is rich in floodplains and marginal lagoons that are used by fish species as a habitat for feeding, reproduction and refuge. Around 8% of the species migrate to reproduce and are considered important commercial fish. These include some Characiformes (*Brycon lundii, S. brasiliensis*, *Leporinus elongatus, Prochilodus affinis* and *Prochilodus marggravii*) and Siluriformes (*Conorhynchos conirostris* and *P. corruscans*) [19].

2. Diversity and biology of Neotropical fish

Neotropical fish comprise approximately 30% of all fish species in the world (5160 species) and are found in only 0.003% of all the freshwater on the planet [20]. The Neotropical region has some of the highest numbers of fish families, and unlike other zoogeographic zones where Cypriniformes predominate, there is a high proportion of endemic families belonging to the Characiformes (~1200 species) and Siluriformes (~1300 species) orders. Despite the predominance of Characiformes and Siluriformes species in the Neotropical basins, the heterogeneity of

species between basins and their unequal distribution are considerable. This is largely due to the formation of lakes, puddles, streams, rapids, rivers and floodplains that have become determining factors for the high diversity of fish species that exist today in the Neotropical region [1, 21].

There are estimates that the number of freshwater fish species in the Neotropical region exceeds 8000 [20]; however, the total number remains unknown. There are many factors that make it difficult to study biodiversity in this region, in addition to problematic taxonomic issues [1] like cryptic species [22]. Furthermore, few institutions [United Nations Food and Agriculture Organisation (FAO) and the Brazilian Institute of Geography and Statistics (IBGE)] provide fishing data statistics that ultimately affects the management of these species.

Despite the low number of fish species described, there was an increase following the advent of molecular biology and cytogenetic techniques. Many single species were re-described as a complex of cryptic species after genetic analyses [23].

Efforts to describe new species of Neotropical fish have focused mainly on the Amazon Basin, with more than half of the fish species described found in this region [5]. Of these, there are around 200 poorly known species that are exploited by commercial and subsistence fishing, and this number may be even greater due to misidentification errors. This makes it difficult to implement fishery management and conservation policies [24, 25].

The diversity of fish species found in the Neotropical basins reflects variations in life-history strategies and exhibition of particular morphological, physiological and ecological attributes. Such attributes are related to the different forms of feeding, life maintenance and reproduction [26–28]. Research into the pattern of life strategies may have practical applications in conservation and is fundamental to fishery management in identifying appropriate measures to reduce the impact from reservoirs and other anthropogenic activities [29].

Neotropical fish species range from colonising and opportunist species to periodic and equilibrium species. Colonising populations, such as those belonging to the genera *Astyanax* and *Phalloptychus*, mostly require energy for reproduction and are generally represented by small, rapidly maturing and short-lived species. Therefore, their numbers are more likely to recover on anthropic impacts. Periodic and equilibrium species are larger, longer-living individuals with few offspring requiring greater parental investment [30]. They use a large part of their energy for somatic growth to ensure their survival to age-at-maturity [31, 32] and generally show late maturation and slow growth that would take longer to recover from high levels of overfishing. The main examples in this group are large, freshwater catfish, such as "piraíba," that belong to the *Brachyplatystoma* genus, followed by other Siluriformes [33]. Neotropical fish have a wide diversity of life histories, with respect to reproduction, migratory patterns and spatial behaviours that can make their classification very difficult [34].

The high degree of variability in reproductive strategies is closely related to their environment and the selective forces present over the life history of the species. As a result, reproductive strategies can be expressed in different ways, including the type of egg fertilisation, differences in age of maturation, parental care, spawning and migratory patterns [35]. Seasonal periods of flooding and dryness influence reproductive strategies, especially in migratory species. However, the connection between reproductive events and fluctuations in hydrometric

level is not entirely clear. The increase in water level combined with changes in temperature, photoperiod and water conductivity may lead to physiological changes in the individual that stimulate gonad maturation, migratory movements, spawning, egg fertilisation and offspring development [36]. Although migratory movements are due to reproductive needs, they have seasonal, trophic and ontogenetic characteristics that are all associated with the hydrological regime of the river [21, 37, 38].

S. brasiliensis, commonly known as "dourado," is a large and carnivorous species found mainly in the Prata Basin. They are vulnerable to indiscriminate fishing and have high commercial value. Their reproductive migration for maintenance of their population stretches over 400 km [39] and can reach 1000 km [40]. In addition, large catfish are known for their remarkably long-distance migrations in rivers such as the Amazon, Paraguay, Paraná and Orinoco [6]. Most of these species have high commercial value due to the taste of their meat and large size. However, stocks of catfish have declined due mainly to overfishing and habitat fragmentation. In the Amazon, goliath catfish such as *Brachyplatystoma rousseauxii*, known as "dourada," cross the entire South American continent from east to west to lay eggs at the headwaters of the rivers near the Andes mountain range, and their migration is considered the longest of any freshwater fish. The larvae, which are less than 6 mm in length, develop along the route that can reach 11,600 km. Their destination is the rich ecosystem of the Amazon River estuary [41]. Neotropical fish may also be responsible for the proliferation of plant species due to their migratory behaviour. The pacu (*P. mesopotamicus*) is a migratory and omnivorous species that travels up to 300 km [42] and plays an important role as a seed disperser for many fleshy-fruited plants of the Pantanal, contributing to seed dispersion for 27% of the tree community existing in the gallery forests [43].

In addition to large migratory species that require long stretches of river and seasonal stimuli to exercise their life strategies, there are also sedentary species that carry out their vital activities in a restricted area and are more influenced by local environmental variations. Due to their small size, they spend their lives associated to a substrate, such as trunks, rocks and aquatic plants where they find protection, food and a suitable surface for egg deposition. The displacement of these species is generally short and occurs in more lentic environments, where there may be occasional variations. The reproductive period of some species occurs during lower levels of precipitation so that eggs and larvae are not dragged to stretches of the river where they will be unable to find suitable conditions for development. On the other hand, some species of sedentary fish such as the lambari (*Astyanax scabripinnis paranae*) are able to prolong their reproductive period throughout the year and overcome the unpredictability of floods in short streams [21].

Some sedentary species also show seasonality in reproduction. *Oligosarcus jenynsii*, popularly known as the "dog fish," is one of the most abundant species in the coastal lagoons of southern Brazil. Spawning starts in the winter months where the water temperature is very low. This strategy can lead to high mortality in the larvae but, on the other hand, it can guarantee food for juvenile individuals since in the months following winter they would be more developed compared with other species that reproduce in spring [44]. Variations in reproductive strategy exist in the same population and between different populations of the same species

due to environmental conditions. For example, the "traíra" (*Hoplias malabaricus*), which has the ability to reproduce throughout the year or to follow a defined seasonal cycle depending on their environment [21].

3. Environmental impacts and risks for Neotropical fish

Neotropical fish are currently being threatened by anthropogenic activities that are showing visible effects on freshwater ecosystems. These effects are related to overfishing; non-native species introduction; dam construction for hydropower; river contamination from mining activities; and industrial and agricultural pollution and deforestation [45].

In Neotropical areas, the limits of exploitation of the majority of commercially valued fish stocks are close to the maximum sustainable yield [46]. Aquaculture would indirectly alleviate pressures on threatened wild stocks and, therefore, needs to be carried out in a sustainable way with the least possible impact on natural populations [47]. In 2015, of the 25 countries with the highest production from aquaculture (97.1% of total production), three Neotropical countries were included (Chile, Brazil and Ecuador). Moreover, with regard to freshwater aquaculture, Brazil leads this ranking, producing 474,300 tons, followed by Chile producing around 68.7 tons and Ecuador 28.2 tons [48].

In 2016, according to IBGE (Brazilian Institute of Geography and Statistics) data, Brazilian aquaculture had continued to grow and reached a total of 580,000 tons, with a production value of R$ 4.2 billion. A total of 77.32% of this production originated from fish farms followed by shrimp farms (21.5%). The development of farming technologies directed at the native species has helped to accelerate fish production, and relieve the pressure exerted by extractive fisheries.

Despite the advantages of aquaculture, uncontrolled fish production and lack of proper inspection by government agencies can be problematic for the Neotropical ichthyofauna. Uncontrolled hybridisation of fish, introduction of non-native species and loading of excess nutrients originating from effluents from aquaculture production can become a serious threat to wild fish populations.

Currently, the production of fish hybrids involves many Neotropical species resulting in viable products of high interest for farmers [49]. Nevertheless, the main threat caused by hybridisation is the genetic introgression on wild populations [50, 51]. If fertile, hybrids can genetically contaminate natural and farmed stocks by genetic homogeneisation and compete with the native parental lineages (in sexual behaviour, territory, food, etc.) [52].

Brazil plays an important role in the conservation of its rich diversity of Neotropical fish. However, policy initiatives have threatened the biodiversity of these species and the functioning of their ecosystems. In some countries, there is specific legislation for hybridisation (in contrast to the Brazilian legislation, which does not require a licence for hybrid production); for example, in the state of California, there are laws prohibiting unlicensed fish hybridisation [53]. In Brazil, most commercial establishments are unlicensed, and there are few legislative proposals to regulate the activity [54]. According to Hashimoto et al., legislation is necessary to guarantee the safety of hybridisation techniques used in Brazil [55].

There is a particularly high concentration of hydroelectric dams in the Upper Paraná and São Francisco rivers (many of the rivers in South America are so heavily dammed they become a chain of reservoirs) [6]. The largest dams in Latin America are Itaipu (Paraná River, Paraguay-Brazil), Guri (Rio Caroni, Venezuela), Tucuruí (Rio Tocantins, Brazil) and Yacyretá (Paraná River, Argentina-Brazil). Currently, 90% of the energy consumed in Brazil originates from hydroelectric plants, with an annual output of 78,000 MW [6]. Dams have been built in almost all hydrographic basins with consequent formation of reservoirs. These reservoirs alter the natural distribution of seasonal flows and nutrients, leading to the formation of new ecosystems with specific structures and functioning [56]. These new ecosystems have several factors that affect the local ichthyofauna have a serious impact on the life cycle of the fish [57]. Dams act as barriers to the natural flow of rivers. They are built mainly to produce electricity, but also to supply water to residential, agricultural and industrial areas. The change and/or loss in water flow impacts the distribution of aquatic life biodiversity [58]. Dams also affect the watershed and lower the water quality, impacting not only the river itself but also its tributaries. This may also be harmful to native species by destabilising the ecosystem and the living communities [57]. For example, migratory fish suffer due to the interruption to their migratory routes and require a different habitat to complete their life cycle. These species generally migrate upstream to spawn during the wet season producing numerous small eggs. These eggs and larvae are transported with the current to nurseries downstream without any parental care, where they find ideal conditions for initial development and protection from predators [59, 60]. The consequences of blocking migratory fish routes is observed in their reproductive cycle for years, leading to the depletion of natural stocks and extinction of the species [61].

The new ecosystem formed modifies the structure of fish communities that inhabit the river, and the establishment of new communities depends on the physical, chemical, hydrological and geomorphological changes as a result of the spatial and temporal redistribution of the river flow [62–65]. Changes in species composition and abundance can increase the numbers of some species and eliminate others, causing collapse of the ecosystem [66].

In order to mitigate such effects, management measures have been put in place to preserve the Neotropical ichthyofauna [67]. Until the 1950s, the main objective of management programmes was to ensure that species could migrate through the reservoirs to complete their life cycle. Transposition mechanisms (fish ladders) were created in the main Brazilian dams. In the 1990s, dozens of fish transposition systems were constructed, even with few studies into the efficacy of the method and despite the costs and effort required [68]. Most of these mechanisms are based on ladders, structures that reduce the velocity and gradient of the water so that fish can climb and pass through the dam [21].

However, these mechanisms have species selectivity and allow the movement of only some species of fish. This divergence between species can cause dramatic imbalances in the population and the Neotropical ecosystem [59]. The main process of passage is recognition of the entrance [69]. If the fish cannot recognise the entrance to the passage, they remain where they are, which delays migration and spawning and interferes significantly with their reproductive process [70].

Storage and repopulation of fish are alternative methods to mitigate the impacts of hydroelectric dams [57]. Several breeding programmes were implemented aimed at the production of fish to restock the reservoirs, mainly to improve fishing activities. Some non-native species were

introduced to southern and southeastern regions of Brazil over 20 years (1970–1990) due to the difficulty of producing native species [71], a trend that has declined in recent years, though it still exists [21]. Hydroelectric companies have begun to produce native species for restocking (repopulation), but for this to be successful, evaluation of the efficiency and genetic quality of the parents is essential [21, 72]. In repopulation programmes, genetic monitoring is a fundamental step, since a reduction in genetic variability reduces the adaptability of the species to different environmental conditions and interferes significantly with the survival of young fish [66, 73]. The use of molecular markers has been shown to be effective for genetic management in order to maximise diversity and reduce inbreeding in the repopulation centres [67, 68, 74].

Aquatic organisms are fragile and sensitive to a wide range of stressors. Reproduction, growth and population survival are highly dependent on water quality. Environmental pollutants such as metals and pesticides present a serious risk to local ichthyofauna. The physiological effects of toxicants include disruption of hormonal, neurological and metabolic systems and elimination of behaviours that are essential to fitness and survival in natural ecosystems [75]. Studies into many Neotropical fish have corroborated this. *Prochilodus lineatus* subjected to 7 days of *in-situ* tests in a contaminated urban stream suffered a series of epithelial lesions, lamellar fusion and aneurysms in the gills [76]. Another study carried out with *Rhamdia quelen* exposed to herbicides (Roundup® Original, Primoleo® and Facet®) showed harmful effects in the gills, liver, kidneys and muscle [77]. *A. altiparanae* and *P. lineatus* exposed to biodegradable detergents in an urban lake showed various changes in gill function such as lamellar fusions, aneurysms, mucous and chlorine cell proliferation [78]. A study involving *A. altiparanae* showed that the level if DNA breaks was most sensitive biomarker to contamination with pesticides [79]. *P. lineatus* showed nickel accumulation in different tissues (kidneys, liver, gills and muscle) with DNA damage [80].

Mining activities impact the aquatic ecosystem in the basins of Upper Paraguay and in the Colombian, Brazilian and Peruvian Amazon [6]. Mercury, the main compound released during gold mining, accumulates in the sediment and in the muscle and tissues of fish (bioaccumulation). This means that through the trophic chain, the predators that are high in the food chain tend to accumulate more metals (biomagnification). Fish in the rivers of Madre de Dios city (Perú), affected by illegal mining, revealed that the species *Calophysus macropterus*, *Pseudoplatystoma punctifer*, *P. fasciatum*, *H. malabaricus*, *Prochilodus nigricans*, *Hydrolycus pectoralis*, *Plagioscion squamosissimus* and *Zungaro zungaro* showed high levels of mercury accumulation in tissues that exceeded the maximum limit permitted by the World Health Organisation (500 μgHg/kg) [81]. The same was found in the Upper Pantanal (Brazil), in the Poconé and Nossa Senhora do Livramento regions (Upper Paraguay River), where piscivorous or detritivorous species such as *Pimelodus maculatus*, *P. fasciatum*, *P. lineatus*, *Salminus maxillosus*, *P. corruscans*, *Acestrorhynchus altus*, *Serrasalmus nattereri* and *H. malabaricus* also had high levels of mercury [82, 83]. Other metals that accumulate in fish organs are nickel and copper [80, 84], causing significant damage to the fish species and its consumers.

Mining can also lead to the collapse of dams, as occurred in Mariana city (Minas Gerais state, Brazil) in 2015 that was considered the biggest environmental disaster in Brazil that released approximately 55–62 million m^3 of mining waste directly into the watershed of the River Doce,

spreading across the Atlantic coast [85, 86]. This affected the ichthyofauna by fragmentation and destruction of habitats, water contamination, change in water flow, impact on estuaries and mangroves at the mouth of the River Doce [87], destruction of fish breeding areas, destruction of the nurseries of the ichthyofauna (feeding areas for larvae and juveniles), disruption of the gene flow between different areas, loss of species with habitat specificity and collapse of fish stocks [85].

4. Genetic applications

Genetic tools are important resources for the conservation of Neotropical fish species. The biology and population dynamics of the species are still unknown due to insufficient research. In spite of the high diversity that characterises Neotropical fish, there are many species with a large geographical distribution and differing population structure. Along a hydrographic basin, one can find many populations, from panmictic populations of long-distance migratory species, characterised by large gene flow, to restricted populations of local organisms with well-defined population structures [88]. Research into the verification of variability and genetic structure of populations belonging to different river basins will aid the construction of policies and management measures for the maintenance of natural populations. In addition, genetic tools are increasingly being used to molecularly identify new species that was previously impossible due to morphological similarities. Furthermore, various anthropogenic activities in aquaculture and pollution have been increasingly studied at the molecular level, particularly with respect to research into hybrid fish and the effects of contaminants.

Biodiversity is conceptualised into distinct biological levels (genetic, species, community and ecosystem) that have each been impacted by human activities. The impact on genetic diversity is one of the biggest concerns, affecting species adaptation and taxa speciation [89, 90]. Knowledge of how the genetic diversity of Neotropical fish is maintained and how the populations are structured is important to determine how these species can be conserved. Many species of freshwater fish display genetic variation with adaptive traits that enhance survival and reproduction in particular environments and increase the capability of the organisms to adapt to environmental changes and anthropogenic activities [90].

Genetic variability in populations can be measured by the allele number and heterozygosity [91]. Intrapopulation variability is influenced by factors such as mutation, genetic drift and natural selection. Genetic variation originates from mutations and decreases in genetic drift that increases the interpopulation differentiation due to a finite population size, with gene flow occurring between populations [92]. Conversely, natural selection can reduce genetic variation by allele fixation [93]. Anthropogenic activities, such as habitat fragmentation, increase the risks of genetic drift and gene flow reduction, diminishing the genetic variability of populations and interrupting flow of the adaptive genes leading to extinction of some species [90]. Molecular genetic markers have emerged as a powerful tool to identify genetic variability in populations [94] and have had a substantial impact on the fields of ecology, evolution and conservation [95].

The identification of cryptic species is an important genetic application for the ecology and conservation of Neotropical freshwater fish. This taxonomic challenge has been overcome due to the advent and availability of rapid DNA sequencing for detecting and differentiating morphologically similar species [22]. The destruction and disturbance of river basins, especially those caused by human interference, have led to the threat of complete extinction of several fish species [96]. However, many species exposed to these threats are still undescribed, and efforts to catalogue and identify these fish are increasingly important. Most species have been described by morphological and typological characteristics [97]. However, speciation is not always accompanied by differences in morphology, and due to the difficulty of identification, the actual number of existing fish species is greater than previously described [22].

DNA sequencing has introduced a new method of species discovery known as DNA barcodes [98]. DNA barcodes are short and standardised sequences from a part of the mitochondrial genome that can be used to distinguish different species. This differentiation can easily be determined when genetic variation between species exceeds that within species [99]. The barcode sequence from each unknown specimen is then compared with a library of reference barcode sequences derived from individuals of known identity. Research has been carried out to evaluate the effectiveness of this technique in identifying cryptic species in insects [100], birds [101] and plants [102]. The diversity Neotropical freshwater ichthyofauna is the richest in the world and make up around 25% of the total freshwater fish fauna on Earth [5]. However, the lack of knowledge of their diversity makes taxonomic identification a great challenge.

Genetic methods facilitate the identification of cryptic species and species with few identifiable phenotypic characteristics. The presumed neutrality of some molecular markers, in conjunction with phylogenetic methods, provides a new perspective on species identification, especially in hierarchical relatedness and relative rates of evolution. The increased frequency with which cryptic species can be discovered with DNA sequence data, and often subsequently confirmed with morphological and/or ecological data, suggests that molecular data should be routinely incorporated into taxonomic research.

Another major problem for the natural populations of Neotropical fish (that can be reduced or controlled using genetic resources) is accidental or deliberate release of non-native fish species [103]. Hybridisation is the mating of genetically differentiated individuals and may involve individuals within a species or between species [104]. Conventional approaches to detect interspecific hybridisation include morphometric and molecular analyses. In recent years, DNA polymorphisms have been used for investigating fish hybridisation [105]. Nuclear genetic markers, in particular, allow hybrid species identification because contributions to the hybrid genome of both the father and mother can be identified [106].

5. Molecular markers

There are a number of molecular markers in Neotropical fish, such as allozyme markers, restriction fragment length polymorphisms in regions of DNA (RFLP), randomly amplified polymorphic DNA (RAPD), randomly amplified polymorphic DNA (AFLP), microsatellites

markers, high genome coverage markers [single nucleotide polymorphisms (SNPs)] and maternal inheritance markers (mtDNA).

5.1. Allozymes

Allozymes were considered the first molecular marker, discovered in the 1960s in enzymes. When DNA sequences of two or more alleles in the same locus are divergent, and the corresponding RNA encodes different amino acids, multiple variants of the same protein are created. However, not every mutation in a DNA sequence results in changes to the amino acid sequences, and this is one of the disadvantages of using an allozyme as a molecular marker [107]. Other disadvantages include heterozygote deficiencies due to null alleles and the amount and quality of tissue samples required [108]. The limitations and disadvantages of these markers led to the development of DNA-based genetic markers.

In the 1980s, the first DNA-based molecular markers were developed. They can be classified into dominant and codominant markers. It is not possible to identify heterozygotes in dominant markers, whereas in codominant markers, this differentiation can be determined, and it is possible to estimate allele frequencies. Molecular markers can also be classified into those with known function (type I markers) or with anonymous regions (type II markers) [108].

5.2. Restriction fragment length polymorphisms (RFLP)

RFLP markers were the first markers discovered that were based on DNA sequences [109]. They are considered codominant markers and are type I or type II. They are based on bacterial enzymes that recognise specific DNA sequences. The DNA is then cut into fragments where these sequences are found. The digestion of DNA by restriction enzymes results in fragments that vary between individuals, populations and species. The fragments can be analysed using the polymerase chain reaction (PCR), and the PCR products are digested by restriction enzymes. RFLP markers have low potential in determining genetic variation when compared to new, recently discovered molecular markers, mainly due to the low level of polymorphism. In addition, sequence information of the specimen is required, making it difficult to determine markers in species without molecular information. However, one advantage of these markers is that they are codominant [108].

5.3. Randomly amplified polymorphic DNA (RAPD)

RAPD techniques use PCR amplification of random anonymous segments of genomic DNA with identical pairs of primers at 8–10 bp in length. Unlike RFLP markers, RAPD does not require any knowledge of DNA sequences of the organism. Therefore, nearly all RAPD markers are dominant, and it is not possible to distinguish whether a DNA segment is amplified from a heterozygous or homozygous locus [110]. The primers used are short and anneal at low temperatures, amplifying multiple products from different loci. Due to the fact that most of the nuclear genome is non-coding, most amplified loci are neutral. Genetic variation is assessed by considering each band as a bi-allelic locus, with the presence or absence of the amplified product generated by PCR. One disadvantage of this technique is the intensity

variation that can occur between bands. They can make it difficult to determine whether bands represent different loci or alternative alleles of a locus. The markers also have a low reproducibility due to low annealing temperature in PCR amplification, and thus have limited application in fisheries science. Despite the disadvantages, the detection of polymorphisms is considered high [108, 111].

5.4. Randomly amplified polymorphic DNA (AFLP)

AFLP is a combination of the RFLP and RAPD techniques, using PCR to randomly amplify anonymous fragments of nuclear DNA (type II marker). The technique involves digestion of DNA using a restriction enzyme, as in RFLP analysis, producing a high number of dominant fragments that, depending on their concentration, are not detected by electrophoresis. The DNA is digested with different types of endonucleases, generating fragments of different sizes. The following steps are similar to the principles of RAPD, where small, known DNA sequences (adapters) are coupled to the ends of the fragments and are annealed with specific primers during PCR [112]. A unique feature of this technique is the addition of known sequence adapters to DNA fragments generated by complete genomic DNA digestion. This allows subsequent PCR amplification of the many fragments generated that are then separated by denaturing polyacrylamide gel electrophoresis [108]. The AFLP technique has some advantages, such as detection of greater numbers of loci generating a higher number of polymorphisms, broad coverage of the genome with high reproducibility (due to high PCR annealing temperatures) and low cost [113]. Like RAPDs, they are considered dominant markers and although there are packages for codominant scoring of AFLP bands, their applicability in population studies is difficult. The major disadvantage of the technique is the need for automated gene sequencers for electrophoretic analysis of fluorescent labels, although traditional electrophoretic methods can also be employed using radioactive labels or silver staining techniques [108].

5.5. mtDNA markers

Mitochondrial DNA (mtDNA) markers were the first widely used DNA markers and are one of the most popular markers for molecular diversity studies in fish [114]. This part of the genome consists of a small, circular, abundant and easy to amplify DNA molecule as there are multiple copies in the cell. Moreover, the mitochondrial gene content is strongly conserved across species, with little duplication, no intronic regions and very short intergenic regions [115]. Studies of vertebrate species have shown a mutation rate that exceeds, by multiple times, nuclear DNA mutation rates that may be due to a lack of repair mechanisms during replication [116]. The complete mtDNA sequences have been sequenced to facilitate analyses of molecular markers in many economically important Neotropical fish species, such as *S. brasiliensis* [117], *P. mesopotamicus* [118] and *L. elongatus* [119].

The DNA of cytoplasmic organelles has a non-Mendelian inheritance, and the mtDNA must be considered a single locus in genetic investigations [94]. Inheritance occurs via the mitochondria of the oocyte from which an animal develops [120]. This maternal transmission gives information on maternal lineages of fish stocks and provides a more sensitive tool for detecting population subdivision, making it an efficient marker when compared to typical nuclear markers such as microsatellites and SNPs [121].

Many studies of mtDNA have focused on the major non-coding region, often called the control region, because of its rapid rate of evolution. The control region includes transcriptional promoters in both strands and the D-loop region. In these non-coding D-loop regions, the evolution rates are higher than the rest of the molecule. These changes lead to the formation of multiple alleles, called haplotypes that can be phylogenetically ordered within the same population and confirm intrapopulation phylogenetic relationships in population studies [94].

5.6. Microsatellites

Microsatellites or simple sequence repeats (SSRs) have been a popular marker in genetic fish research due to their abundance in the genome in all regions of the chromosome. There can be a small number to a few hundred copies of tandem repeat sequences of mono-, di-, tri- and tetranucleotide motifs. They are codominant and mostly type II markers, with abundancy in all species of fish with an estimated occurrence of one in every 10 kb in coding genes, intronic regions and regulatory sequences [122, 123].

These markers are useful in evaluating structure and genetic diversity between different populations due to high polymorphisms that give a high power in analyses of population genetics [114]. The polymorphisms are identified by size differences, resulting in varying numbers of repeat units in alleles of a single locus [108]. Mutation rates have been detected as high as 10^{-2} per generation [124].

There have been many studies of wild fish stocks using microsatellites that allowed the analysis of historical population structures, colonisation histories and connectivity between populations [125]. These population characteristics are generally controlled by environmental effects [126–128] or by anthropogenic intervention [129–131] that can induce the structuring of fish populations with a reduction in gene flow exchange and genetic variability.

However, the use of microsatellite markers has some drawbacks. They require a large investment of time and laboratorial effort due to the genotyping step [108]. Moreover, they require a species-specific marker, where there is a high potential for null alleles and imperfect repeats due to polymerase slippage during replication, and genotyping errors that impact population studies by providing unreliable genetic information for conservation biology, molecular ecology and population genetic research [132].

5.7. Single nucleotide polymorphisms (SNPs)

SNPs are type I or type II polymorphisms caused by point mutations that generate different alleles for a given nucleotide belonging to a specific locus. These molecular markers are unique nucleotide substitutions of a sequence at a single site and have been well characterised since the beginning of DNA sequencing [108]. SNPs are the main focus in molecular marker development as they constitute the most abundant polymorphism in any organism's genome, with a frequency estimated at approximately 1 SNP per 200–500 bp [133]. This marker is adaptable to the automation of genotyping and reveals hidden polymorphisms that are not detected by other markers and methods [108]. Moreover, they can be efficiently identified in any organism without the need for genomic information.

Theoretically, the SNP of a particular locus can contain up to four alleles (A, T, C and G). In practice, however, most SNPs are usually limited to two alleles (often two C/T pyrimidines or two A/G purines) with codominant inheritance [108]. The level of polymorphism is not as high as in microsatellite markers (multi-alleles), but this disadvantage is counterbalanced by its abundance in the genome [133]. Therefore, to be considered an SNP, it is necessary for the least frequent allele to have a frequency of 1% or higher [134].

These characteristics demonstrate that this marker is ideal for several biological studies because they allow complex genomic analyses with high yield and coverage. This marker has been revolutionary in fish population research. The SNP markers have already been used in comparative studies of evolutionary genomics, population genomics, identification of interspecific hybrids, identification of sex-related sequences, genomic selection, mapping of genes by linkage maps and detection of alleles associated with economically important characteristics in aquaculture [135–139].

For the routine use of SNPs, genotyping platforms for analysing a large number of markers and samples, in a fast and economical manner, are fundamental. For low-throughput SNP genotyping, candidate loci can be tested using different methodologies. In summary, each platform uses a specific detection chemistry, which generates differences in the cost of genotyping, price of equipment, number of markers, expertise for use, sample volume analysis and automation [140].

One of the greatest barriers to the routine use of SNPs is the characterisation and discovery of these markers. Historically, numerous approaches to SNP discovery have been described, primarily from the comparison of specific locus sequences. Direct sequencing (Sanger) of candidate genes was considered the simplest, though expensive, strategy for SNP discovery. On a larger scale, the comparison of sequences of cloned fragments, particularly expressed sequence tag (EST) designs using different types of tissues, is the best alternative [134]. However, in addition to the high costs, a considerable amount of laboratory work, time and expertise is required for this type of analysis.

5.8. Next generation sequencing (NGS) in molecular marker discovery

Next generation sequencing (NGS) allowed researchers to generate a large amount of sequencing data at relatively low cost as compared with other methods such as Sanger sequencing. To identify a greater number of gene-associated markers, a greater yield of sequence readings is required. Next generation sequencers are particularly adapted to produce high precision sequence coverage [141, 142]. Furthermore, NGS provides an enormous number of reads, which allows entire genomes to be sequenced at a fraction of the cost for Sanger sequencing [143] and is inclusive of non-model organisms [144]. Therefore, NGS technologies have become useful for *de novo* sequencing (sequencing without a reference genome) of eukaryotic genomes [145]. When using NGS technologies, the absence of a reference genome is one of the greatest barriers to discovery of molecular markers in non-model organisms. In these cases, from a sequencing project, individual reads can be assembled into consensus sequences called *contigs* that may serve as a pseudo-reference genome [146]. There are two

alternative techniques for genome reduction to acquire sets of redundant *contigs*: transcriptome sequencing (RNA-seq) and restriction site-associated DNA (RAD-seq) [144].

Transcriptome sequencing of genomes is one of the most common analytical approaches. Complementary DNA (cDNA) is produced from the mRNA of a specific tissue or life stage. Thus, whole mRNA sequences (cDNA library) from a specific tissue or set of tissues can be aligned to a reference genome (or reference transcripts) or assembled *de novo* [147]. This approach allows data to be obtained for a single nucleotide variation profile, as well as transcriptome characteristics and gene expression levels, in a cost-effective way [148]. Additionally, transcriptome sequencing allows gene-associated SNP studies, depending on the exact genomic location and functional role that are inserted [149].

RAD-seq is an important method of genome reduction in non-model fish for identifying and genotyping SNPs, and unlike RNA-seq, uses genomic DNA as a template. The technique uses the principles of RFLP by reducing the complexity of the genome by subsampling at sites defined by restriction enzymes [150]. This technique consists of digesting the genomic DNA with restriction enzymes, followed by mechanical fragmentation to reduce the size of the fragments making them suitable for sequencing. The digested fragments are then attached to adapters with single barcodes for each individual so they can be multiplexed in a pool of samples. Thus, the regions adjacent to the restriction sites of multiple individuals are sequenced simultaneously in a single run [151]. There are numerous variations of the RAD-seq technique with single restriction enzyme cut sites (original RAD, 2bRAD) or with two restriction enzyme cut sites (GBS, CRoPS, RRL, ddRAD) that promise to increase the number of loci assayed at low cost and effort in ecological and evolutionary studies [152].

The identification of SNPs using the RAD-seq method has the advantage of avoiding unequal gene expression problems that may impair the discovery of SNPs using transcriptome sequencing [144]. Another advantage of the RAD-seq technique is the possibility of identifying DNA barcodes for individual samples or pools of samples during the preparation of DNA libraries, thus reducing costs [153]. However, alongside transcriptome analysis, the ability to identify true SNPs is hampered by the occurrence of errors caused by high-throughput sequencing. To mitigate this problem, a sufficient sequence read depth is necessary for both techniques [154].

RNA-seq and RAD-seq techniques have allowed the detection of many microsatellite markers [155, 156] and SNP markers [154, 157, 158] in model and non-model fish species around the world. Although they have been increasingly used in the aquaculture industry for Neotropical fish, microsatellites have been identified and characterised for research in the field of biology and conservation [159–162]. In previous studies, microsatellites loci in closely related species have been identified. These include species belonging to the Anostomidae [163], Characidae [164, 165], Cichlidae [166], Pimelodidae [167], Prochilodontidae [168–170] and Serrasalmidae [171] families.

With respect to SNP identification, few studies have been carried out in relation to the conservation of Neotropical freshwater species. Researchers have focused on valorous species such as the tambaqui (*C. macropomum*) and the pacu (*P. mesopotamicus*) of the Serrasalmidae family

that are considered one of the most captured species in the Neotropical region due to their high commercial value and potential in aquaculture [172, 173]. Other studies on SNPs (identified by the Pool-seq technique) refer to the evolutionary adaptation of species such as *Poecilia mexicana* in waters with high hydrogen sulphide concentrations (H_2S) in Mexico [174] and studies regarding the identification of SNPs in the sex chromosomes of *Characidium gomesi* by the RAD-seq technique with the aim of differentiating males and females [175].

6. Application of molecular markers

6.1. Identification of Neotropical cryptic species

mtDNA has been a marker of choice for reconstructing historical patterns of population demography, admixture, biogeography and speciation [88, 176] and can help identify cryptic individuals in many Neotropical fish species [177]. However, the main problem in genetic studies aimed at the maintenance of biodiversity is the difficulty of developing a method of species identification, since there are millions of unidentified and unknown species. The use of DNA barcodes, segments of approximately 600 bp of the mitochondrial gene cytochrome oxidase I (COI), has been considered an efficient technique to catalogue all biodiversity. The Neotropical freshwater ichthyofauna is considered the most diverse in the world, and very few fish species have been identified. It has been estimated that 30–40% of species have not been described, and genetic identification is a challenge, even with molecular techniques [176, 177].

Barcode research has already been performed in the São Francisco River Basin and provided evidence of the effectiveness of barcodes to catalogue the diversity of Neotropical basins by discovering new species and genera (*Hisonotus* sp.), expanding the range of known species (*Knodus moenkhausii*) and identifying overlooked species (*Bryconamericus stramineus*, *Piabina argentea* and *Poecilia* sp.) [178]. Analyses on 254 species of fish from the Upper Parana River Basin in Brazil correctly identified 252 species using their barcode sequences, including a large number of closely related species [179]. Moreover, comparative analyses using traditional morphological taxonomy and DNA barcoding of Neotropical ichthyoplankton from the Upper Paraná and São Francisco Rivers showed no conflicting results between the two techniques [180].

Advances in the use of barcodes have also been achieved in the Pampas plain region of Argentina and have shown that specimens of *S. brasiliensis*, *R. quelen*, *H. malabaricus*, *Synbranchus marmoratus*, *Australoheros facetus*, *O. jenynsii* and *Corydoras paleatus* differed by more than 3% from their conspecifics in other parts of South America. Overall, this study was able to highlight the likely occurrence of cryptic species, showing evidence of hidden diversity in the Neotropical region [181]. Although these results are important, more barcode research studies in Neotropical ichthyofauna are needed.

6.2. Genetic variability

Levels of genetic diversity between individuals in the same population and between populations are essential for species conservation in the face of environmental changes. In general,

most of the wild populations tend to have high levels of genetic diversity [182]. This is largely due to formation of these groups by migratory fish, representing panmictic populations, since high gene flow and the size of the population reduce the effects of genetic drift [183].

Several factors that may interfere in the fragmentation of populations, or their migratory potential, may cause a population bottleneck and decrease the genetic variability. Bottlenecks reduce population size by making individuals subject to genetic drift and inbreeding, thereby reducing the species evolutionary potential [184].

Several studies carried out in the Paraná River Basin have already demonstrated a decline and genetic homogenisation among fish populations in this basin [185–188]. These studies indicate that the fragmentation of the basin due to the large hydroelectric dams installed in the Paraná River Basin, mainly in the Upper Paraná region, is one of the major factors affecting these populations.

Brazil is the third largest producer of hydroelectric power, accounting for up to 10% of total world production. The conversion of free-flowing tropical rivers into the regulated systems associated with hydroelectric dams is one of the major concerns for the conservation of freshwater Neotropical fish. In addition to the impact on water velocity and temperature, hydroelectric dams block the natural river flow that affects freshwater fish populations due to habitat fragmentation, with increased risks of population isolation and consequent destruction of gene flow. This has already been reported using microsatellite markers for *Prochilodus argenteus* in the São Francisco River [168] and *Brycon insignis* in the Brazilian southeast [189].

In order to mitigate the damage caused by hydroelectric dams, programmes to reintroduce affected species are a potential solution. However, lack of knowledge about the genetics of local species can have the opposite effect. Analyses of restocking programmes for *P. argenteus* indicate differences between stock populations and wild populations, and this differentiation represents a risk and interrupts the diversity of local genes [190].

In addition to hydroelectric dams and inappropriate programmes for genetic restocking, the inadequate management of cultivated populations may also interfere with the genetic variability of species. Fish escaping from aquaculture facilities may influence the level of genetic diversity in natural populations living in the vicinity of fish farms. The introduction of cultivated individuals to wild populations may result in a mixture of populations with different genetic characteristics that reduce the average genetic diversity (Wahlund effect), as has already been observed in many fish populations [191, 192].

6.3. Genetic structure

As mentioned previously, Neotropical ichthyofauna is subject to many environmental factors that may affect their rate of retention in the environment of origin, including the destruction of their habitat and consequent fragmentation of populations. The effects on the spatial distribution of fish populations may result in genetic processes that affect gene frequency, including dispersive processes, gene oscillation and founder effects. These genetic processes intensify systematic migration, mutation and selection. Due to the high levels of polymorphisms and abundance throughout the genome, molecular markers are useful for genetic

structure analyses in different populations [193]. Studies directed towards the verification of structures of Neotropical populations using microsatellite markers are concentrated on populations affected by the construction of dams.

Many freshwater fish species that inhabit Neotropical rivers have migratory behaviour and reproduce during the rainy season, when water levels increase and temperatures rise. Normally, fish migration occurs in the main river or its tributaries for the spawning of eggs that are subsequently carried downstream to the floodplains, where they find suitable conditions for development [19]. This ability to migrate long distances suggests that these fish species constitute a single panmictic population, as reported in several studies of *Prochilodus mariae* [194], *Brycon hilarii* [195] and *P. mesopotamicus* [196] using microsatellite analysis. Many studies have found genetic structuring on microsatellite loci in different migratory species. This is not necessarily a spatial structure, but a temporal structure between individuals that share the same habitat with overlapping reproductive periods, as shown for *Prochilodus costatus* [197] and *S. brasiliensis* [198] in different Neotropical hydrographic basins.

Some research has also been carried out using mtDNA for population structure analyses. D-loop regions were used to infer structural analyses of populations of pacu (*P. mesopotamicus*) from the Paraguay River and in four other main tributaries, showing high gene flow and the formation of a single panmictic population. This is due to the flood cycle that regulates the interconnectivity between different environments and allows gene flow between populations, forming metapopulations [42].

There are few studies that use SNPs for the conservation of Neotropical freshwater species, and there are insufficient data to evaluate the genetic structure of natural stocks. More genetic studies using SNP markers for species identification need to be conducted in order to better understand population structure and to develop management measures and conservation policies [172, 173].

6.4. Identification of Neotropical hybrid fish

In the Neotropical region, particularly in Brazil, serrasalmid and pimelodid hybrids represent important advances in aquaculture. Hybrid fish originating from serrasalmid species such as the pacu (*P. mesopotamicus*), tambaqui (*C. macropomum*) and pirapitinga (*P. brachypomus*), or from pimelodid species such as the pintado (*P. corruscans*) and cachara (*Pseudoplatystoma reticulatum*), are considered commercially valuable and have high growth rates and have other characteristics that are useful to the commercial sector such as resistance and better reproductive performance [199]. The interest in hybrid production may be due to the lack of knowledge about the pure species, mainly in genetic breeding approaches [200]. The tambacu (*C. macropomum* female crossed with *P. mesopotamicus* male) and patinga (*P. mesopotamicus* female crossed with *P. brachypomus* male) correspond to a large part of Brazilian aquaculture production [201]. However, the uncontrolled production of fertile hybrids, such as the patinga, can have negative consequences for the environment. Improper management may result in fish escaping into the rivers and threatening the genetic integrity of pure species with free occurrence of backcrossing.

Genetic technologies for hybrid fish identification include cytogenetic methods and PCR techniques. The morphological similarity of fish hybrids to their parental species, mainly in juvenile

stages or post-F1 generations, means they can only be differentiated using molecular marker techniques. Initially, non-PCR-dependent molecular methods (using allozymes and RFLPs) were used to identify hybrids of serrasalmid species [200]. These markers are not currently used due to the advantages of PCR techniques. Mendelian codominant PCR markers, such as microsatellites and SNPs, are suitable for hybrid identification and introgression events. However, more studies are required to define genetic markers, such as SNPs, that are essential for the identification of fish hybrids, together with production monitoring and management measures, particularly in detecting escaped fish hybrids in the natural environment [55].

Alternative and less costly techniques, such as PCR-RFLP and multiplex-PCR, are easier to carry out and have proved to be efficient methodologies that can be quickly and inexpensively executed, allowing the identification by simple PCRs based on single nucleotide polymorphisms [55]. The PCR-RFLP method allows the analysis of DNA variation. Base substitutions in specific fragments formed at the enzyme recognition sites result in patterns of restriction fragments [202]. Multiplex-PCR uses species-specific primers for determining loci that differ between the analysed species by a few nucleotide substitutions, and two or more reactions can take place in the same tube [203]. PCR-RFLP and multiplex-PCR techniques are well established for the identification of hybrids between Neotropical species [55, 204]. However, genetic monitoring of hybridisation programmes should be applied in a routine way to verify whether the trade and management of hybrids are being performed correctly in fish breeding farms.

6.5. Traceability of Neotropical fish

Traceability is the ability to identify species and their origin. It is considered important for the conservation of natural stocks and for certification of food quality. DNA-based methodology of traceability has greater reliability and accuracy and is an important tool for the conservation of threatened stocks of Neotropical fish. Furthermore, SNP arrays for species identification, or for identification of a specific population, can be used in processed fish samples that have been frozen, salted, cooked and canned with a high attribution power. This makes it possible to identify the origin of the fish consumed and avoid commercial fishing in places with threatened stocks. However, the fish traceability test alone is not sufficient to reduce the decline in fish numbers; rather, traceability techniques should be used in conjunction with sustainable fisheries, by-catch reduction and management-based policies [125, 154, 205]. Despite traceability research in fish populations worldwide to avoid predatory and indiscriminate overfishing, there is still a lack of important studies related to DNA traceability markers in freshwater Neotropical fish species.

7. Conclusion

The methodological advances and the development of sequencing technologies can enable an efficient applicability of molecular markers in the conservation of Neotropical fish. Despite the negative impact that human activities have had on fish from the Neotropical region (such as deforestation, construction of dams, overfishing and non-native species introduction to the basins), there are few genetic studies into population structure, genetic variability and hybrid identification.

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Chapter 13

Status of the Important Bioresources of Girwa River with Special Reference to Ganges River Dolphin (*Platanista gangetica gangetica*) in Katerniaghat Wildlife Sanctuary, Uttar Pradesh, India

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Additional information is available at the end of the chapter

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Abstract

The Girwa River in India supports a rich variety of fauna including the endangered Ganges River Dolphin and critical endangered gharial. Due to rising conservation concerns, biologists in the country have conducted a great deal of research over the past few decades on the status of these species in its distribution range. However, in the Girwa River such studies are still lacking, both to inform conservation efforts and to help address broader concerns related to biodiversity conservation. In light of the above statement, the present study was conducted in the ca.18 km of the Girwa River in Katerniaghat Wildlife Sanctuary in Uttar Pradesh. During the survey, dolphins, crocodile and aquatic birds were encountered along most of the river with the exception of ca. 1.5 km section below the international border and a ca. 2 km section above the Girijapuri barrage. Based on the best estimate, Low-best-high figures of 27-35-41 dolphins, with an encounter rate of 1.94 dolphins/km were estimated. Besides dolphin, 65 gharial, 20 mugger crocodile and 64 species of aquatic birds were counted. Actual growth may be higher because of possible population under estimation during the present survey. Increasing anthropogenic activities such as dam and barrage, coupled with mortality in fishing nets, are likely to affect the future survival of these populations. Recommendations for management and research are made to ensure the effective conservation of these species in the Girwa River.

Keywords: Girwa, river, dolphin, gharial, mugger, birds, status, platanista, gangetica

1. Introduction

Freshwater ecosystems are fragile environment, are rich in biodiversity and are believed to be at risk than other freshwater animals. To understand the mechanism, driving losses in aquatic biodiversity, is important to the conservation and restoration of freshwater environments worldwide [1].

The Ganges River dolphin is distributed in the Ganges, Brahmaputra, Karnaphuli-Sangu, and Meghna river systems and their tributaries, from the foothills of the Himalaya to the limits of the tidal zone in India, Bangladesh and Nepal [2, 3]. It has already become extinct from most of its earlier distribution ranges and even in its present day distribution range the population is purported to be declining [4]. Extensive population fragmentation has resulted from the widespread construction of dams and barrages [5]. The Ganges River Dolphin however, is a true River Dolphin; it occurs only in fresh water, and is considered endangered [6]. The Government of India declared it as the "National Aquatic Animal" [7] and it has received protection in nine protected areas (PAs); of these the Vikramshila Ganges River Dolphin Sanctuary near Bhagalpur is specifically known for the conservation of the Ganges River Dolphin. They have also received some protection in the Girwa and Chambal River, specially created for the protection of the gharial [8]. In recent years several workers estimated the population of Ganga river dolphin in different segments of Ganga River and its tributaries e.g. In Ganges River ca.1200 km (Bijnor to Varanasi), with an encounter rate of 0.21 dolphin/km. In Yamuna River ca. 400 km (Pachnada-Allahabad), with an encounter rate of 0.07 dolphin/ km [8]. In River Girwa, ca. 18 km, with an encounter rate of 1.56 dolphins/ km. In Ghaghara river with an encounter rate of 0.45 dolphins/km. In Saryu River, ca. 30 km with an encounter rate of 0.51 dolphin/km. In Rapti River ca. 30 km with an encounter rate of 0.26 dolphin/km. However, no dolphin was recorded in Ken river ca. 30 km and Betwa River ca. 29 km [8]. In Chambal river ca. 425 km (Pali-Pachnada), the encounter rate of dolphin was 0.19 dolphin/km [9].

The north Indian State of Uttar Pradesh includes a large extent of the present day distribution of crocodile species, which occurs in the several large rivers flowing through the State viz. the Ganga [10], the Yamuna [11], the Chambal [11], the Ghagra, the Gandak [12] and the Sone River [13]. Early records reveal that these aquatic reptiles were, at one time, very abundant throughout their distribution range [14]. However, due to commercial exploitation and habitat destruction, populations of Crocodile species have been reduced to near extinction. In many habitats, Crocodile populations have been totally wiped out [15]. Considering their vulnerability, the gharial is now listed as Critically Endangered and the Mugger is listed as endangered on the International Union of Conservation of Nature (IUCN) Red List [16].

In Girwa River dolphin prefers to stay in deep water in and around the confluence of rivers, shares its habitat with few indicator species such as crocodiles, freshwater turtles and aquatic birds many of which are fish eaters and potential competitors with dolphins [17]. Studying indicator species could create the basis for a sustained research program to see

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how the changes of the said species can be related to the health of Indicator species in the river. This would help to implement various programes for restoration of the river system. This was the first survey of its kind in recent times, where an attempt was made to objectively assess the status of important bio resources of the Girwa River in Katerniaghat Wildlife Sanctuary.

2. Study area

The Karnali River arises in Nepal and bifurcates into two rivers, the Girwa and the Kauriala which reunites again in India to form Ghagra (WWF-India unpublished, 2001). The Girwa River, in the Katerniaghat Wildlife Sanctuary ca.18 km in length is bounded upstream by the Nepalese border and downstream by the Girijapuri barrage in India (**Figure 1**). The river is home to large aquatic animals such as endangered Ganges river dolphin (*Platanista gangetica gangetica)* (**Figure 2A**), critically endangered gharial (*Gavialis gangeticus)* (**Figure 2B**)., mugger (*Crocodilus palustris)* (**Figure 2C**)., Smooth coated otter *(Lutragale perspicillate),* several freshwater turtle species and aquatic birds (**Figure 2D**).

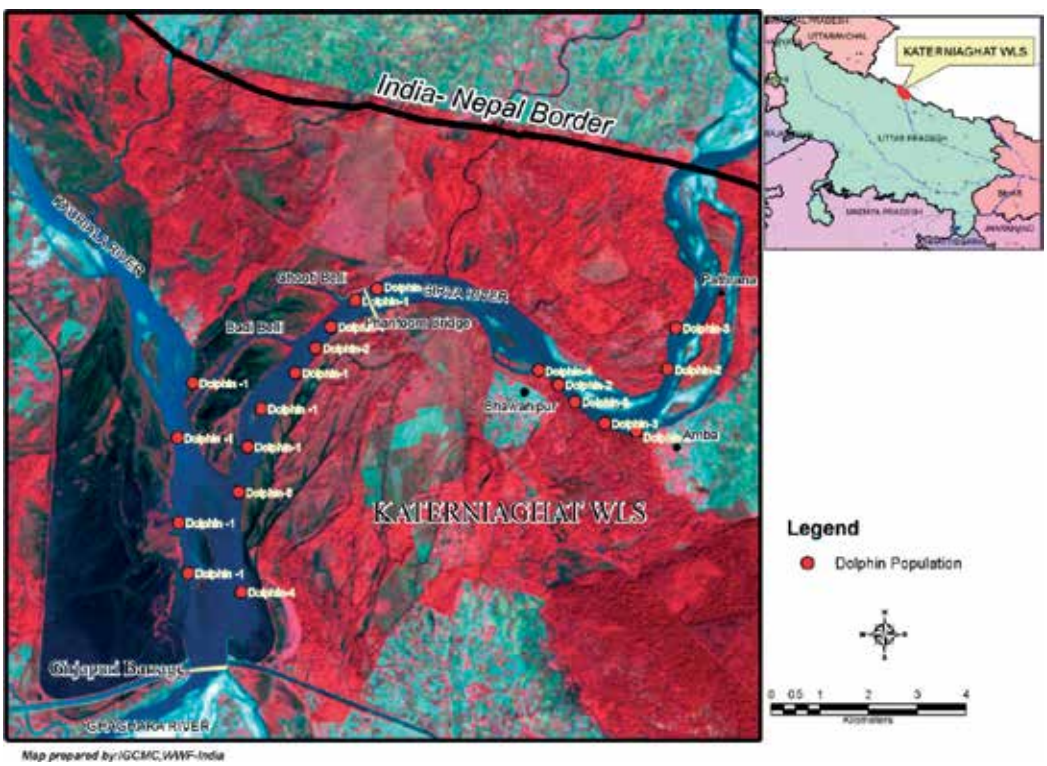

Figure 1. Study area showing dolphin sightings in different locations of Girwa River. (*Map Source: WWF-India*).

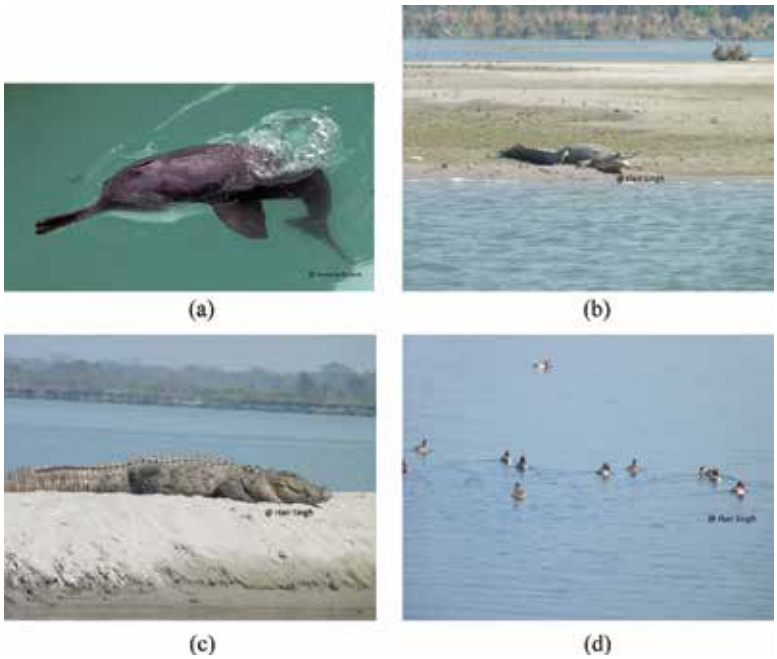

Figure 2. Gangetic dolphin _2A, Gharial_2B, Mugger _2C, and Red Crested Pochard_2D.

3. Methodology

To know the status of key aquatic fauna in the Girwa River, a vessel-based visual survey from Indi-Nepal border to Girijapuri Barrage (N 28° 33′.875; E 81° 12′.981) including the section of Kauriala River (N 28° 28′.640; E 81° 08′.308) in Katerniaghat Wildlife Sanctuary (**Figure 1**) was conducted in a ca.18 km of the river during 8th to 9th February 2013. The survey was conducted by using a motorized boat with an average speed of 4-5 km^{-1} both in upstream and downstream directions. A single transect line close to one river bank only was followed during the survey.

Methods for dolphin survey, estimation of absolute and relative abundance and study of Asian river dolphins, have been considered and described in detail by [2]. In these method two primary observers, one each on the right and left sides of the vessel searched accurately in a 90° one in front of the vessel. Two independent observers positioned behind the primary observers recorded dolphin sightings missed by the primary team. A Global Positioning System was used to record the distance traveled and the geographical coordinates of dolphin sightings. Group sizes were evaluated with a best, high, and low estimate of numbers to incorporate a degree of uncertainty [3]. The low group size estimate was considered a minimum count and the high estimate a maximum count. Estimates of the total number of individuals and of group size were calculated from the "best" estimates of group size, while the high and low estimates were used to evaluate the uncertainty of the observers about the accuracy of their best estimates [18].

During the survey a separate observer searched for gharial, mugger and aquatic birds (in addition to Dolphins) using the naked eye and binoculars [9]. Data were recorded whenever basking gharial, mugger and aquatic birds were sighted. Identification and status of birds was done using field guides [19], and for conservation status and common and scientific names Bird Life [20], was followed.

4. Results and discussion

4.1. Present and past estimates of key aquatic fauna in Girwa river

All sections of the Girwa River were searched both in up and downstream. Certain sections affected by water storage due to the barrage however comprised wide channel. A single transect line close to one river bank only was followed during survey of these sections. The population estimates in these sections could therefore be biased downward. All published and unpublished counts of Crocodile and Dolphin populations are presented in **Table 1**. [21] have reported the numbers of dolphins sighted in two detailed surveys of the Girwa River, one upstream and one downstream, conducted in 19 to 25 February 1994. The low-best-high figures totaled 20-24-29 and 13-15-16 for the two surveys respectively. Basu and Sharma, (Unpublished Report 2000) estimated a population of 25 dolphins for the entire river in the Katerniaghat Wildlife Sanctuary. During 2001, the encounter rate of dolphins in the river based on "best" estimate of 30 dolphins was 1.67dolphins/ km river length. Low-best-high estimates in the river were 23-30-44 (WWF-India unpublished Report 2001). In 2006, the encounter rate of dolphins based on best estimate of 39 dolphins was 1.95 dolphins/ km. The Low-best-high estimate of dolphin in the river was 31-39-54. Based on best estimate in 2009, a total of 49 dolphins with an encounter rate of 2.22 dolphins/km of river length are estimated. The Low-Best-High estimate was 40-49-62 (WWF-India unpublished Report 2009). In 2012, the encounter rate of dolphins was 1.56 dolphins/ km. The Low-Best-High estimate was 29-39-44 [9]. During present survey, the encounter rate of dolphins based on "best" estimate of 35 dolphins was 1.94 dolphins/km river length where the boat was moving at a speed of 4–5 km^{-1}. The Low-best-high estimates totaled 27-35-41 (**Table 1**).

Evaluation of status of dolphins in the Girwa River, compared to the best estimates of [21], indicates that in the 23 year period following their work, the encounter rate of dolphins is higher than the earlier population record. During the present survey, dolphins were encountered along the entire length of the Girwa with the exceptions of ca. 1.5 km section immediately below the international border entirely of boulder bed riffles and too shallow (< 0.5 m depth) to be habitable by dolphins and a 2 km section just above the barrage. The effect of water storage by the barrage that became operational in 1975–1976, is perceivable up to ca. 10 km upstream of the barrage (WWF-India unpublished 2001).

Earlier count of crocodile in Girwa river was 49 gharial and 14 mugger in 2006, 70 gharial and 16 mugger in 2009 and 65 gharials and 20 mugger in the year 2013 (**Table 1**). The first gharial population survey in Katerniaghat Wildlife Sanctuary was in 1975–1976 followed by [22, 23] 105 individual in 2008 by [24], WWF-India unpublished (2006, 2009) and present survey in 2013 (**Table 1**).

S.No.	Location	Dolphin			Crocodile	
		Low	Best	High	Gharial	Mugger
7th–10th February 2001 (Reference: WWF-India unpublished)						
Indo-Nepal border to Katerniaghat pontoon bridge						
I	Pathrana-Amba	7	9	12	NA	NA
II	Amba-Bhawanipur	4	7	13	NA	NA
III	Phantoom bridge-Girjapuri barrage	5	7	10	NA	NA
IV	Geruwa-Kauriala Confluence	7	7	9	NA	NA
Total		**23**	**30**	**44**		
11th–12th December 2006 (Reference: WWF-India unpublished)						
I	Bhawanipur to Amba	11	14	20	NA	NA
II	Amba to Pathrana	4	5	5	NA	NA
III	Bhawanipur to Girijapuri barrage	16	20	29	NA	NA
IV	Geruwa-Kauriala Confluence	5	5	7	NA	NA
	Total	**36**	**44**	**61**	**49**	**14**
9th–10th December 2009 (Reference: WWF-India Unpublished)						
I	Katerniaghat to Girija Barrage	9	9	14	NA	NA
II	Kauriala to Katerniaghat	6	7	9	NA	NA
III	Katerniaghat to Amba	20	27	30	NA	NA
IV	Geruwa-Kauriala Confluence	5	6	9	NA	NA
	Total	**40**	**49**	**62**	**70**	**16**
5th–7th October 2012 (Reference: Behera *et al.* 2014)						
I	Katerniaghat to Girija Barrage	5	7	9	NA	NA
II	Kauriala to Katerniaghat	7	8	10	NA	NA
III	Katerniaghat to Amba	13	19	20	NA	NA
IV	Geruwa-Kauriala Confluence	4	5	5	NA	NA
	Total	**29**	**39**	**44**	**NA**	**NA**
8th–9th February 2013 (Present study)						
Indo-Nepal border to Katerniaghat pontoon bridge						
I	Katerniaghat to Girija Barrage	5	7	8	9	10
II	Kauriala to Katerniaghat	6	8	8	30	3
III	Katerniaghat to Amba	11	14	19	26	7
IV	Geruwa-Kauriala Confluence	5	6	6	0	0
	Total	**27**	**35**	**41**	**65**	**20**

Legend: NA = Data not available.

Table 1. Distribution of dolphin and crocodile in Girwa River.

S.No.	Common name	Scientific name	Status
1	Asian open billed Stork	*Anastomus oscitans*	LC
2	Black-necked Stork	*Ephippiorhynchus asiaticus*	NT
3	Black Stork	*Ciconia nigra*	LC
4	Black-Bellied Tern	*Sterna acuticauda*	EN
5	Black-headed Ibis	*Threskiornis melanocephalus*	NT
6	Black-tailed Godwit	*Limosa limosa*	NT
7	Black-winged Stilt	*Himantopus himantopus*	LC
8	Brahminy Kite	*Haliastur indus*	LC
9	Bronze-winged Jacana	*Metopidius indicus*	LC
10	Brown-headed Gull	*Chroicocephalus brunnicephalus*	LC
11	Comb Duck	*Sarkidiornis melanotos*	LC
12	Common Coot	*Fulica atra*	LC
13	Common Greenshank	*Tringa nebularia*	LC
14	Common Kingfisher	*Alcedo atthis*	LC
15	Common Moorthen	*Gallinula chloropus*	LC
16	Common Pochard	*Aythya ferina*	VU
17	Common Redshank	*Tringa totanus*	LC
18	Common Sandpiper	*Actitis hypoleucos*	LC
19	Common Teal	*Anas crecca*	LC
20	Cotton Pygmy-Goose	*Nettapus coromandelianus*	LC
21	Darter	*Anhinga melanogaster*	LC
22	Egyptian vulture	*Neophron percnopterus*	EN
23	Eurasian Curlew	*Numenius arquata*	NT
24	Eurasian Spoon bill	*Platalea leucorodia*	LC
25	Eurasian Wigeon	*Mareca penelope*	LC
26	Ferruginous Pochard	*Aythya nyroca*	NT
27	Gadwall	*Mareca strepera*	LC
28	Garganey	*Spatula querquedula*	LC
29	Great cormorant	*Phalacrocorax carbo*	LC
30	Great thick-knee	*Esacus recurvirostris*	NT
31	Green Sandpiper	*Tringa ochropus*	LC
32	Gray heron	*Ardea cinerea*	LC
33	Indian Skimmer	*Rynchops albicollis*	VU

S.No.	Common name	Scientific name	Status
34	Indian Spot-billed Duck	*Anas poecilorhyncha*	LC
35	Intermediate egret	*Ardea intermedia*	LC
36	Kentish Plover	*Charadrius alexandrinus*	LC
37	Large egret	*Ardea alba*	LC
38	Little cormorant	*Microcarbo niger*	LC
39	Little Egret	*Egretta garzetta*	LC
40	Little Ringed Plover	*Charadrius dubius*	LC
41	Mallard	*Anas platyrhynchos*	LC
42	Marsh Sandpiper	*Tringa stagnatilis*	LC
43	Northern Pintail	*Anas acuta*	LC
44	Northern Shoveler	*Spatula clypeata*	LC
45	Osprey	*Pandion haliaetus*	LC
46	Painted Stork	*Mycteria leucocephala*	NT
47	Pallas gull	*Ichthyaetus ichthyaetus*	LC
48	Pheasant-Tailed Jacana	*Hydrophasianus chirurgus*	LC
49	Pied Avocet	*Recurvirostra avosetta*	LC
50	Pied Kingfisher	*Ceryle rudis*	LC
51	Red-Crested Pochard	*Netta rufina*	LC
52	Red-Wattled Lapwing	*Vanellus indicus*	LC
53	River Lapwing	*Vanellus duvaucelii*	NT
54	River Tern	*Sterna aurantia*	NT
55	Ruddy Shelduck	*Tadorna ferruginea*	LC
56	Sarus Crane	*Antigone antigone*	VU
57	Small Pratincole	*Glareola lactea*	LC
58	Spot-Billed Duck	*Anas poecilorhyncha*	LC
59	Stork-Billed Kingfisher	*Pelargopsis capensis*	LC
60	Tufted Duck	*Aythya fuligula*	LC
61	Whiskered Tern	*Chlidonias hybrida*	LC
62	White-Breasted Waterhen	*Amaurornis phoenicurus*	LC
63	White-Throated Kingfisher	*Halcyon smyrnensis*	LC
64	Wooly-necked Stork	*Ciconia episcopus*	VU

Legend: LC = least concern; VU = vulnerable; EN = endangered; NT = near threatened.

Table 2. Checklist of avifauna species recorded in Girwa River.

At least sixty-four species of birds, most of them aquatic or semi-aquatic, were observed during the survey. Of the species listed 49 species are least concern (LC), 09 near threatened (NT), 04 vulnerable (VU), 02 endangered (EN) (**Table 2**). The list is not complete because of failure to identify some related species such as ducks, teals, snipes, terns and certain raptors. The only avifaunal record from Girwa and Ghagra river was 57 species by WWF-India unpublished (2001), and 151 species in Katerniaghat Wildlife Sanctuary [25]. Although, comparison of the result of the earlier surveys and the present one though not fully valid due to difference in study site. Also non-aquatic birds were not studied during the present survey. It is felt to safely indicate that Girwa River supports great avifaunal diversity.

5. Conservation constraint

The major cause of concern about the future survival of the species was gross hydro ecological changes that may occur in the stream characteristics of the Girwa River resulting from the construction of the Chisapani high dam, in Nepal and Girijapuri Barrage in India. Beside this, every year the entire barrage gates of the Girijapuri Barrage on the Girwa River, are opened at once for maintenance. When this is done in summer months of April/May, water flow in the river is at its lowest. The entire stretch of the river within the sanctuary up to (and beyond) the Nepal border is "drained" and there is very little flow left, with much of the river only knee deep or less with large number of fish left stranded and dying. The River dolphins and the gharial and whatever fish that survives have to congregate in the few remaining pools of water. This is not only in the interests of saving the habitats for endangered riverine fauna such as gharial and river dolphin but also for the important fish stocks and other commercially valuable species (WWF-India unpublished). Fishing was observed in the Girwa River at several locations. Also, reports of dolphins being intentionally caught in gill nets being used close to dolphins surfacing (WWF-India unpublished).

To understand the health of river ecosystem available information of important key aquatic resources is urgently needed to effectively understanding the conservation needs. These are reliable indicator species that is threatened by human activities. In light of the results of the present study, conservation management recommendations are suggested. These include highlighting the need for habitat management, control of illegal activities, and long-term monitoring program.

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Chapter 14

Closed Aquaculture System: Zero Water Discharge for Shrimp and Prawn Farming in Indonesia

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Additional information is available at the end of the chapter

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Abstract

This chapter focuses on the development and application of zero water discharge (ZWD) system, which has become an alternative solution to conventional methods of aquaculture production. With this system, it is expected to answer many issues in aquaculture cultivation, such as environmental damage, disease outbreak, and land-use change, and to create a sustainable aquaculture cultivation system. ZWD system is an improved batch system with an emphasis on microbial manipulation in rearing tank. The principle of microbial selection is based on the role of each microbial component in nutrient cycle in the rearing tank. This chapter contains in detail how methods and stages are performed in order to conduct this system, including design of construction system, cultivation of microbial components, initial conditioning of this system, and microbial manipulation. The performance of the system was tested in crustacean culture such as white shrimp and giant freshwater prawns, and it showed that the system can increase the average survival rate of 10–20%. In addition, the technical and economic feasibility of this system was evaluated to illustrate the production efficiency upon the application of this system in the industry.

Keywords: closed aquaculture system, zero water discharge, white shrimp, prawn, microbial loop, microbial manipulation

1. Introduction

Driven with the increment of human population in the world, trend of total world fishery production has increased from 2009 to 2014 with an average growth rate of about 2.77% per year [1]. This growth mostly came from aquaculture sector instead of capture practice with

annual growth rate of 8.8% [2]. Global aquaculture production has reached 73.8 million tons in 2014 with an estimated value of USD160.2 billion. It shared about 44.14% of total fishery production. In the next decade (2025), FAO predicted that aquaculture sector would share 52% of the total fishery production [1]. Along with the prediction, Indonesia has a great potential to develop the aquaculture sector. Indonesia is one of main producers of both capture and aquaculture fishery commodities because it is supported by its geographical condition. Indonesia is an archipelagic country that has great potential in fisheries sector. It consisted of 17,500 islands and located between two big oceans, Pacific and Indian Ocean. Moreover, Indonesia is a country crossed by equator line and ranked as world's 4th longest coastline, which indicates a high diversity of aquatic organisms, including marine biota [3]. So, there are many fishery commodities grown in Indonesia. Currently, Indonesia ranks as the second top both capture and aquaculture producers after People's Republic of China, contributing 6.48 and 14.36 million tons, respectively, to worldwide production [1]. One of main commodities is crustaceans that produced both capture and aquaculture practices. In fact, most of productions were obtained from aquaculture. In 2014, shrimp capture production only contributed about 30% of the total shrimp production or approximately 273,133 tons [4]. Shrimp commodities rank as the top by annual total aquaculture production from aquatic animal.

Most of shrimp production is dominated by white shrimp (*Litopenaeus vannamei*) which is also exported to several countries in the world, such as United States of America, Japan, People's Republic of China, United Kingdom, Malaysia, etc. [5]. Trend of white shrimp production has increased significantly with an average growth of 22.46%. This increment production was due to ease of cultivation practice, in case of availability of seed, cultivation period, and more resistance to environmental changes. Another species, giant freshwater prawn has the opportunity to become a main commodity due to high economic value. In 2013, prawn production reached approximately 3.171 tons, which has been cultivated in several site, such as West Kalimantan, Bali, West Java, and East Java [6]. Although it is still small in number compared to white shrimp, production volume continued to rise in recent years. Ministry of Maritime Affairs and Fisheries Republic of Indonesia seriously began promoting the cultivation of prawn, started in 2015, they have allocated a national capital budget for prawn production up to Rp 275.2 billions [7].

However, a high production scale does not ensure sustainability of shrimp aquaculture industry, because currently most shrimp farms use conventional culture practices, such as batch or flow-through system. It is true that conventional shrimp rearing strategies are still widely applied and profitable due to its simplicity and acceptable production cost, but since the cultivation relies on natural environment with less control to water quality and disease or predation, this condition leads to unpredictable culture performances [8]. Furthermore, the accumulation of harmful substances in culture water from uneaten feed and excretion (e.g., ammonium and nitrite) is very likely exceeds the tolerance limits, causing a decrement of culture survival rate and thus affecting overall shrimp productivity in conventional culture system [9]. Besides, the system is considered as not environmentally friendly, because untreated effluent water can pollute the surrounding aquatic environment [8]. In term of space requirement, the system occupies a large production area and requires close distance to coastal area to ensure seawater access. These circumstances contribute to impractical shrimp farming

industry and its sustainability in the near future. These problems urge an improvement of better aquaculture technology, which can support the culture's sustainability, with regard to water quality and culture performance, good hygiene, as well as high culture efficiency in terms of space utilization, water sources, and feed.

One alternative technology called zero water discharge (ZWD) system has been developed to resolve the above-mentioned problems [10–13]. ZWD is a sustainable intensive culture technology, which is environmentally friendly as it maintains water quality, therefore prevents pathogen spreading as well as wastewater discharge, which is rich in nutrients, to the environment [14]. The ZWD system allows limiting or reducing water usage, by implementing microbial consortium with various important roles, such as recycling nitrogen compound in the culture water and cleaning harmful nitrogen substances prior partial or total reuse of the water.

2. ZWD principle

Water body is habitat for all aquatic animals, including shrimp and prawn. Consequently, the key for success cultivation is to keep the habitat favorable for shrimp to grow. So that, it is crucial to maintain water quality in tolerance range for shrimp growth. Water quality includes physical, chemical, and biological parameters particularly temperature, dissolved oxygen, and toxic nitrogen substance concentrations [15]. Temperature and dissolved oxygen parameters can be manipulated by physical treatment such as using aerator and water heater, while toxic nitrogen substances have dealt with biological treatment system usually utilizing microbial-based treatment.

Toxic nitrogen substances produced from excretion activity of shrimp and their feed residue, such as ammonium and nitrite, disturb metabolic balance of the shrimps, making them more prone to disease that causes several disadvantages, including reduced body weight, increased mortality, and eventually decrease production yield [16–18]. As this has become one major problem in aquaculture, ammonium and nitrite removal management is a major concern in ZWD system. In natural aquatic ecosystems, microorganism present in water body maintains a balance concentration of each nitrogen compounds. As ammonium and nitrite concentration in intensive aquaculture systems build up much faster than in natural ecosystems, we cannot rely on naturally occurring microorganisms in the ponds. Their low population size cannot cope with the rate of ammonium accumulation, and therefore, addition of microorganism is needed.

This system uses the principle of microbial loops adapted from natural ecosystems. Toxic nitrogen substances present in ammonium and nitrite form can be converted into nitrate which is less toxic substance through consecutive nitrification microbial process. ZWD system aims to improve water quality through recycling chemical waste [19]. While conventional system (e.g. flow-through) requires a continuous new water supply to avoid waste accumulation in the culture, ZWD recycles ammonium, nitrite, and nitrate using microorganism consortia, and therefore, it reduces water usage significantly. Ammonium, nitrite, and nitrate level can be maintained using addition of heterotrophic bacteria, nitrifying bacteria, and microalgae, regularly [13].

2.1. State of the art

Based on the principle explained earlier, the most crucial thing is the selection of microbial components that have functions in maintaining water quality and are harmless to the animals being cultivated. In addition, selected microbes may act as probiotics such as to counteract pathogenic attacks from *Vibrio* sp. in shrimp farming [19]. Since this system refers to nutrient cycles in aquatic habitat, the selected microbes should have a role in the alteration of toxin substances into harmless substance produced in the cultivation system. The system emphasizes nitrogen nutrient cycle because nitrogen toxin is very dangerous if it accumulates excessively.

Figure 1 shown below is an example for the estimation of nutrient cycle and microbial loop that occur in the ZWD system [13]. The greatest accumulation of toxic compounds in cultivation is from animal feed and feces. These compounds are mostly organic matters, which can be degraded by heterotrophic bacteria into inorganic compounds. Inorganic compounds, such as ammonium and nitrite, which became the focus, have to be removed. The ammonium and nitrite should be converted into less harmful compounds such as nitrate by oxidation. Microbes that can do the oxidation process from inorganic compounds are litoautotrophic bacteria [20]. There are two stages of the oxidation processes: (1) the conversion from ammonium to nitrite and (2) nitrite to nitrate. Ammonium-oxidizing bacteria (AOB) convert ammonium to nitrite, for example, Nitrosomonas, and nitrite-oxidizing bacteria (NOB) convert nitrite to nitrate, such as Nitrobacter [21]. Even though nitrate is a harmless substance, the tolerance range in aquaculture system is no more than 200 ppm [22]. Therefore, it is necessary to search microbes that can utilize nitrate. Some microalgae can use nitrate as a source of nitrogen, so that addition of microalgae is important in this system. In addition, at the trophic level, microalgae act as

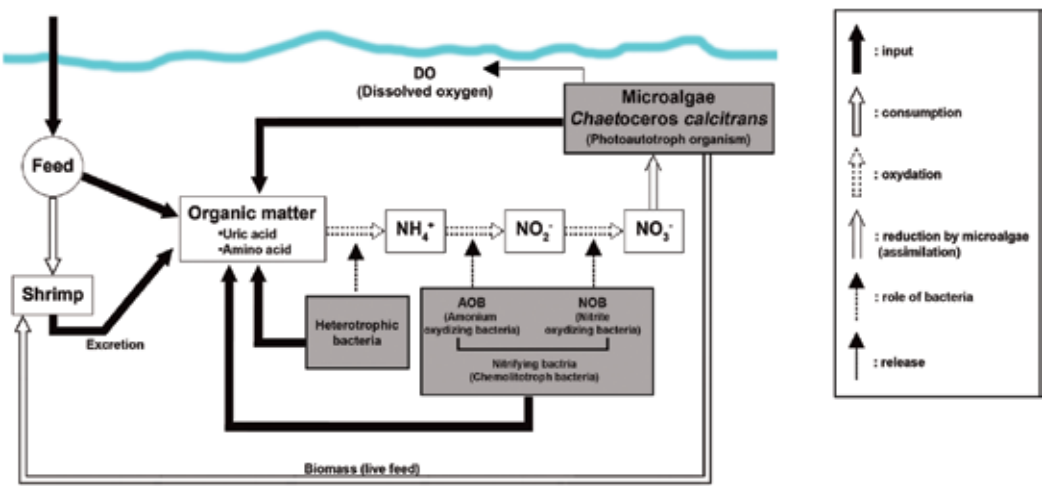

Figure 1. Schematic of nutrient cycle in ZWD system [13].

producer that also provides oxygen that can raise the DO level, and their biomass may be possible as food source for cultivated animals in the system.

Because the ZWD system relies on microbial components added to the system, different animal cultures will have different microbial components added, and the system must be favorable for microbial components to live. For a simple example, we have to consider about native microbial habitat; marine microbes will be suitable for marine animal farming and freshwater microbes for freshwater animal farming. **Figure 1** is an example of ZWD system in the cultivation of white shrimp, so the microbial components used are marine heterotrophic bacteria, marine AOB, marine NOB, and marine microalgae (*Chaetoceros calcitrans*).

2.2. Distinctive characteristics in ZWD system compared to other microbial-based systems

As the aquaculture industry grows rapidly in the world, it encourages research to create technology that leads to the sustainability in aquaculture industry. One of main research areas is the utilization of microbes that are now widely used in aquaculture industry. It can play a role as a food source such as microalgae for the larval phase [23, 24], maintain water quality such as using ammonium as a source of nitrogen for microbial metabolism [21, 25, 26], fight against disease such as immunostimulant that trigger antibodies or directly interact antagonistically with pathogen [19, 27, 28].

The application of microbes in aquaculture is conducted into microbial-based closed systems, such as the ZWD system. The term of zero water discharge has many versions; it can be zero water exchange [14, 29–31], limited water discharge [32, 33], minimal discharge system [34], minimal effluent discharge [35], minimal exchange system [36], etc. All such systems have the same principle that is minimizing water use and re-recycling water used by involving the role of microbes. ZWD system is an improvement from batch system with an emphasis on microbial manipulation in rearing tanks. ZWD system can be interpreted as no water discharge during culture period, additional water that put into the system is to balance water level due to water losses caused siphoning and evaporation. It is approximately 2% of culture volume in every 6 weeks [13].

So far, the existing microbial form used may be in consortium, biofilm, periphyton, biofloc forms or has separated compartments such as biofilter in recirculation aquaculture system (RAS). In ZWD system, the form of microbial used is consortia that have been added regularly to the system during cultivation period. The purpose of additional microbial consortia regularly is to control microbial loop works in appropriate way. In addition, the presence of microbial control is to keep dominancy of selected microbes that play a role in predicted microbial loop. However, to maintain the availability of microbial cultures, the system must be equipped with separated microbial cultivation facilities. Consequently, there is control to maintain microbial culture from contamination and to keep the microbes in their optimum growth. **Table 1** below is a summary of the characteristics of each microbial-based closed system.

Microbial-based. closed system	Characteristics	References
Zero water discharge	– Low/no water discharge – Improved system from batch system – Emphasize in microbial manipulation – Nitrogen toxic compound removal by microbial loop system – Microbial consortia added regularly to the system – Microbial component is kept dominant in the system – Need additional compartment for separated microbial cultivation	[13]
Biofloc	– Low/no water discharge – Improved system from batch system – Add carbon source to enhance heterotrophic bacteria consortium – Emphasize in C/N ratio in the system – 'waste' Nitrogen is converted to high concentration of total suspended solid (microbial biomass) that can act as highly protein feed for cultured animal – Consider well mixing and aeration to compensate BOD in the system	[26, 37, 38]
Periphyton	– Low/no water discharge – Improved system from batch system – Need organic substrate i.e. bamboo to periphyton attachment – Input organic matter i.e. manure and chemical fertilizers to trigger periphyton growth – Sometimes, needs additional carbon source to maintain C/N ration in the system – Periphyton acts as nitrogen toxic removal system and food source for cultured animal	[39–41]
Biofilm	– Low water discharge – Improved system from batch system – Nitrogen toxic compound removal was done by formed biofilm during culture period – No control of microbial consortia in biofilm – Biofilm can also be a food source for cultured animal	[18]
Defined biofilm	– Biofilm production needs additional reactor and attachment substrate – Defined microbial consortia in biofilm (predominantly nitrifying bacteria) – Main purpose is to remove nitrogen toxic substance in system – Can be applied in the system or in external unit i.e. biofilter	[42, 43]
RAS	– No water discharge – Many treatment process involved including physical and chemical treatment – Microbial compartment is in biofilter – Biofilter has defined microbial consortia – Isolated and clear-water system – Main purpose is biologically secured and hygiene aquaculture product – Investment cost and operational cost is higher than other systems	[44–46]
Green water technique	– Low water discharge – Use batch system – Mostly autotrophic microalgae used as microbial component in the system – Utilized chemical fertilizer and organic waste to trigger phytoplankton grow – No control in microbe community in the system – Main purpose is to provide natural food for cultured animal	[47, 48]

Table 1. Characteristics of microbial-based system.

3. Preparation of ZWD system

Proper designed systems and good microbial management are important parts to optimize production efficiency in intensive cultivation using ZWD system. This section will describe the

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main and supporting facilities of ZWD systems for crustacean cultivation, particularly white shrimp and giant freshwater prawn. Besides, the selection of microbial components that are suitable for shrimp and prawn culture and how to prepare the cultivation will be explained in this section.

3.1. Design construction and facility

As ZWD system is an improvement system of batch culture, the main facilities provided are similar to batch system. ZWD system installation can be constructed in a rectangular or circular culture tank that is equipped with several basic utilities commonly used in aquaculture. Here is the list of facilities for ZWD system.

1. Culture tanks for nitrifying, heterotrophic bacteria, and microalgae culture. Separation is necessary for easier maintenance purpose. Proper maintenance is critical to keep optimum performance of microbial components. Tank sizes of nitrifying bacteria and microalgae are suggested to have minimal capacity about 20% from culture tanks, while tank size of heterotrophic bacteria is suggested to have minimal capacity about 2.5% from culture tanks.
2. Aeration equipment including aerator, silicon hose, and air stone. The aerator provides continuous oxygen supply with airflow rate of 28 L/min [49]. Proper aeration is critical, not only to provide oxygen to shrimp for effective feed utilization and growth, but as importantly to oxidize liquid, solid, and gaseous waste in the system. Oxygen level in water must be maintained between 4 and 6 ppm [44]. However, 6 ppm is recommended to support optimum growth. With high inputs of feed, there is higher demand for oxygen by shrimps and by the microbial community in the water.
3. A net covering is used to avoid pollutant entry to culture pond, and it is more important to reduce water evaporation, which can affect salinity level significantly. In addition, covering reduces light penetration through the water column to suit intensity level for the microalgae population in water.
4. A thermometer to monitor daily culture temperature.
5. Feeding trays to administer and control sufficient daily feed amount.
6. $CaCO_3$ and gravel, as a substrate for nitrifying bacteria attachment as well as a buffering agent.

In addition, shelter is required for some crustacean such as prawn (*Macrobrachium rosenbergii* De Man). Prawn is much more aggressive than shrimp. There is a risk of cannibalistic behavior that emerges when prawns are cultured at high density, especially during their grow-out phase and molting period [11]. Without shelter, they do not have enough niche for each individual. Several shelters that have been proved in previous studies were textile vertical substrate [11] and cubical bamboo shelters [51]. These shelters have been proven to improve prawn culture productivity. **Figure 2** below represents the components of ZWD system.

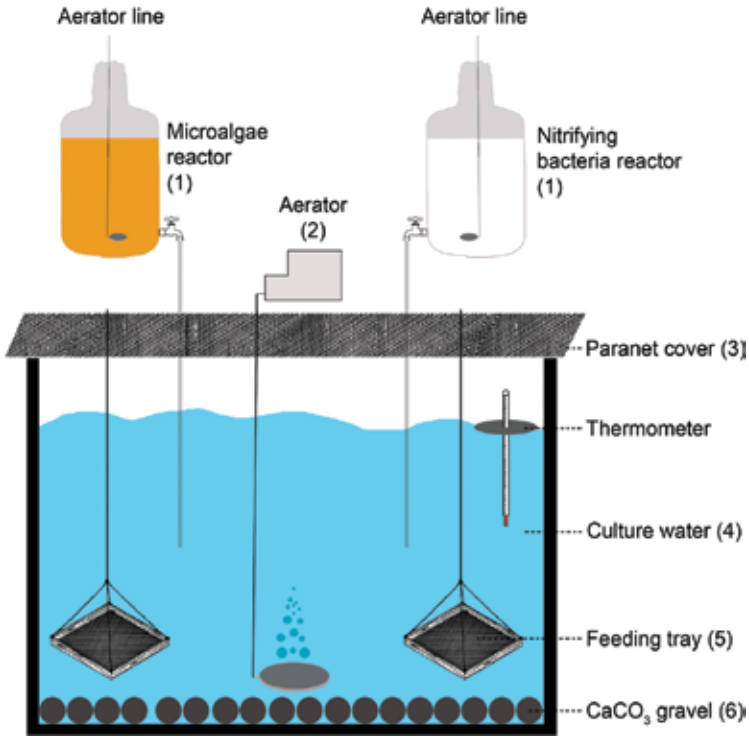

Figure 2. Basic facilities of ZWD system [13, 50].

3.2. Microbial components

The microbial component of ZWD system can be understood through three key functional groups: nitrifying bacteria, autotropic microalgae, and heterotrophic bacteria. When managed correctly, a diverse healthy microbial community contributes directly and indirectly to shrimp nutrition and growth while processing excess nitrogen waste in the system. Once established, the community becomes stable, competitively excluding harmful opportunistic pathogen and therefore improving health and immune competence of shrimps. The key to maximize these benefits is in understanding and managing the microbial community in the system.

3.2.1. Nitrifying bacteria

Nitrifying bacteria live in a wide variety of habitats, including soil, freshwater, seawater, rocks, and sediment. Nitrifying bacteria are widely used in aquaculture practice and usually in the form of ammonium-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). AOB derive energy through the process of catabolism of ammonium into nitrite; the bacteria included are genera *Nitrosomonas, Nitrosococcus, Nitrosospira, Nitrosolobus*, and *Nitrosovibrio*. While NOB oxidize nitrite to nitrate, the bacteria included are genera *Nitrobacter, Nitrococcus, Nitrospira,* and *Nitrospina*. The bacteria are classified into litoautotrophic bacteria because they use inorganic compounds as a source of energy and CO_2 as a carbon source [52].

According to previous studies, the most common nitrifying bacteria that were stable during cultivation period were *Nitrosomonas* sp. and *Nitrobacter* sp. [13]. *Nitrosomonas* and *Nitrobacter* are Gram-negative and aerobe obligate that is used as final electron acceptor. The bacteria can grow and multiply as individual units or in the form of biofilms. *Nitrosomonas* reproduces with binary fission, while *Nitrobacter* reproduces with budding. *Nitrosomonas* and *Nitrobacter* have different generation time, *Nitrosomonas* is every 8 h, and *Nitrobacter* is every 12 h. After 72 h, the population size of *Nitrosomonas* will be eight times greater than the population size of the *Nitrobacter* [53].

Several inhibition factors affect the nitrification process. Inhibitors may be short-term or long-term impact to their enzymatic activity. Some factors that can inhibit the rate of nitrification, there are alkaline pH, temperature, oxygen, salinity, organic and inorganic compounds, substrate for attachment and sunlight [53]. From mentioned factors, sunlight is an important factor to be taken into attention because sunlight can decrease the activity of bacteria *Nitrosomonas* and *Nitrobacter* in oxidizing ammonium and nitrite compounds [54].

The cultivation of nitrifying bacteria was performed in Winogradsky medium and in strong aeration with no light conditions (covered with black plastic). At the beginning of cultivation, bacterial culture of nitrification is activated by adding 10 ppm of ammonium. Ammonium levels are measured daily until it reaches 0 ppm. Furthermore, the activity of nitrifying bacteria is enhanced by continuously increasing the ammonium level up to 50 ppm. Later, the culture was scaled up to 10 L and then up to 500 L. The culture substrate used was $CaCO_3$ and gravel. **Figure 3** below shows schematic reactor for nitrifying bacteria cultivation.

3.2.2. Autotrophic microalgae

Besides nitrifying bacteria, microalgae are also an important component in ZWD system. Through their metabolism, microalgae take up nitrate obtained from final nitrification process by nitrifying bacteria as a nitrogen source [13]. Microalgae have the ability to conduct photosynthesis, which captures energy from light to synthesize organic carbon from inorganic carbon (CO_2). It accumulates organic carbon in forms of starch or other carbohydrates. Along

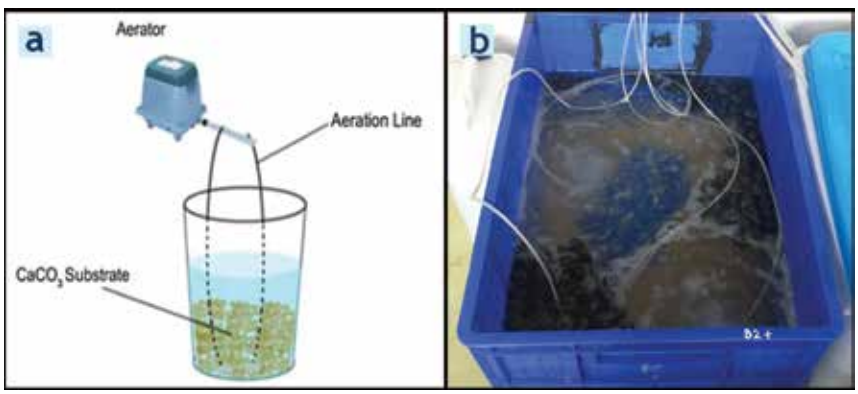

Figure 3. (a) Schematic diagram of nitrifying bacteria reactor [11, 13]; and (b) real nitrifying bacteria culture.

with other physiological processes, microalgae produce high-quality vitamin and minerals [58]. Moreover, microalga also has a good nutritional composition for aquaculture animals. Microalgae usually serve as a live feed at larval or early juvenile stage. Selected microalgae must have rapid growth rates, can cultivate to mass culture, and are stable growth to any environmental fluctuations.

Several species of microalgae that have been documented to success cultivation since 1997 are *Isochrysis* sp., *Pavlova lutheri, C. calcitrans, Chaetoceros muelleri, Chaetoceros gracilis, Thalassiosira pseudonana, Skeletonema* spp., *Tetraselmis suecica, Navicula* spp., *Nitzschia* spp. [55–59]. In practice, diatoms such as *C. calcitrans, C. muelleri,* or *C. gracilis* proved to increase shrimp productivity. Moreover, cell wall of these microalgae contains silicate, which is an important mineral for building exoskeleton of shrimps [60]. In addition, as microalgae cell density increases throughout their growth, it reduces light penetration to water body, so shrimp is not directly exposed to light (i.e., shading effects). Shading effect improves the production of shrimp, even though their exact mechanism of action remains unclear [61].

Microalgae begin to be cultivated from small scale (1 L) to large scale approximately 500 L. The cultured microalgae are diatoms (*C. calcitrans, C. muelleri,* and *C. gracilis*) for white shrimp and *Chlorella* sp. for prawn. For diatoms, medium used is f/2 medium [62] for stock culture up to 1 L, while medium used for *Chlorella* stock culture is Bold's Basal Medium [63]. Commercial media consisted of chemical fertilizer are used for large scale. Fertilizer must comprise a source of nitrogen, phosphate, silicate, and a small portion of the mineral. Examples of fertilizers used are NPK, Urea, ZA, mineral concentrates, etc. The cultivation uses batch system with condition parameter as follows: temperature is at interval 25–30°C, light intensity 3000–5000 lux, pH between 7.0–8.5, and aeration rate 3 L/min. Initial density is 10^5 CFU/mL and is incubated for 7–10 days until density reaches approximately 10^6 CFU/mL. **Figure 4** shows 1 L stock culture and 500 L scale-up culture for diatom *C. calcitrans*.

3.2.3. Heterotrophic bacteria

Heterotrophic bacteria can also uptake ammonium and nitrate as their nitrogen source [64]. The main advantage of adding particular heterotrophic bacteria is related to growth rate of heterotrophic bacteria that exhibit much faster than nitrifying bacteria [65]. Just like nitrifying

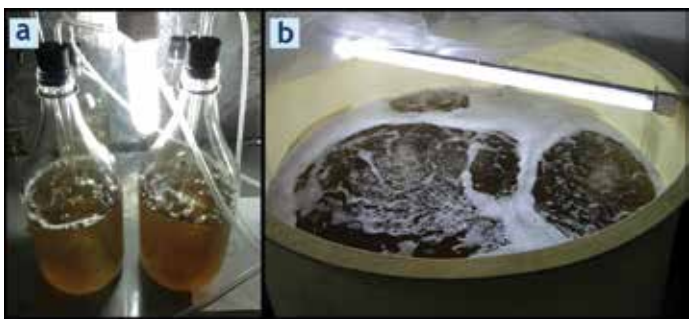

Figure 4. (a) Small scale and (b) large scale of *Chaetoceros calcitrans* in batch system.

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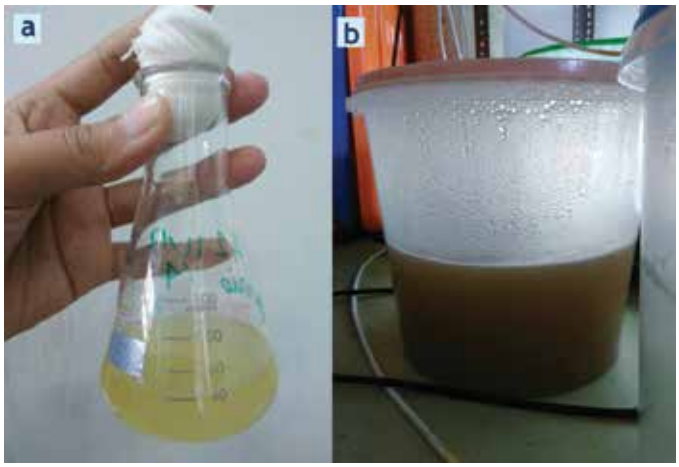

Figure 5. (a) Culture stock and (b) large scale cultivation of *Bacillus megaterium*.

bacteria, species of heterotrophic bacteria varies among different psychochemical conditions. Most common predominant species are *Bacillus megaterium* and *Bacillus flexus* [66]. *B. megaterium* is an example of well-studied bacteria for aquaculture application. The bacteria secretes high amount of extracellular enzymes, such as protease, carbohydrolase, and lipase, that can increase feed intake and digestibility in shrimp [67]. Other species that are also beneficial are *Shewanella algae* and *Halomonas aquamarina*. These natural-occurred bacteria have been studied and found to increase shrimp weight significantly [19].

Recent research progress proved that several heterotrophic bacteria genus (e.g. *Bacillus* sp. and *Pseudomonas* sp.) associated with microalgae through a mutual symbiosis. Although these heterotrophs have no direct role in controlling nitrogen cycle, their presence was proven to suppress growth of *Vibrio* spp. Mechanism of pathogenicity was done by quorum sensing [68]. This pathogen causes shrimp's mortality only when their population reaches a certain number of cells at least 10^6 CFU/mL [69]. Therefore, dominance of friendly heterotrophic bacteria can avoid pathogenic bacteria growth in the water, minimizing the risk of pathogen infection to the shrimp's gut.

Medium for culturing heterotrophic bacteria is nutrient broth medium for stock culture, while commercial medium is made from beef broth and ammonium chloride for large-scale cultivation. Heterotrophic bacteria were cultivated up to 15 L and then incubated for 24 h or until they reached the cell density of 10^7 CFU/mL. **Figure 5** below shows heterotrophic bacteria *B. megaterium* that are cultivated for culture stock and large-scale reactor.

4. Conditioning of ZWD system

There are several steps in conditioning of ZWD system; they are (1) microbial maturation in culture animal tanks; (2) acclimatization and stocking of shrimp or prawn; (3) feeding management; and (4) microbial manipulation that will be described in detail below.

4.1. Microbial maturation in rearing tank

Rearing tank conditioning was started by adding limestone ($CaCO_3$) and gravel at the bottom of the tank as substrate for nitrifying bacteria and then fill with disinfected seawater or freshwater (depends on cultured animal habitat). Afterward, 2% v/v of nitrifying bacteria consisted of AOB, and nitrite NOB at 10^6 CFU/mL is inoculated in the tank. 0.3 gr NH_4Cl (approx. 1 ppm of NH_4^+) is added per tank as ammonium source. After NH_4^+ and NO_2^- concentration was about 0 ppm, NO_3^- concentration was rising. It shows that AOB and NOB activities to convert ammonium to nitrate works. About 2% v/v of microalgae, i.e., *C. calcitrans* at 10^6 cell/mL and 0.05% v/v of heterotrophic bacteria, i.e., *B. megaterium* at 10^7 CFU/mL are inoculated into each tank. After the water turns to brownish color, the tank was ready to be used.

4.2. Acclimatization and stocking shrimp and prawn

After microbial maturation, we entered to the next steps or we can acclimatize shrimp or prawn in the same time with tank maturation. White shrimp PL-10 was acclimatized at room temperature (25 ± 1°C), while prawn was acclimatized from PL-40. Afterward, white shrimp was usually stocked at intensive farming using stocking density of 400 ind/m^3 and prawn at stock 60 ind/m^2. White shrimp and prawn were usually conducted for 90 and 60 days culture period, respectively.

4.3. Feeding management

Feed management was done by creating an estimation of daily feed (blind feeding; **Table 2**). The amount of feed was determined according to the mean body weight, estimated survival rate, and feeding rate, where:

$$Daily\ Feed\ (gr) = SD \times ABW \times FR \times SR \tag{1}$$

With: SD is the stocking density (Ind/tank), ABW is the average weight of shrimp (gr), SR is survival (%), and FR is feeding rate.

The feed was placed on the feed tray (ancho) and then checked to gain daily information of feeding accuracy (**Table 3**).

Average body weight (gr)	Feeding rate (%)	Survival rate (%)	Feeding tray monitoring intervals (h)
<1	10.0	100	3.5
1–3	8.0	98	3.5
3–5	6.0	96	2.5
5–7	5.0	94	2
7–9	4.0	92	2

Table 2. Blind feeding in super intensive white shrimp cultivation at 25 ± 1°C.

Ancho	Uneaten feed				Results	Decision
	1	2	3	4		
A	0	0	0	0	4/4	Add more 5–10%
B	0	0	0	+	3/4	Sufficient
C	0	0	+	+	2/4	Subtracted 5%
D	0	+	+	+	1/4	Subtracted 10–15%
E	+	+	+	+	0/4	Subtracted 20–30%

Table 3. Strategy to measure feeding efficiency following common feeding procedures created by Shrimp Club Indonesia in Lampung [personal communication].

Total consumption of feed and shrimp condition can be monitored via ancho based on the remaining amount of feed in the ancho. Feeding frequency was four times a day, given at 09:00, 12:00, 16:00, and 21:00 [13].

4.4. Microbial manipulation

This stage explores the characteristic of ZWD system that is similar to green water technique. Microbial components consisted of nitrifying bacteria, microalgae, and heterotrophic bacteria are added into the system every 2 weeks. The additional of microbial components is to maintain water quality and to balance microbial cycles in the system. In addition, microbial components are also given into the tank, if the ammonium concentration significantly rises. Similar to maturation step, nitrifying bacteria, microalgae, and heterotrophic bacteria were also added in the same volume to maintain the system performance.

5. Monitoring ZWD system

Monitoring is to evaluate the performance of ZWD system. Some parameters in the ZWD system that must be monitored are water quality and the growth performance of shrimp or prawn.

5.1. Water quality parameter

Maintaining water quality is important in aquaculture system because water is habitat of aquatic animal so it should be monitored periodically. Factors that affect water quality are temperature, dissolved oxygen, pH, and inorganic nitrogen concentrations. Water quality parameters are divided into two groups: psychochemical and microbiological parameters.

5.1.1. Psychochemical parameters

5.1.1.1. Temperature

The optimum range of water temperature allows aquatic organisms to perform metabolism and growth. Temperature is an important water quality parameter, because it can affect the

amount of dissolved oxygen budget in water and increase the rate of chemical reaction. If temperature value exceeds the tolerance limit of the cultures animal, then it leads the animal die. A good temperature range for aquaculture is 25–32°C for the tropics [70]. The critical temperature for living water organisms ranges from 35 to 40°C. Various regions in Indonesia have average air temperature during the day between 12.8–38°C, and the difference depends on the elevation above sea level.

5.1.1.2. Dissolved oxygen (DO)

Dissolved oxygen is one of limiting factor for aquatic animals. Changes in oxygen concentration can have a direct effect to their respiration, if the oxygen is insufficient, the animal will be death. The number of organic compounds present in water body influences the amount of dissolved oxygen content. Organic compound is produced by microorganisms, consequently increasing biological oxygen demand (BOD), so that oxygen concentration is reduced in water body [71]. The minimum DO value that can be tolerated by crustacean is 4 mg/L, if less than that number, the shrimp will die. The recommended DO range for cultivation is 4–6 mg/L [44].

5.1.1.3. pH

pH is a value for expressing the concentration of hydrogen ions (H^+) in water. Water with a pH less than 4 and higher than 9.5 can cause death to living creatures and reduce aquatic productivity [71]. Water pH fluctuates with dissolved CO_2 and has an inverse relationship pattern; the higher the CO_2 content of the water, the pH will decrease and vice versa. This fluctuation will decrease when water contains $CaCO_3$ salt. The tolerance for aquatic life to pH depends on many factors including temperature, dissolved oxygen concentration, variations of differentiated anions and cations, species, and biota life cycle. The nonoptimal pH of water affects the growth and reproduction [72].

5.1.1.4. Inorganic nitrogen compounds

Inorganic nitrogen compounds are often found in water body in the form of ammonia, ammonium, nitrite, and nitrate. These compounds are strongly influenced by the oxygen content in water, when oxygen decreases, the ammonia formation increases. Naturally, the ammonia present in water is the result of animal metabolism and the decomposition of organic matter by bacteria. Ammonium tolerance for shrimp does not exceed from 3.95 ppm [9].

Another nitrogen form is nitrites. Naturally, such compounds are usually found in very little amount in water, because nitrite is unstable when there is oxygen. Other compounds are nitrate, which is the main nitrogen form in natural waters. Nitrate is one of the important compounds in the process of protein synthesis in animals and plants. The concentrations of nitrite and nitrate suggested in aquaculture were less than 25.7 ppm [73] and 200 ppm [22], respectively.

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5.1.2. Microbiological parameter

Aquatic microbes have an important role in aquatic ecosystems. These microbes can affect the health of aquatic animals and occupy key positions in the food chain by providing edible nutrient for the next higher trophic level of aquatic life. In addition, the microbes assist the biochemical reactions that recycle most of the elements in the aquatic environment as well as in the soil. The amount of microbes in the water depends on the amount of organic compounds present in water body that usually interprets in biological oxygen demand (BOD) and chemical oxygen demand (COD) index. Higher organic matter causes dissolved oxygen content to be smaller because microbes use oxygen to oxidize organic matter.

5.2. Growth performance of shrimp or prawn: biological parameters

Production performance is also evaluated by a number of biological parameters. However, the list describes only for most priority parameters; there are survival rate (SR), average daily growth (ADG), total biomass (Wt), and food conversion ratio (FCR). Here are the formulas to calculate important biological parameters.

a. Survival rate is a survival index of cultured animals in a cultivation process from the beginning of the animal stocked until the animal harvested. Survival was calculated using equation:

$$SR = \frac{Nt}{No} \times 100\% \tag{2}$$

Where, SR = survival rate (%), No = initial shrimp number (ind), Nt = final shrimp number (ind), t = culture period (day).

b. Average body weight represents the average of individual weight for the entire shrimp population, it can be done by measuring individual shrimp weight that the numbers follow statistical rule (n). ABW was calculated as follow:

$$ABW = \frac{\sum_{i=1}^{n} Wi}{n} \tag{3}$$

Where, ABW = average body weight (gr), Wi = body weight of the i-th shrimp (gr), n = number of shrimp or prawn measured (ind)

c. Average daily growth is average weight gained each day. ADG was calculated as follow:

$$ADG = \frac{Wt - Wo}{t} \tag{4}$$

Where, ADG = average daily growth (gr/day), Wt = total biomass at harvest (gr), Wo = total initial biomass at stocking (gr), t = culture period (day).

d. Total biomass is total weight of cultured animals at harvest. The total biomass was calculated as follows:

$$Wt = \sum_{i=1}^{n} Wi \tag{5}$$

Where, Wt = total biomass (gr), Wi = body weight of the i-th shrimp (gr)

e. Food conversion ratio (FCR) indicates a ratio of efficiency of feed, which is converted into animal body mass. FCR was calculated as follow:

$$FCR = \frac{Total\ feed\ given\ during\ culture\ period\ (kg)}{Total\ biomass\ (kg)} \tag{6}$$

6. ZWD performance in shrimp and prawn cultivation

Commodities used in this ZWD system were white shrimp (*L. vannamei*) and giant freshwater prawn (*M. rosenbergii* De Mann). The ZWD system was examined in pilot scale for both commodities, but the system was only applied in white shrimp at industrial scale. Here are the detailed explanations of each performance.

6.1. White shrimp cultivation

6.1.1. Pilot scale

Research on the performance of ZWD system on white shrimp nursery at pilot scale has been done [13]. The ZWD system was compared to batch system as control based on performances of water quality and biological parameters in the same stocking density (approx. 400 ind/m^3). Microbial components used were nitrifying bacteria consortia (*Nitrosomonas* sp. and *Nitrobacter* sp.) and microalgae *C. calcitrans*.

After 90 days culture period, all growth performance including average body weight, total biomass, survival rate, and specific growth rate was significantly higher in ZWD than those of batch systems (**Table 4**). In contrast, food conversion ratio in ZWD system was significantly lower than that of conventional culture system (1.27 and 4.10, respectively). Based on data, the FCR value of ZWD system was still tolerance range of shrimp (1.5–2.6).

Parameter	Batch	ZWD
Final ABW (g)	5.45 ± 0.28	8.24 ± 0.84*
SR (%)	27.22 ± 2.09	90.82 ± 2.5*
Total biomass (g)	160.48 ± 6.62	923.38 ± 42.15*
SGR (%)	7.24 ± 0.05	7.7 ± 0.11*
FCR	4.10 ± 0.66	1.27 ± 0.29*

*Significant difference $p < 0.05$.

Table 4. Biological performance during 90-day cultivation period [13].

Water quality during 90-day cultivation period was still in tolerance levels for white shrimp in both systems, as seen in **Table 5**. As a concern, threshold value of ammonium in ZWD systems was higher than batch system, it was 0.69 ppm compared to 0.59 ppm, but it was not significantly different ($p > 0.05$). High ammonium level was caused by higher feed input in the ZWD system, it was about 44% compared to the batch system. A large feed input would affect the increment of ammonium level in culture, but ZWD system has rapid ammonium breakdown capacity that was accomplished through microbial manipulation. Moreover, nitrite level also did not differ significantly in both systems. These levels suggested that the ammonium and nitrite breakdown capacity of ZWD systems are higher than batch system. In addition, better ammonium and nitrite breakdown capacity were shown through nitrate level that was higher in ZWD system after 90-day culture period. The nitrate level in ZWD system reached 42.9 mg/L, while in batch system, it was 14.17 mg/L.

Besides the benefits of nitrifying bacteria and microalgae in maintaining water quality, they also indirectly inhibit pathogenic bacteria *Vibrio* spp. growth. Microbiological assessment shown that excessive organic matter did not increase the number of *Vibrio* spp. Previous research reported that marine diatom, such as *C. calcitrans,* has the ability to secrete fatty acids and esters, antibacterial compounds which inhibit several heterotrophic bacteria growth, such as *Vibrio* spp. [74]. Another analysis of predominant bacteria found in shrimp cultivation using ZWD system reported that the water contained following species, based on the most dominant bacteria found *B. flexus, Geobacillus stearothermophilus*, five species of *Bacillus* sp., *Pseudomonas oleovorans, Pseudomonas peli,* and *Xenorhabdus nematophilus* [66]. *Bacillus* sp. known as probiotic bacteria suggested that ZWD system not only achieved acceptable psychochemical parameters, but also microbiological parameters support shrimp growth as well.

6.1.2. Industrial scale

Research conducted for grow out white shrimp cultivation using ZWD system at industrial scale has been applied in UD. Populer, Gresik, East Java [50]. The research used PL-17 white shrimp as cultured animal at low salinity water (5 ppt) in ZWD system. Culture period was 70 days for three different stocking densities; there were 200, 300, and 400 ind/m^3 further referred as SD200, SD300, and SD400.

Parameter	Batch	ZWD	Tolerance range
Temperature (°C)	25.96–30.63		25–32°C for tropic area [70]
pH	7.63–8.80		4–9.5 [71]
DO (mg/L)	7.42 ± 0.52	6.81 ± 0.5	> 4 mg/L [44]
NH_4^+ (mg/L)	0.20–0.59	0.07–0.69	< 3.95 mg/L [9]
NO_2^- (mg/L)	0–3.20	0–3.15	< 25.7 mg/L [73]
NO_3^- (mg/L)	1.38–14.17	1.04–42.9	< 200 mg/L [22]

Table 5. Water quality measurement during 70-day culture period [13].

Based on the research, biological parameters have been documented as shown in **Table 6**. Interestingly, survival rate has a value inversely to total biomass. Treatment SD400 has the lowest survival rate of 70.59 ± 6.15% but has the highest total biomass, which is 44.13 ± 4.44 kg. It was because in higher stocking density has a higher stress level than lower density culture, such as space competition, higher cannibalism level, etc. If the total number of living individuals was calculated, SD400 has the largest number of about 280 individuals, and this number was larger than SD200 and SD300, which were 186 and 237 individuals, respectively. In addition, given feed was the highest proportion compared to other two treatments, so that nutrient sources were greater too, it can be seen from its size distribution, SD400 has a size of 100–150 ind/kg of 95.85%. Specific growth rate and FCR did not differ significantly. Based on the value of productivity, three treatments have reasonable value for cultivation conducted in Indonesia that was 1.72–2.0 kg /m^3 for 100–120 days of culture on stocking density of 60–300 ind/m^3.

For water quality parameters, all parameters had acceptable tolerance limits. Among all treatments, there was no significantly difference ($p > 0.05$). **Table 7** showed the measurements for water quality for 70 days of culture period.

6.2. Prawn cultivation

The performance of ZWD system on the nursery phase and grow-out phase of giant freshwater prawn (*M. rosenbergii* De Mann) was evaluated using addition of two types of shelters: three-dimensional cubical bamboo shelters (on nursery phase experiment) [51] and vertical textile shelters (on grow-out phase experiment) [11].

6.2.1. ZWD system and additional bamboo shelters

Research was conducted at laboratory scale using PL40 giant freshwater prawn taken from commercial hatchery in Sukamandi, West Java [51]. Microbial components used were nitrifying bacteria and *Chlorella* sp. that inhabit in freshwater. The research used ZWD system with addition of cubical bamboo shelter that has dimension of 0.6 x 0.6 x 0.2 m. Experimented

Parameters		**Stocking Densities (ind/m^3)**		
		200	**300**	**400**
SR (%)		93.52 ± 3.32^a	79.11 ± 5.81^b	70.59 ± 6.15^b
SGR (%)		4.64 ± 0.14^a	4.22 ± 0.24^a	4.40 ± 0.25^a
FCR		1.05 ± 0.07^a	1.06 ± 0.08^a	1.14 ± 0.14^a
Total biomass (kg)		27.7 ± 1.55^a	36.25 ± 3.01^b	44.13 ± 4.44^c
Size distribution (%)	100–150 ind/kg	86.77^a	91.93^{ab}	95.85^b
	150–250 ind/kg	13.23^a	8.07^{ab}	4.15^b
Productivity (kg/m^3)		1.39	1.81	2.21

Note: Means of values with same superscript along rows are significantly different ($p < 0.05$).

Table 6. Biological parameter measurement during 70-day culture period [50].

Parameter	SD200	SD300	SD400	Tolerance range
Temperature (°C)	29.3–30.1	29.8–30.1	30.4–30.9	25–32°C
pH	7.61–8.27	7.71–8.36	7.27–8.38	4–9.5
DO (mg/L)	4.90–8.50	5.00–8.00	5.60–7.80	> 4 mg/L
NH_4^+ (mg/L)	0–0.5	0–3.0	0–0.30	< 3.95 mg/L
NO_2^- (mg/L)	0–5.0	0.2–3.0	0.2–5.0	< 25.7 mg/L
NO_3^- (mg/L)	5.0–35.0	5.0–30.0	5.0–25.0	< 200 mg/L

Table 7. Water quality measurement during 70-day culture period [50].

treatments in the system were differences in the number of bamboo shelters installed in culture tank (2 x 1 x 0.4 m) and variation of stocking density. There were no shelter as control (C), two shelters (20% of water volume) (CB1), and four shelters (40% of water volume) (CB2) (**Figure 6**). Stocking density in sheltered tank was two times higher than control, and it was 60 ind/m^2. The cultivation was conducted for 28 days.

At the end of cultivation period, growth performance of prawns with shelters has better performance than control (no shelter). Treatment of CB2 produced final total biomass of 196 ± 0.09 gr, specific growth rate of 8.24% gr/day, final mean body weight of 2.17 ± 0.89 gr, and final mean body length of 6.50 + 0.91 cm. Shelter installment also resulted in a significant higher survival rate that was significantly different to control, as seen in **Table 8**. It was due to the availability of a larger territory area, vertically and horizontally, which will reduce contact possibility of prawns that was reared at high stocking densities, and at minimum contact with each other, cannibalistic behavior was suppressed. In addition, culture productivity increased with system using shelter, both CB1 and CB2 (30 and 39%, respectively). This suggests that shelters installation with the addition of microbial consortium improves prawn cultivation productivity.

Based on water quality parameters, ZWD system balances dissolved nitrogen concentration in water during cultivation period. pH value, dissolved oxygen (DO), ammonium nitrogen (NH_4^+),

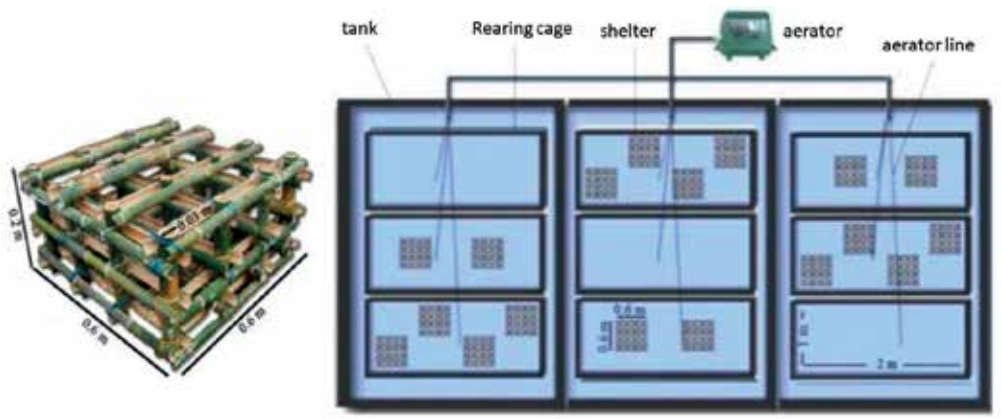

Figure 6. Cubical-bamboo used as shelters (left); cultivation scheme for prawn cultivation using bamboo shelters (right) [51].

Parameters	Control	CB1	CB2
Total biomass (gr)	141 ± 0.03^a	183 ± 0.05^b	196 ± 0.09^b
SGR (gr/day)	7.74	7.88	8.24
Survival rates (%)	77	90	92
Productivity increase compared to control (%)	—	30	39

Note: Different letters in the same column denote a significant difference ($p < 0.05$).

Table 8. Growth performance during 28-day cultivation period [51].

nitrite nitrogen (NO_2^-), and nitrate nitrogen (NO_3^-) remain stable during the culture period, keeping it in tolerance range of prawns (**Table 9**).

6.2.2. ZWD system and additional textile vertical substrate

Prawn (*M. rosenbergii* De Man) cultivation on the grow-out phase takes a period of 60 days, longer than nursery phase, which is only 14 days. For longer period of water quality maintenance, several inoculation of nitrifying bacteria suspension (10^5 CFU/mL) was needed. Prawn cultivation was conducted using five different stocking densities: 30, 40, 50, 60, 70 ind/m^2 into each pond (2 x 1 m^2) [11]. Shelter used in this research was textile vertical substrate that was placed in culture tank as seen in **Figure 7**.

Bodyweight, body length, specific growth rate, and survival rate were measured during 60-day cultivation (**Table 10**). As stocking density increases, prawn growth rate and survival rate decrease, due to competition of resources and risk of cannibalism. In final measurement, the highest mean bodyweight and body length were achieved by 70 ind/m^2 stocking density (11.46 ± 4.52 g and 10.70 ± 1.50 cm, respectively). Specific growth rate and survival rate from all treatments range between 1.393–2.569%/day and 67.1–76.3%, respectively. Overall, biological performance did not differ significantly among other stocking densities ($p > 0.05$).

From these results, the presence of a microbial component in rearing tank, mostly attached on solids, such as $CaCO_3$, and the textile vertical substrate surface directly improves water quality. In addition, textile vertical shelters reduce aggressive behavior of prawns due to secured spaces for each individual.

Parameters	Control	CB1	CB2
pH	7.47–8.45	7.40–8.07	7.13–7.96
DO (mg/L)	6.43–8.10	6.37–747	5.87–7.63
NH_4^+ (mg/L)	0.041–0.121	0.033–0.022	0.044–0.116
NO_2^- (mg/L)	0.011–0.156	0.012–0.237	0.011–0.210
NO_3^- (mg/L)	13.33–53.22	5.00–44.97	8.33–37.491

Table 9. Water quality parameter during 28-day cultivation period [51].

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Ammonium concentration during cultivation was maintained acceptable for prawn culture. On early stage of cultivation period, ammonium reached to almost undetectable level but gradually increased to 0.09–0.12 mg/L, observed in all treatments. The highest ammonium concentration (0.12 mg/L) was obtained in the highest stocking density (60 ind/m^2), followed respectively by the lower stocking densities (**Table 11**). Nitrite concentration during cultivation remained below the tolerance limit of freshwater prawn (1 mg/L). Nitrite concentration was gradually decreased during the cultivation from 0.064–0.066 mg/L to 0.015–0.032 mg/L in all treatments, showing nitrification process of conversion of nitrite to nitrate. Nitrate concentration remained stable between 53.9–71.0 mg/L in all treatments. Overall, DO levels during

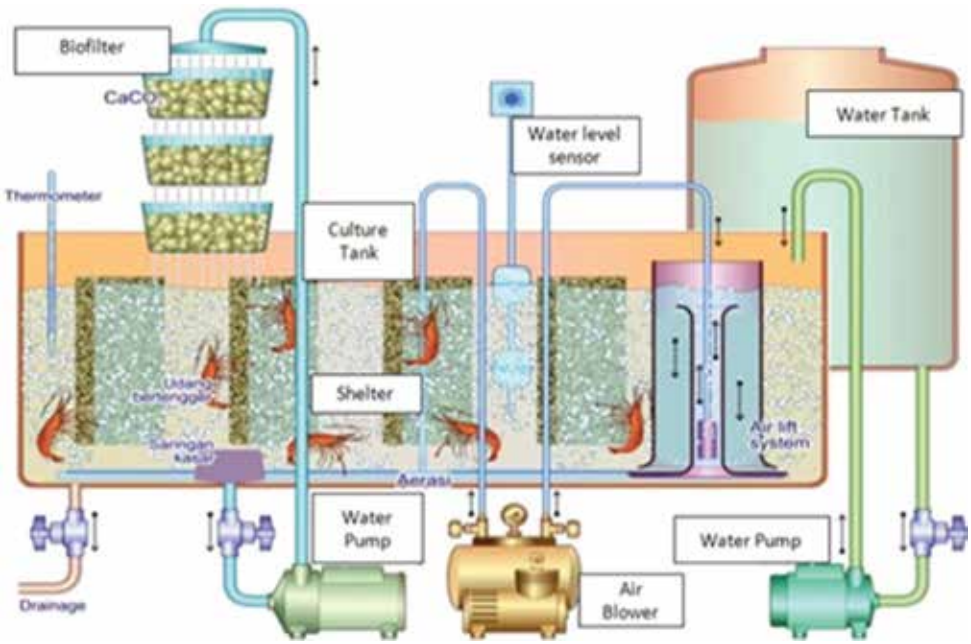

Figure 7. Cultivation scheme for prawn cultivation on grow-out phase using vertical textile shelters [11].

Parameters	Treatment (ind/m^2)				
	30	**40**	**50**	**60**	**70**
Mean body weight (g)	11.37 ± 4.96	9.34 ± 3.82	10.80 ± 5.62	10.98 ± 5.86	11.46 ± 4.52
SGR (% per day)	2.569	1.393	2.105	1.916	1.893
Survival rate (%)	78.3	76.3	70.0	70.0	67.1
Total biomass (g)	534.48	532.57	734.06	922.3	974.37
Total feed (g)	526.75	625.87	864.49	1080.5	1334.14
Feed conversion ratio (FCR)	0.99	1.18	1.18	1.17	1.37

Table 10. Prawn growth performance during 60-day cultivation period [11].

Parameters	Stocking densities (ind/m^2)				
	30	40	50	60	70
pH	6.74–8.42	6.74–8.15	6.31–7.91	6.42–7.95	6.52–7.96
DO (mg/L)	5.6–8.3	5.15–8.3	5.2–8.4	4.3–8.5	3.7–8.4
NH_4^+ (mg/L)	0.044–0.089	0.045–0.101	0.044–0.116	0.042–0.123	0.044–0.092
NO_2^- (mg/L)	0.015–0.066	0.017–0.064	0.021–0.066	0.027–0.066	0.032–0.065
NO_3^- (mg/L)	23.5–56.3	22.9–54.0	30.6–63.0	36.6–71.0	25.8–56.6

Table 11. Water quality parameters during 60-day cultivation period [11].

cultivation remained within tolerance range of prawn (min. DO is 4 mg/L) [44]. DO level decreased with response to increasing prawn growth, and the lowest level was 3.7 mg/L in 70 ind/m^2. pH level was relatively the same among all treatments, ranged between 7.71–7.96. From these parameters, it can be concluded that water quality during cultivation is suitable for the grow-out phase of prawns.

Based on microbiological parameters, there was a significant difference in microbial diversity between batch and ZWD system. In batch system, the water contained various species during cultivation period such as *Xenorhabdus japonica, Bacillus megaterium, Micrococcus luteus*, and *Bacillus amyloliquefaciens*, whereas in ZWD system, *B. megaterium* dominated from second week to the end of cultivation period. Batch system showed a fluctuation on bacterial dominance, which probably due to various abiotic and biotic factors, and therefore, tanks were prone to pathogen infection. In contrast with ZWD system, *B. megaterium*, which has been proven to benefits prawn growth, constantly dominated water during cultivation. Its dominance limited other bacteria domination, including pathogenic bacteria as well. Total pathogen bacteria particularly *Vibrio* spp. was counted. *Vibrio* spp. in rearing tank reached 10^0–10^3 CFU/mL during cultivation in both systems. Based on the results, *Vibrio* spp. abundancy below 10^6 CFU/mL was safe for prawn [69].

It was proven that the use of nitrifying bacteria can maintain good water quality, and textile vertical substrate can support a higher stocking density, better growth, larval survival rate, and profit of prawn *M. rosenbergii* de Man during the grow-out phase.

7. Technical and economic feasibility analysis

7.1. Technical feasibility analysis

Technical feasibility is an assessment of supporting factors in cultivation. It is concern in natural, social and cultural factors for successful cultivation [50]. The technical feasibility of parameters using ZWD system consists of site selection in terms of topography and structure, quantity and quality of water sources, accessibility, available of production facilities such as electricity source, seed producers, and distance to government research facility.

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Research in technical feasibility has been conducted in white shrimp culture using ZWD system at grow-out stage. Based on criteria, north coastal areas of East Java Province are suitable for white shrimp urban aquaculture using ZWD system, such as Tuban, Lamongan, Gresik, Sidoarjo, Pasuruan, Probolinggo, and Situbondo, etc. The sites were included in the following criteria:

- Geographically, the sites are adjacent to the sea, therefore easy to get seawater
- Environmental condition is suitable for shrimp growth such as temperature and humidity ranged between 22 and 34°C and 50–86%, respectively
- Land topography is included into lowland making it possible to construct shrimp farming
- The sites are close to domestic market in Surabaya as capital cities of East Java province and Lamongan that had many cold storage companies (19.8%) and fish processing units (23.7%) compared to total available units in Indonesia
- There is no social conflict of interest

7.2. Economic feasibility analysis

The economic feasibility was analyzed to calculate the overall cost and profitability from real implementation of ZWD system at industrial scale in Gresik (East Java, Indonesia) [50]. White shrimp juveniles were stocked on different stocking densities, providing that varying stocking densities can affect financial calculation, considering operational as well as investment expenses. Assumptions used were to produce 1000 kg shrimp/cycle during 10 years production period.

The results showed that the best biological feasibility was in stocking density at 400 ind/m^3. The lowest operational and investment cost was stocking density at 400 ind/m^3, because it needed the least area to produce 1000 kg/cycle. So, this economic feasibility takes the best performance in 400 ind/m^3. Based on calculation, operational cost consisted of shrimp seeds, feed, labor, electricity, seawater, algae and probiotics, chemical and disinfectants, harvesting, packaging and delivery, and depreciation costs that has the proportion as seen in **Figure 8**. The operation cost at 400 ind/m^3 reached Rp 44,227,125, while the highest component contributions to the investment costs were production ponds cost (36–42%) and land purchasing (21–24%) and total cost reached Rp 318,230,000.

Financial projections were calculated to predict the break event point. Profit could be calculated by subtracting the total revenue with production cost. Assumed that there were four production cycles per year, in which 1000 kg shrimps were produced per cycle with duration of 3 months, in 1 year, the farm would produce 4000 kg of fresh shrimp with total revenue Rp 240,000,000. The production cost in 400 ind/m^3 stocking density was achieved Rp 40,227,125 per cycle or Rp 160,908,500 per year and has the highest profit Rp 79,091,500 among all stocking density treatment. A total of 400 ind/m^3 stocking densities treatment will achieve a payback period after 4 years of operation.

To assess the economic feasibility of ZWD system, financial ratios were calculated. Financial ratio analyzed consisted of NPV, IRR, B/C ratio, and Pay Back Period (PBP). Based on financial

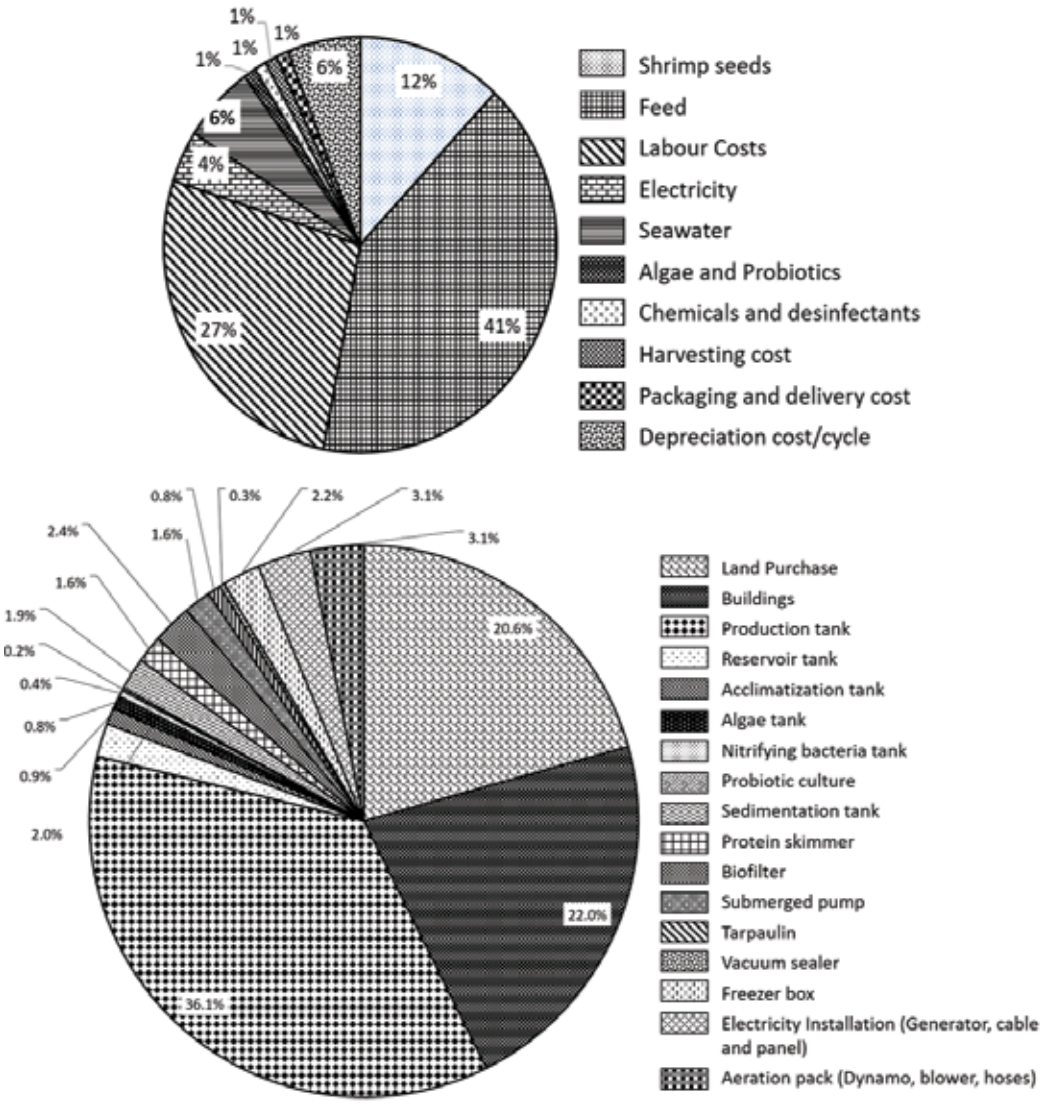

Figure 8. (above) Diagram of operational cost and (below) diagram of investment cost components (in percentages) at 400 ind/m^3 [50].

analysis, the project was financially feasible if the NPV is positive, IRR value is higher than discount factor, and B/C ratio value is higher than one [75]. The financial ratio calculations were presented in **Table 12**. It can be clearly seen that 400 ind/m^3 stocking density was financially feasible, because it has positive NPV (Rp 69,439,955), IRR value that was higher than discount factor (15.49%), and B/C ratio that was higher than 1 (1.22).

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Parameter	SD (400 ind/m^3)
Investment cost	Rp 318,230,000
Revenue	Rp 60,000,000
Production cost/cycle	Rp 44,227,125
Profit/cycle	Rp 15,722,875
Profit/kg shrimp	Rp 15,773
BEP (kg)	**2804**
Net present value (NPV)	**69,439,955**
B/C ratio	**1.22**
Pay back period (year)	**4.02**
Internal rate of return (IRR) (%)	**15.49**

Table 12. Financial ratio calculation of ZWD system at 400 ind/m^3 stocking density to produce 1000 kg shrimp/cycle using low salinity [50].

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The book is divided into two sections and represents the current trend of research in aquatic bioresource. In the section "Biology, Ecology and Physiological Chemistry", high-impact articles are contributed on reproduction, population genetics, evolution, biodiversity, biology and ecology of different aquatic faunas. Physiological chemistry of lipid, bioactive pharmaceuticals and chemical ecological aspects of aquatic organisms were discussed. In the section entitled "Conservation and Sustainable Management", authors highlighted conservation- and management-related issues of various bioresources in different regions of the earth. The book mentions the biological, ecological, physiological and genetic significance of aquatic organisms with resource potential. The authors stressed on rational utilisation and management of bioresource ensuring minimal damage of the aquatic ecosystem. This book would provide a direction towards sustainable ecological management of bioresource.

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