Mariana Fonseca Hinzmann

STUDIES ON IMMUNITY OF FRESHWATER BIVALVES (UNIONIDAE FAMILY): CHARACTERIZATION, ANTIBACTERIAL ACTIVITY AND APPLICATIONS AS BIOMARKER

Tese de Candidatura ao grau de Doutor em Ciências Biomédicas submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

Orientador – Doutor Jorge Pereira Machado
Categoria – Professor Associado
Afiliação – Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

Co-orientador – Doutor Manuel Vilanova
Categoria – Professor Associado
Afiliação – Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

Co-orientador – Doutor Jürgen Geist
Categoria – Professor Associado
Afiliação – Faculdade Ciências da Vida da Universidade Técnica de Munique

This work was partly funded by grants from the projects: PTDC/MAR/098066/2008, AMB/117688/2010 CONBI and PTDC/MAR/098066/2010 SYNERG, from the Portuguese Foundation for Science and Technology (FCT) and also by the projects NORTE-01-0145-FEDER-000035, supported by North Portugal Regional Operational Programme (NORTE 2020) and COST Action TD0903 both under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF).

The author received a PhD scholarship (SFRH/BD/76265/2011) from the Portuguese Foundation for Science and Technology (FCT) financed by European Social Funds (Programa Operacional Potencial Humano (POPH) and Programa Operacional Capital Humano (POCH)).

Dedicated to:

my daughter my biggest achievement and

my soul mate.

"*To remain indifferent to the challenges we face is indefensible. If the goal is noble, whether or not it is realized within our lifetime is largely irrelevant. What we must do therefore is to strive and persevere and never give up.*"

Dalai Lama XIV

Acknowledgments

My thanks go first to my supervisors Dr. Jorge Machado, who introduce me into the world of freshwater mussels, a journey that started almost 10 years ago and I hope to continue. I also what to acknowledge Dr. Manuel Vilanova for his wise advices, new approaches, suggestions and important knowledge transmitted in the immunology field and Dr. Juergen Geist for his unconditional support and for the exceptional work done in ecology and conservation of this endangered faunistic group, all were fundamental in the development of this work.

I express my sincere gratitude to Manuel Lopes Lima for his help these past years, he was a co-mentor for this project, and I hope to continue to work with him in the future.

I express my deeply thanks and gratitude to Lucinda Bessa, Alexandra Correia and Sebastian Beggel, for their unconditional support.

I could not forget my field companions, big mussel's lovers: Dr. Amílcar Teixeira, Dr. Simone Varandas and Dr. Ronaldo Sousa, always very enthusiastic and dedicated to the preservation of these animals.

I would like to thanks to Dr. Paulo Martins da Costa for being always readily to collaborate and enriched my works with its microbiology expertise and to Dr. José Gonçalves, for providing the best conditions to keep my mussels and also for allowing me to grow as a teacher.

During my thesis, in different phases, I could always count with technical support from: Elsa Oliveira, Madalena Santos, José Andrade, Bruno Ramos and Madalena Rodrigues, that I here recognize and thank, for the valuable knowledge transmitted and friendly assistance. My laboratory friends and companions: Sandra Araújo, Filipa Antunes, Filipe Cerca, Anabela Lopes, Jessica Tajdari for supporting me technically, physically and mentally.

The students that I had the opportunity to guide, that help me to grow academically and as a person: Mónica Nogueira, Catarina Santos, Joana Lameiras and Vitor Bompastor.

I could not forget to acknowledge my friends and co-workers: Magda, Eloi, Susy, Sonia, Sara and Diana that helped to keep the mood animated, especially during lunch break, with always a pleasant talk, they were my relaxation pause.

To Zé my husband, who has accompanied me for the last sixteen years, for his unconditional support and dedication, without him this would not be possible. To Leonor, my beautiful daughter, that makes me laughs like no one and as the capacity to recharge me with just a smile.

To my mother, for being the first person to believe and encourage me to follow my heart, becoming anything that I wanted to. I dedicate this work also to Antonio, wherever you are, who always was a fan of my work.

To finish, I have to acknowledge my Leiria family, which has welcomed me as if they had known me forever: Isabel and Carolina, thank you so much for all the help, support and above all friendship.

I really hope that the work started here may proliferate with others and together we can gain tools to stop the devastation chain of extermination of naiades.

Legal details

I hereby declare that the author of this thesis participated actively in the creation and execution of the experimental work leading to the results here presented, as well as for their interpretation and writing for this manuscript and respective publications, with the collaboration of the remaining authors.

Published papers and in preparation:

In international index journals:

- Hinzmann, M.F., Lopes-Lima, M., Gonalves.J, Machado, J., 2013. Antiaggregant and toxic properties of different solutions on haemocytes of three freshwater bivalves. Toxicological & Environmental Chemistry 95 (5), 790-805.
- Antunes, F., Hinzmann, M., Lopes-Lima, M., Vaz-Pires, P., Ferreira, S., Domingues, B., Machado, M., Martins da Costa, P., 2014. Antibacterial effects of A*nodonta cygnea* fluids on *Escherichia coli* and enterococci multi-drug-resistant strains: environmental implications. Toxicological & Environmental Chemistry 96 (6), 880-889.
- Hinzmann, M.F., Lopes-Lima, M., Bobos, I., Ferreira, J., Domingues, B., Machado, J., 2014. Morphological and chemical characterization of mineral concretions in the freshwater bivalve *Anodonta cygnea* (Unionidae). Journal of Morphology 276 (1), 65–76.

Papers in preparation:

- Hinzmann, M., Beggel, S., Machado, J., Geist, J., 2016. Synergistic impact of acute ammonia exposure and temperature stress on the freshwater mussel *Unio pictorum.* Hydrobiologia (submitted).
- Hinzmann, M., Bessa, L., Teixeira, A., Martins-da-Costa, P., Machado, J., 2016. Antimicrobial activity of unionid mussels from the North of Portugal. Fish and Shellfish Immunity Journal (submitted).
- Hinzmann, M., Lopes-Lima, M., Cerca, F., Correia, A., Machado J., Vilanova, M., 2016. Morphological and cytometric characterization of haemocytes from freshwater bivalves: *Anodonta cygnea* and *Anodonta anatina* (in preparation).

Table of contents

List of Abbreviations

AMP	Antimicrobial peptides
BrdU	5-bromo-2'-deoxyuridine – marker to detect cell proliferation
CAT	catalase
CFU	Colony forming unit
Cg-Pro	proline-rich antimicrobial peptides from *Crassostrea gigas*
Cg-Def	defensin from *C. gigas*
Con A	carbohydrate-binding protein originally extracted from the jack-bean
DAPI	4',6-Diamidino-2-Phenylindole, Dihydrochloride
DNA	Deoxyribonucleic acid
FFAs	Free fatty acids
GSH	Reduced glutathione
HPA	*Helix pomatia* agglutinin
IUCN	International Union for Conservation of Nature and Natural Resources
L15	Medium Leibovitz with L-glutamine
M199	Medium 199, Earle's Salts
MTs	Metallothioneins
NEM	N-ethylmaleimide
NMs	Nanomaterials
PAHs	Polycyclic aromatic hydrocarbons
PBS	Phosphate buffer saline
PHA-L	Biotinylated *Phaseolus Vulgaris* Leucoagglutinin
PNA	Peanut agglutinin
proPo	prophenoloxidase activating system
RCA	*Ricinus communis* (castor bean) agglutinin
ROS	Reactive oxygen species
SEM	Scanning electron microscopy
SOD	superoxide dismutase
TEM	Transmission electron microscopy
FTIR	Fourier transform infrared spectroscopy
TRITC	Tetramethylrhodamine
TSB	Tryptic Soy Broth medium
WGA	Wheat germ agglutinin

Abstract

The Unionidae family, or commonly referred as freshwater pearly mussels or naiades, are one of the most impaired groups of the Animalia kingdom. Although it includes hundreds of different species all over the world, it is an unknown group for many, but for the minority that spend their lives studying these organisms, they are quite amazing and surrounded by many open questions. They are extremely interesting from a biological perspective; first, as important providers of aquatic ecosystem services; secondly, they require a host for larval development and dispersal; third, they are powerful ecotoxicological bioindicators; and were already used until exhaustion for commercial purposes (pearl and button industry).

Freshwater mussel's immunology is a field with great potential, since little is known. However, the lack of suitable culture media and cell markers greatly difficult the achievement of more functional knowledge, concerning the immune elements and understanding how the immune system operate. The aim of this thesis is to contribute with new strategies to evaluate the fitness of freshwater mussels, by demonstrating the important relationship between immunity and ecology for these organisms. Their capacity to adapt is closely link to the well-functioning of their immune system. The more we know about their immune system, better tools can be provided to preserve them.

The work presented here has a multidisciplinary approach integrating different scientific areas like immunology, cell biology, toxicology and microbiology. In this perspective, this work explores immune elements, like the blood cells (haemocytes), fluids (plasma) and micropearls, to stablish the best conditions to study them and explore their potential as complementary biomarkers when analysing the health status of the bivalves. From these studies stands out the potential source of new antimicrobial compounds, present in the plasma from these species, probably similar to the ones already identified in marine species.

Haemocytes are the cells responsible for the cellular immune response and were key elements in the different studies conducted. Their concentration, ratios, cell types and viability, vary greatly among organisms and under experimental conditions. Thus, they firstly were well characterized to become helpful elements in posterior studies. Besides the morphological features, the functional capacity to have affinity with specific lectins like WGA, found only for one cell type (granulocytes) in the two species studied here (*Anodonta cygnea* and *Anodonta anatina*) was a new characteristic found, important to distinguish between haemocytes.

Micropearls or calcium carbonate concretions, are a peculiarity to highlight when studying these animals, can easily be extracted and analysed by multiple techniques, involved in the biomineralization process and can also function as bioindicators of the habitat conditions, since many toxic compounds can be trapped inside, even their origin may be closely linked to the haemocytes.

Unionids are an important faunistic group. Their ecological relevance should be by itself important to promote their preservation. Nonetheless these animals may also be a source of new antimicrobial compounds, adding further interest to their protection. The antibacterial capacity of the fluids and haemocytes of several unionid species is shown here. Even if the elements involved are still unidentified, their action is proved with the inhibition of bacterial growth and biofilm formation, using human opportunistic gram negative bacteria: *Escherichia coli* and *Pseudomonas aeruginosa* and gram positive bacteria: *Staphylococcus aureus*.

Ecotoxicological assays give important information surrounding the threats that these animals are constantly being exposed to, and the implications in terms of behaviour, fitness and immune response. Although the results obtained in the combined ecotoxicologic and immunologic study were not very consolidated, however this multifactor approach is more realistic, these organisms tend to have a large plasticity among them, giving rise to inconclusive results, the effects noticed were not always in the same direction, but reflect imbalance instead of homeostasis.

The combination of immunological and ecological knowledge will thus greatly benefit on the measures to implement to deal with factors threatening the naiades.

Resumo

A família Unionoidae, vulgarmente designados como mexilhões ou bivalves de água doce ou naiades, são um dos grupos mais ameaçados do reino Animália. Embora inclua centenas de diferentes espécies em todo o mundo, é um grupo desconhecido para muitos, mas para a minoria que passa as suas vidas a estuda-los, eles são bastante surpreendentes e rodeados por muitas questões em aberto. Estes animais são de interesse prático, realçado devido ao seu ameaçado estado de conservação em todo o mundo. Eles também são extremamente interessantes do ponto de vista biológico; em primeiro lugar, como importantes fornecedores de serviços aos ecossistemas aquáticos; em segundo lugar, por exigirem um hospedeiro para o desenvolvimento larval e dispersão; terceiro, eles são poderosos bioindicadores ecotoxicológicos; além de já terem sido utilizados até a exaustão para fins comerciais (cultivo de pérolas e indústria dos botões).

A imunologia dos bivalves de água doce é um campo com grande potencial, uma vez que pouco se sabe nesta área. No entanto, a falta de adequados meios de cultura e marcadores celulares específicos, dificulta um conhecimento mais funcional, relativamente aos elementos imunológicos isoladamente e da compreensão integrada de como o sistema imunitário destes animais funciona. O objetivo deste trabalho é contribuir com novas estratégias para avaliar a condição física dos bivalves de água doce, demonstrando a importante relação entre a imunidade e ecologia para estes organismos. A sua capacidade de adaptação está estreitamente ligada ao bom funcionamento de seu sistema imunológico. Quanto melhor se conhecer o seu sistema imunológico, melhores ferramentas conservacionista podem ser estabelecidas para preservá-los.

Este trabalho tem uma abordagem multidisciplinar que integra diferentes áreas científicas, como a imunologia, biologia celular, toxicologia e microbiologia. Nesta perspetiva, este trabalho seleciona elementos do sistema imunológico, como as células do sangue (os hemócitos), respetivo fluido (plasma) e micropérolas, para tentar estabelecer as melhores condições para estudá-los e explorar seu potencial como biomarcadores, no sentido de avaliar o estado de saúde dos bivalves. Destes estudos destaca-se a possível existência de novos compostos com atividade antimicrobiana no plasma destes bivalves, fundamentais na resposta imunológica, que deverão ser semelhantes aos já identificados para espécies marinhas.

Os hemócitos são as células responsáveis pela resposta imune celular, e foram elementos-chave nos diferentes estudos realizados. A sua concentração na hemolinfa, proporções,

tipos celulares e viabilidade, variam muito entre os organismos e sob condições experimentais. Assim, em primeiro lugar foram bem caracterizados para tornarem-se elementos úteis em estudos posteriores. Além das características morfológicas, a capacidade funcional detetada através da afinidade com lectinas específicas, como WGA, encontrada apenas para um tipo de célula (granulócitos) nas duas espécies estudadas (*Anodonta cygnea* e *Anodonta anatina*), tratando-se de uma característica nova encontrada para distinguir hemócitos.

As micropérolas ou concreções de carbonato de cálcio são um elemento que se destaca quando se estudam estes animais, podem facilmente ser extraídas e analisadas por várias técnicas, envolvidas no processo de biomineralização, mas também podem funcionar como bioindicadores uma vez que as características de seu habitat ficam aprisionadas nos seu interior, deixando assim um rasto caso haja exposição a compostos tóxicos, a própria origem delas pode estar associada aos hemócitos.

Os Unionoida são um grupo faunístico importante, a sua relevância ecológica deve ser suficientemente elevada para promover a sua preservação. No entanto estes animais podem também ser uma fonte de novos compostos antimicrobianos, aumentando ainda mais o interesse para a sua proteção. A capacidade antibacteriana dos fluidos e hemócitos de várias espécies de Unionidae contra várias espécies bacterianas é aqui demostrada. Mesmo que os elementos envolvidos ainda não estejam identificados, a sua ação é aqui provada com os ensaios de inibição do crescimento bacteriano e formação de biofilme, utilizando bactérias gram-negativas oportunistas em humanos: *Escherichia coli* e *Pseudomonas aeruginosa* e bactérias gram-positivas: *Staphylococcus aureus*.

Os ensaios ecotoxicológicos são uma fonte de informação importante em torno das ameaças que estes animais enfrentam, cujas implicações vão desde o comportamento, o "*fitness*" e respetiva resposta imune. Embora os resultados obtidos do estudo combinado ecotoxicológico e imunológico não terem sido muito claros, a abordagem multifatorial é a mais realista, contudo estes organismos tendem a ter uma grande plasticidade entre eles, dando origem a resultados inconclusivos, apesar de os efeitos notados não seguirem sempre a mesma direção, refletem um desequilíbrio em vez de homeostasia.

A combinação do conhecimento imunológico e ecológico, pode contribuir muito sobre as medidas a implementar para lidar com os fatores que ameaçam as naiades.

Chapter 1

General Introduction

Statement of problem

Freshwater bivalve molluscs of the order Unionoida have shown severe declines during the last decades with many species now facing extinction, their extirpation will likely alter ecosystem functioning in many rivers (Bogan, 1993). Given the high biomass and the high original abundances, these species have important roles in particle processing, nutrient release, and sediment mixing. Freshwater mussels have a particular set of traits that render them especially vulnerable to habitat disturbance. These animals have extended life spans. Maturity is delayed (6-12 years of age), they have reduced ability of dispersal, poor juvenile survival, and long turnover times. Adults are sedentary filter-feeders that may remain in the same location for their entire life span (Bauer and Wächtler, 2012). The larvae (glochidia) are obligate parasites on the gills or fins of fishes. Nonetheless their environmental relevance, there is a lack of information concerning their complex biology, which is closely associated with their rapid declines (Geist, 2010; 2015; Lopes-Lima et al., 2016).

Pollution is generally regarded as one of the most important causes of aquatic ecosystems decay. Freshwater mussels assimilate and concentrate heavy metals, contaminants and whatever is in the water column, making them good indicator organisms to evaluate environmental pollution and stress (Bogan, 1993). There is a developing awareness that perceived increases in gross abnormalities and disease in fish and shellfish populations are linked with an escalation of aquatic pollution. Recent studies increasingly support links between environmental contaminants and non-infectious diseases in aquatic organisms. However, there is a scarcity of scientific data to support the hypothesis that contaminant induced suppression of the immune system leads to enhanced susceptibility to infectious disease agents in bivalves. Also, there is a number of invasive freshwater mussel species which currently expand their distribution ranges and which seem to be more tolerant to pollution. The ability to filter large quantities of water, together with the sedentary habitat, ensures that mussels are exposed to a wide range of potentially toxic pollutants, many of which they accumulate (Parry and Pipe, 2004). During feeding, bivalves may ingest bacteria of many species, which can provide a substantial proportion of carbon and nitrogen requirements in their diet. However, some bacterial species can be also pathogenic to immune suppressed animals. Species of *Vibrio* are an important cause of disease in cultured fish and juvenile shellfish, with reports of vibriosis in commercial operations referring almost to infections on juvenile bivalves, demostrating that the organisms become more resistant to infection as they grow up (Sindermann, 1990; Pruzzo et al., 2005; Ciacci et al., 2009; Canesi

et al., 2016c). Physiological responses of bivalve molluscs to environmental and biological alterations are mediated, in part, by cells, collectively referred as haemocytes, which circulate within an open vascular system and across the epithelia. Two fundamental classes of haemocytes have been identified, granulocytes and hyalinocytes (Cheng, 1981; Hine, 1999), this classification is not consensual. These cells are involved in many processes: internal defence, tissues repair, shell repair, transport and digestion of nutrients. Humoral factors such as agglutinins, ROS, antimicrobial peptides (AMP) and lysozymal enzymes play also a fundamental role in defence. So, an evaluation of critical functions of the immune status in bivalves may help us to determine the relationship between exposure to environmental contaminants (that can be of chemical or biological origin) and immunotoxicological effects (Blaise et al., 2002).

The freshwater mussels – Naiades or Unionoida

Ecology, distribution and conservation

Freshwater mussels include species belonging to the Order Unionoida and are known also as river mussels or naiades (associated to mythology term meaning water nymph), including species that can reach big length and weight (up to 20 cm and 500 g), live more than 100 years and present a complex life cycle (Bauer and Wächtler, 2012).

Naiades use to be very diverse and abundant, existing in all freshwater habitats, nowadays their occurrence is becoming more restricted and in smaller populations, a problem that repeats itself all over the world (Strayer, 2008; Lopes-Lima et al., 2016).

Most bivalves are predominantly sessile or sedentary and can be found in streams, rivers, lakes, ponds, and even artificial reservoirs or canals. They use their large muscled foot to burrow into the substrate or to move. By burrowing or attaching to the substrate, bivalves are able to maintain their position in high-flow environments, such as rivers and tidal zones. Freshwater mussels are well known for their long life expectancy: for example, the freshwater pearl mussel, *Margaritifera margaritifera* (Linnaeus, 1758), can live more than 100 years (Bauer, 1987), while most common species may reach ages around 10-15 years old.

The filter-feeding behaviour makes them key elements of the ecosystem, reducing plankton (phyto and zooplankton) and bacteria populations, increasing water clarity, and promoting better conditions for surrounding plants, invertebrates, fish, birds and humans populations.

In Portugal, only a dozen of freshwater bivalves species occur (Table 1 and Figure 1)(Reis, 2006), the new genetic approaches helped clarify some species, since morphologically they can be quite similar, being the Unionids the more studied and the ones possessing worse conservation status, from which one is already considered critically endangered, *Margaritifera magaritifera* (Young et al., 2001) and another *U. tumidiformis* as vulnerable (Reis, 2006), others are in threat situations, like *U. delphinus* or endangered like *Potomida littoralis,* even if the global status is not consider critical some populations in specific locations are in danger of disappearing. While these native species seem their diversity and populations decrease, invasion mussel species from a different Order (Veneroida) seem to proliferate easily. As an example, *Corbicula fluminea*, native from Asia, as a simpler life cycle and ecophysiologic advantages, is able to proliferate even in slightly estuarine environments, adapting quickly to new environments that conquers (Frances et al., 2013).

Table 1 - Species of freshwater bivalves with confirmed occurrences in Portugal (Reis, 2006), and conservation status by the IUCN.

		Species	IUCN Status
Order Unionoida	Superfamily Unionoidea	Family Unionidae - *Anodonta cygnea*	Least Concern (LC)
		Anodonta anatina	Least Concern (LC)
		Potomida littoralis	Endangered (EN)
		Unio delphinus	Near threatened (NT)
		Unio tumidiformis	Vulnerable (VU)
		Family Margaritiferidae - *Margaritifera margaritifera*	Critically Endangered (CR)
Order Veneroida		Family Sphaeriidae - *Pisisdium subtrucatum*	not yet been assessed
		Pisidium personatum	not yet been assessed
		Pisidium nitidum	not yet been assessed
		Pisisium milium	not yet been assessed
		Pisidium henslowanum	Least Concern (LC)
		Pisidium casertanum	Least Concern (LC)
		Pisidium amnicum	not yet been assessed
		Musculium lacustre	Least Concern (LC)
		Sphaerium corneum	Least Concern (LC)
		Family Corbiculidae - *Corbicula fluminea*	Least Concern (LC)
		Family Dreissenidae- *Dreissena polymorpha* (not recorded yet in Portugal)	Least Concern (LC)

Figure 1 – Portuguese species from the Order Unionoida, in the map of Portugal is represented thought a colour gradient the numbers of different species that can be found (from 1 to 4) (map adapted from Reis, 2006).

The list of reasons for the decline of unionids populations is large and continuously increasing, being the focus of several conservation studies (Bogan, 1993; Bauer and Wächtler, 2001; Young et al., 2001; Farris and Van Hassel, 2006; Bogan, 2008; Bauer and Wächtler, 2012; Smith et al., 2015). The occurrence of other freshwater mussels, like *Dreissena polymorpha* (zebra mussel) (Figure 1 left) and *Corbicula fluminea* (Figure 2 right), with invasion characteristics, are one of the biggest treats on native populations all over the world, causing not only ecologic impacts but also economic losses. *Corbicula fluminea* is already one of the main species occurring in the biggest Portuguese rivers (Douro, Tejo and Minho), reaching very high biomass changing the surrounded ecosystem (Sousa et al., 2008).

Figure 2 – Invasion freshwater mussels, on the left is the native mussel *Anodonta anatina* covered in the apical region by the invasion species *Dreissena polymorpha*, from river Danube, on the right are several specimens of *Corbicula fluminea*, from Minho River.

Zebra mussels infest native unionids, attaching to the outside shell, interfering with feeding mechanisms, growth and locomotion (Grizzle and Brunner, 2009).

Declines in Unionids, in mussel diversity and population size, result from an integrative action of diverse stressors (Table 2) that have been challenging them in the last century.

The Veneroida order also includes the Sphariidae family, with several native species of very small dimensions that occur in Portugal, but yet little studied, even through the Europe, their status as not been assessed.

Table 2 – Main stressor's directly affecting freshwater mussel's occurrence (adapted from Farris and Van Hassel, 2006).

Categories	Stressor
Infrastructure impacts	Bridges and roads Dams Drainage, irrigation, channelization Gravel and sand removal
Waterbody use	Navigation Sport and recreation Cooling and hydro energy production
Resource use	Commercial shelling Historic removal for pearls or button manufacture Cultured pearl industry Surveys to assess water body condition
Large-scale disturbance	Climate warming Flood and fire events Sedimentation from above Criminal intent or eco/bioterrorism
Responsibility and oversight	Resource damage assessment Agency obligation for species of concern Permitted discharges Site remediation Treatment of pests Registration of products Spill, dump, or exceedance response Control of nuisance and introduced species

In general terms anthropogenic factors lead the list of reasons that may be implicated in the decline of unionid bivalves, with the constructions of dams, alterations on streams, pollutions of variable sources and intense harvesting for commercial purposes (buttons and pearl industry). Together with abiotic alterations: climate changes, flood and dry events, physicochemical properties of the water (pH, temperature, composition) and biotic factors: disappearance of host fishes, alterations in landscape composition, introductions of new species with invasion, competitor or predation characteristics (fish, mussels and others)(Strayer et al., 2006; Strayer and Malcom, 2007b; a; Strayer, 2008; Sousa et al., 2012; Fritts et al., 2015) and even pathogens or parasites as inducer of diseases (Farris and Van Hassel, 2006).

The shell, more concretely the nacre (pearly white), deposited in the inner shell layer has gemmological and economical value, freshwater mussels used to be harvested for the buttons industry and also for the pearl industry (Cummings and Graf, 2015).

Globally is believed that 70% of native unionid species are under some special concern by the IUCN- red list and around 20 are already considered as extinct, making emergent the

application conservation measures and complete information about life history of the remaining species fundamental, in order to preserve them, in Europe the scenario is not different (Figure 3), concerning status of the native species and main treats.

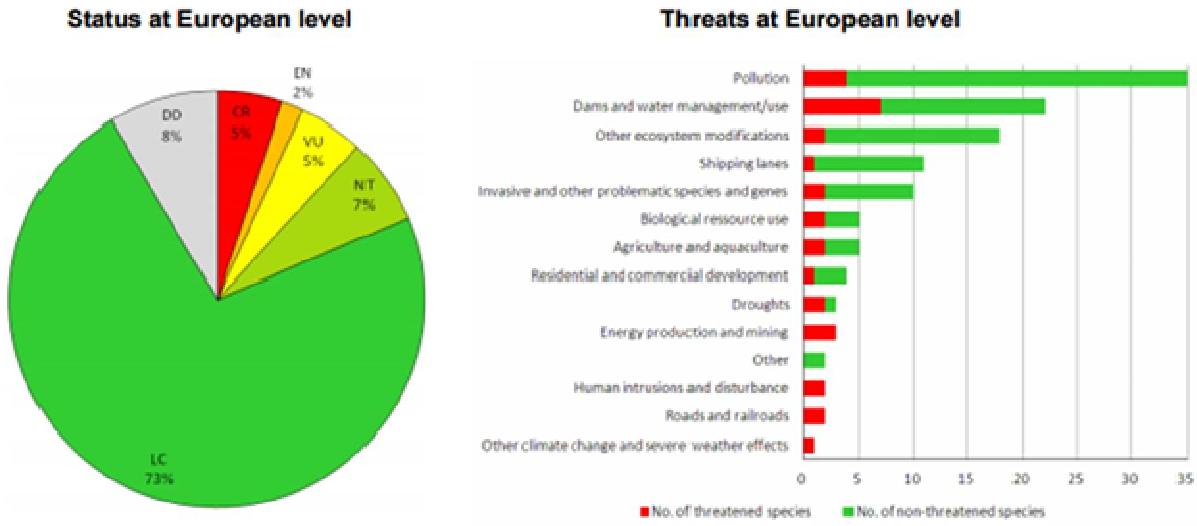


Figure 3 – Status by the IUCN – red list of European freshwater mussels (source: IUCN https://cmsdata.iucn.org/downloads/portugal_s_biodiversity_at_risk_fact_sheet_may_2013.pdf).

However, even if the environmental conditions are adequate for the occurrence of naiads, their mortality rate continues to be very high, due to constraints linked to their life cycle, mainly in the stage of host infection (Bauer, 2001; Bauer and Wächtler, 2001). During reproduction one million larvae are released, only 100 to 10 will successfully attach to the specific host and transit from the parasitic development stage to the juvenile, this transitional stage also carries a high mortality, associated with immune mechanisms from the host. The transition between juvenile to adult also results on a high decrease on individuals linked more to the environmental suitable conditions (Bauer, 2001). The adult has a much higher viability chance, the problem is reaching this stage and the environmental factors must be adequate.

The habitat challengers include natural predators like worms, crayfish, frogs and turtles of juveniles and smaller species; small mammals like muskrat, raccoon or the otter that can also predate adult mussels (Neves and Odom, 1989; Kopij, 2011; Bauer and Wächtler, 2012; Cummings and Graf, 2015). Parasites and pathogens are other sources of stressors that can diminish physiologic functions and initiate diseases.

The biology of freshwater mussels

Freshwater mussels are bivalves, the second biggest class of the phylum Mollusca. Their name – bivalves - comes from their bilateral symmetry and a shell formed by two valves, are characterised by a body compressed latterly, enclosed-valves shell, that are articulated dorsally though the hinge and ligament, the shape is usually elongate-ovate. The valves functioning are responsibility of the adductor muscles, attached in the inner surface of each valve (Brusca and Brusca 2003). When the muscle is relaxed the shell opens, when contracted or compressed, the shell closes. The well-functioning of this aperture/closed behaviour is a good indicator of the health status of the organisms, since has they become weak this capacity becomes looser.

The inner shell is almost fully coated by the mantle tissue, formed by two layers, the extrapallial layer in contact with the shell and the intrapallial layer in contact with the interior body, like a skin protecting the soft tissues that lays inside the mantle cavity, with functions closely associated with the shell formation (Machado et al., 1988; Coimbra et al., 1988), but also with respiratory and nutrition, since between these layers circulate the majority of their circulatory fluids (haemolymph and extrapallial fluid) (Figures 4 and 5). The cavity created serve also as a reservoir for fluids exchange and discharges via internal orifices (anus, nephridiopores, and gonopores), that are then carried away by the current of water generated by the gills (Cummings and Graf, 2015).

The siphons, an inhalant and exhalant, help water exchange mechanisms with their ciliated movement. Interiorly a pair of large gills (demibrachs or ctenidia), formed by two lamella, each made of several individual parallel tubular filaments that end up merging by junctions (Cummings and Graf, 2015). The gills besides being fundamental for the breathing and nutrition (filtrating the water and retaining the suspended particles) also function has marsupia, usually the outer gill that is also called marsupial gill, since during reproduction it will host the eggs during breeding and allow them to develop inside in the interior of the filaments, until the stage of glochidia, when fully mature are released into the water. Another function of the gills is storage of calcium micropearls fundamental for the biominerilazion, of the mother shell and also of the offspring.

Their bigger body portion is occupied by the foot, with a terminal part formed mainly by muscle tissue, very stiff to allow articulation and animal movement, and anterior part more soft filled with the organs associated with the reproductive system and digestive tract. The digestive system consists of a mouth, esophagus, stomach, intestine, and rectum, the food

particle enters the mouth assisted by the labial palps, located just near the adductor muscle (Cummings and Graf, 2015) (Figures 4 and 5).

The kidney is composed by a pair of nephridial ducts connected in a mesh and located above the pericardium in connection with the mantle cavity (Figures 4 and 5) (Brusca and Brusca, 2003).

Osmoregulation, is a big challenge for freshwater bivalves, the concentration of ions are much lower in the external medium than in the inside the tissues, the natural gradient is toward outside due to the hypotonic environment, this makes their body fluid with low ions concentration (low osmotic potential), however they possess mechanisms for scattering specials ions like calcium and sodium, by selective reabsorption and antigradient concentration, thought epithelium transport systems (Lopes-Lima et al., 2012; Cummings and Graf, 2015).

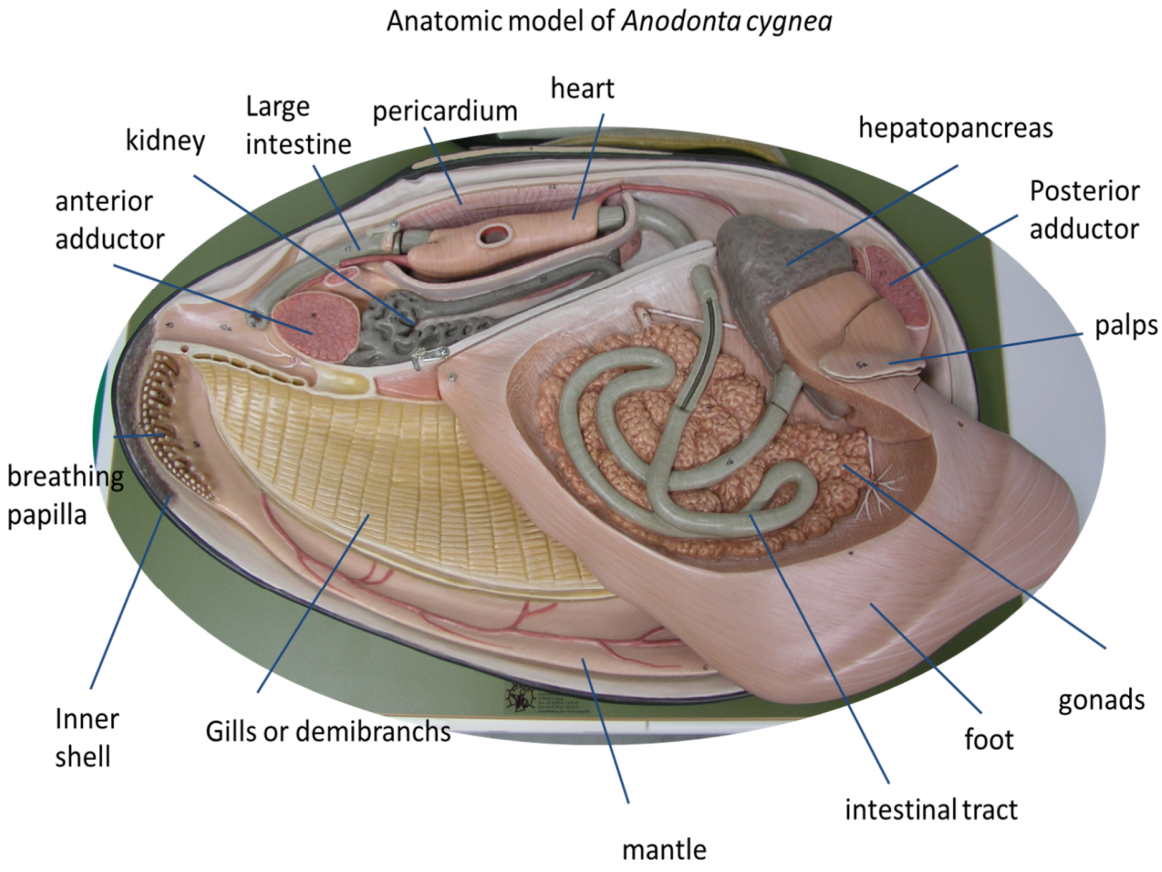


Figure 4 – Anatomic model of the freshwater mussel *Anodonta cygnea* (from SOMSO Mussel Model Zos 119).

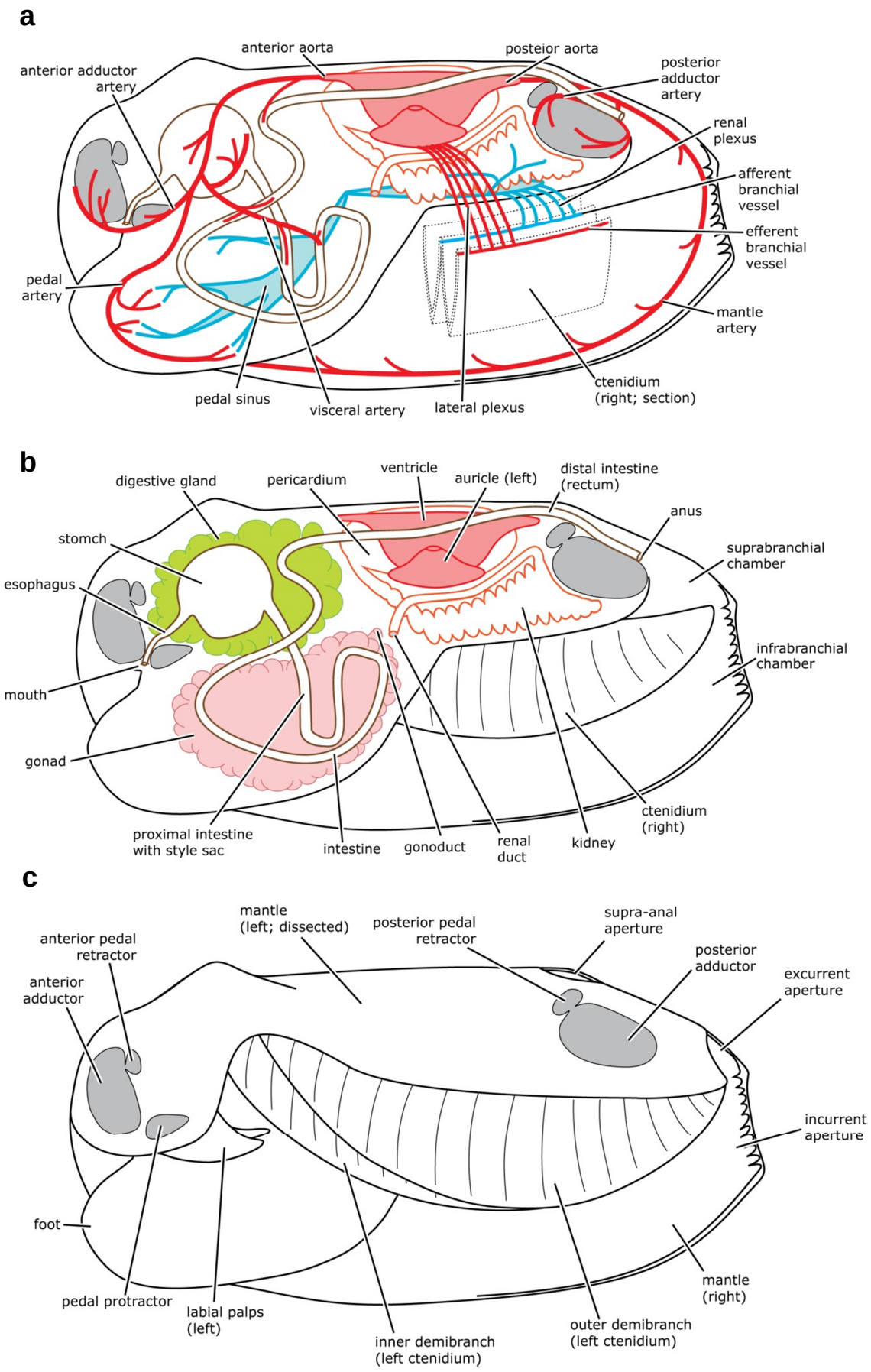


Figure 5 – Freshwater bivalves' anatomy: a – circulatory system, b – internal organs and c – external structures from (adapted from Cummings and Graf 2015).

The reproduction is one of the main aims of these naiades, to guarantee their continuity, and it starts as soon as the organisms reach the adult phase. The reproductive system, occupies a big portion of the foot, surrounding the digestive tract (Figures 4 and 5), the gonads are organized in clusters of acini, here the respective oocytes and spermatozoa maturate until being release to the gonoduct, and fertilization is internal. Freshwater mussels tend to be dioecius, although in some cases hermaphroditism has been observed (Heard, 1975; Grande et al., 2001; Hinzmann et al., 2013a; Cummings and Graf, 2015). The sexes can be determined macroscopically by the coloration of the gonads and size of the gametes (Cummings and Graf, 2015). Gametogenesis follows usually an annual cycle that can differ between species, depending on maturation of the individuals and environmental conditions (temperature, food availability, and photoperiod). However mature gametes, sperm and oocytes are only produced in specific periods and for a shorter period, some weeks before spawning, and this can be of two types, according with the spawning period and time of brooding: bradytictia (long term brooding) and tachtictia (short term brooding)(Cummings and Graf, 2015).

What is unique in unionids is that they have a parasitic life stage during their life cycle (Figure 6). The fertilized eggs mature in the gill (marsupial gill) of females into a special larvae called glochidia, that when mature is released into the water column and using different strategies try to find its specific host fish, attaching into the fish gill and fins as a parasite, what can take between days to several weeks (from 10 days up to 10 months), until by metamorphosis reach the stage of juvenile, at this stage it can already live autonomous in the sediment like an adult mussel (Figure 6) (Kat, 1984; Bauer and Wächtler, 2012; Kovitvadhi et al., 2007; Cummings and Graf, 2015).

Glochidia present unique features that allow species identification, specific morphology and ornamentation. The size range can vary from 50–500 µm (Bauer and Wächtler, 2012), only one adductor muscle, already two calcareous valves, the shape can vary between oval to triangular and some present a characteristic hook to attach themselves to the host fish (Cummings and Graf, 2015).

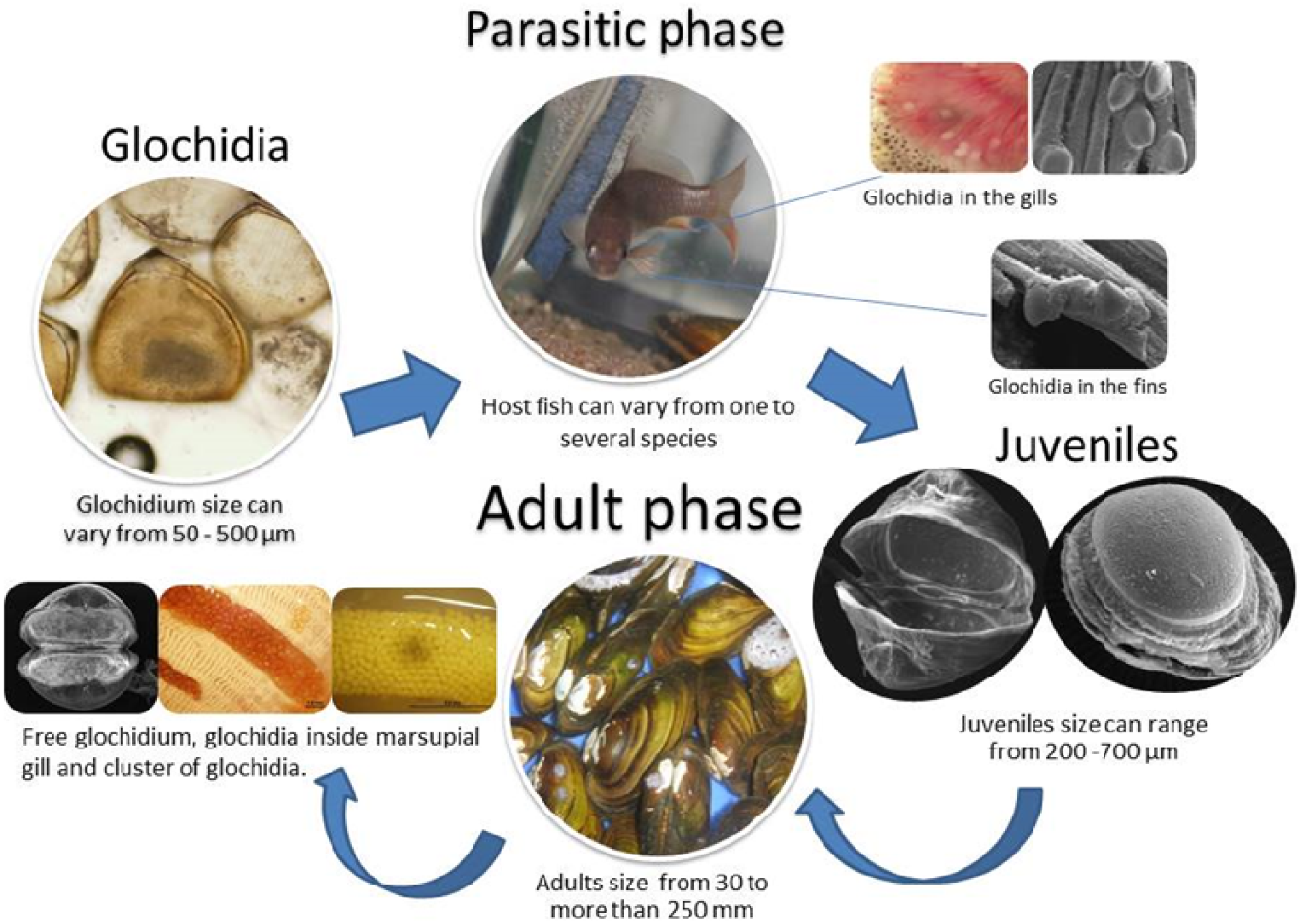


Figure 6 – Complete life cycle of naiads, before reaching the adult phase freshwater mussels must first pass a parasite stage in a host called glochidia, after released become juveniles, already similar to the adults but much smaller.

Bivalve Physiology – Immunity and circulatory system

All animals, independently of their complexity are born with their innate immune system, it was the first to appear during evolution, and for invertebrates is only one they have. Adaptive immune systems appeared 500 million years ago with the ancestors of cartilaginous fishes, and gave vertebrates other tools, producing receptors by recombining somatic genes, allowing fine specific recognition of antigens and also is the emergence of immunological memory. Nevertheless, the first line of defence always relies in innate immune mechanisms, which broadly recognize foreign organisms and trigger the response that can be mediated by the innate mechanisms or by adaptive immunity mediated by B- or T-cells (Canesi et al., 2006; Jiravanichpaisal et al., 2006; Canesi and Procházková, 2014; Canesi et al., 2016a).

Invertebrates are a highly successful group, making 97% of the animal kingdom, that inhabit all habitats, presenting a great diversity of shapes, symmetries, locomotion, skeletons, feeding mechanisms, so innate immunity is not synonym of maladjustment or weakness.

The study of bivalve immunity has developed rather recently due to the mass diseases attacking marine species; however in terms of freshwater mussels the knowledge is still very poor.

The first immune barrier that bivalves have is their own shell that protects them from bigger predators and gives shelter, is composed mainly by calcium carbonate ($CaCO_3$) in a structured mineral arrangement that is continuously being deposited following the bivalve growth (Cummings and Graf, 2015).

The second barrier is the mucus that moist all the interior surface and in terms of internal organization bivalves possess what is called an open circulatory system, in which their blood - the haemolymph, pass through the open ends of arteries, moistens all the organs before returning to the hearth by the sinus and gills, its volume can reach up to 50% of total water volume (Cheng, 1981; McMahon and Bogan, 2001). The haemolymph cells - haemocytes, are the key elements implicate in the response against pathogens. Haemocytes are responsible for mechanisms such as phagocytosis and encapsulation in the fight with strange substances or particles (Song et al., 2010). Additionally in the plasma, are humoral factors also important in the defence response like: antimicrobial peptides, opsonins and enzymes.

The most complete revision work made on haemocytes of bivalves, by Cheng, dates from 1981, where their homeostatic functions are highlighted: wound repair, nutrient digestion and transport, excretion and cellular immunity. The haemolymph circulation is partially heart dependent. This organ receives it from the venous sinuses and pumps it out thought the arteries by its movement, composed by three chambers, one auricle and a ventricle at each side, all enclosed by a pericardium membrane, the intestine pass through the middle of the heart (Cheng, 1981).

The haemolymph flows from the auricles to the ventricle, which pumps it to the anterior aorta, this vessel divides into several arteries and supplies the organs (mantle, stomach, intestine, muscles and foot) (Figure 6) directly in a bath, these regions are called the sinuses. The sinuses, constitute the hemocoel, the internal cavity surrounding the muscles and body parts; the major sinuses are the renal sinus, surrounding the kidney near the heart, the visceral sinus, the adductor muscle sinus and the mantle sinus; functioning as respiratory exchange places and has also the function of support, giving rigor to the several body constituents (Gosling, 2008; Cummings and Graf, 2015). Haemolymph from the sinuses is then purified in the kidney and from there is oxygenated by the efferent branchial veins in the gills (Figure 7) (Cheng, 1981; Jones, 1983; Gosling, 2008). Contrarily to what occurs in other molluscs, freshwater bivalves carry the oxygen dissolved in the haemolymph without the help of respiratory pigments. This absence is compensated by the large respiratory surface of the gills and large volume of fluids (Cummings and Graf, 2015). The mantle is also used as additional oxygenation place, due to its large extension and being the biggest haemolymph deposit. In the haemolymph circulate the blood cells, the haemocytes, these cells are mobile and can be found out of the sinuses in other tissues (Gosling, 2008).

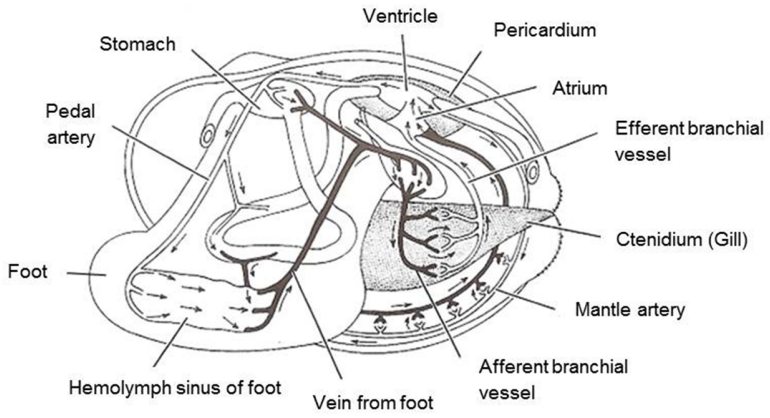


Figure 7 – Circulatory system of a bivalve (adapted from Brusca and Brusca, 2003).

The haemopoiesis (origin and formation) of haemocytes in bivalves is still unknown, in opposition to other Mollusca classes (gastropod and cephalopods) where specific regions and cells where already associated with this phenomena (Pila et al., 2016); even so, these cells are key immune elements, responsible for different functions such as infiltration, aggregation, cytotoxic reactions, wound repair, shell repair (early stages) and formation (Mount et al., 2004) phagocytosis, encapsulation or apoptosis (Gestal et al., 2008; Gosling, 2008). Haemocytes also support other systems: transporting oxygen and other metabolites, structural support for locomotion, mobilize calcium and excretion (Cummings and Graf, 2015). There is no specific organ to form them, it is believed that they arise from the differentiation of the connective cells (Cheng, 1981).

The cell classification is also controversial, the basic separation, being consensual, was made in granulocytes and agranulocytes (Cheng, 1981; Hine, 1999; Pila et al., 2016), with different morphologies and sizes. Further subdivisions are made according with the bivalve species and the techniques followed, but the subsequent divisions are based on this terminology. Based on dimensions haemocytes can also be divided in large cells which could be granulocytes or fibrocytes and in the smaller cells, dominantly agranular that were designated by hyalinocytes (Cheng, 1981).

The granulocytes, usually are the predominant population, characterised by the presence of several granules in the cytoplasm, with different shapes and composition, are associated the function of phagocytosis of bacteria, algae, cellular debris and protozoan parasites. These cells also have the capacity to move, active migration by projecting pseudopods (Cheng, 1981; Hine, 1999; Cummings and Graf, 2015). The ratio nucleus - cytoplasm (N/C) is low, usually are uninucleated and the nucleus has an eccentric position and centric to oval shape. The properties of these cells may vary between species, as well as the capacity to bind to specific lectins and the way of reaction in the presence of pathogens (Hine, 1999; Tame et al., 2015).

The agranular haemocytes, or hyalinocytes are usually less abundant with few or no granules inside; a lower phagocytic capacity is attributed to these cells, although in the literature they can be a quite heterogeneous group. The nucleus - cytoplasm ratio is high, with a larger, round, central nucleus and almost no cytoplasm, due to this characterization it is also attributed the name of blast-like cells. However bigger agranulocyte cells can be found, with a higher cytoplasmic content and a more eccentric nucleus (Hine, 1999). In terms of their function, these cells are believed to have a more enzymatic way of action in

the immune response (Hine, 1999; Soares-da-Silva et al., 2002), can migrate but without forming pseudopods, only small lobopodia.

Other different types of cells can also be found like the brown cells, which occur in molluscs prevenient from polluted waters that are associated with a detoxification role (Hine, 1999).

The classification problem gave rise to different nomenclatures, and revealed that the study of molluscan haemocytes based only in morphologic features is insufficient, even when resourcing to electron microscopy (Cheng, 1981), since the techniques used can by themselves alter the cell characteristics (size, granules, organelles), many fixation proceedings can cause degranulation of the haemocytes, or even the size change in relation to live cells (Cheng, 1981; Burkhard et al., 2009).

In terms of staining affinity granulocytes can be divided in acidophilic, basophilic or neutrophilic considering the properties of the cytoplasm granules, although these characteristic may reflect only the maturation stage of the cell, from basophilic to acidophilic.

There is a theory that postulates that each of these cell types has a precursor cell (Figure 8), in the case of the granulocytes it is the granuloblast and for the hyalinocytes is the hyalinoblast. Both follow a series of transformations, with different transitional stages, giving to the cells different characteristics before acquiring all mature functions (Figure 8) (Cheng, 1981). There is a third line of cells that will originate the brown cells.

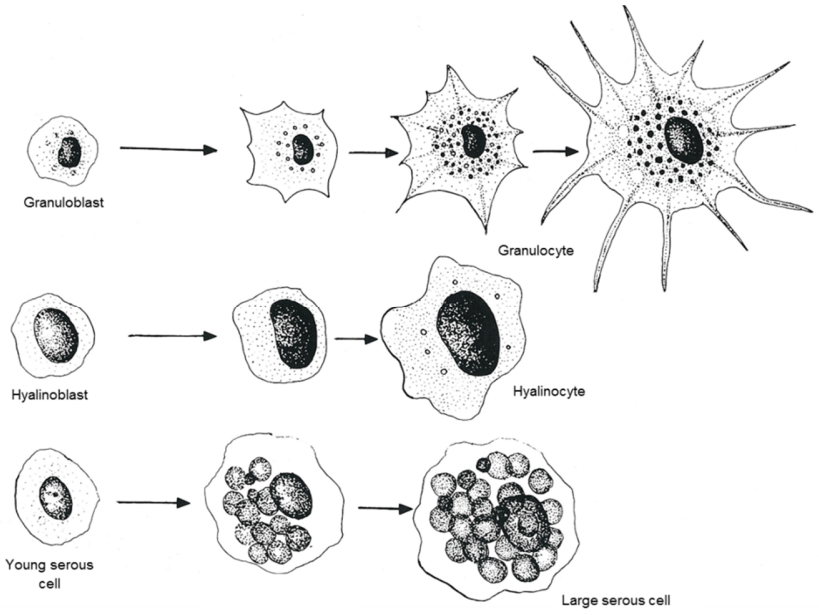


Figure 8 – Theoretical maturation process of haemocytes of bivalves proposed by Cheng 1981 (adapted from Cheng 1981).

Cellular immunity

Clotting

The first immune response is the clotting mechanism, to avoid haemolymph hemorrhage. The haemocytes have the capacity of clumping, avoiding loss of haemolymph, presenting a high tendency to aggregate using their filopodia, forming aggregates of thousands of cells very quickly (Cheng, 1981; Muta and Iwanaga, 1996). Clotting process is the basis of the wound repair mechanisms, initiated with the migration of the haemocytes to the affected area, followed by aggregation and filling up of the area. Then the healing process begins with the substitution of the cells from the affected region by new cells. Collagen is used has replacement medium until new cells are formed. In the end the haemocytes still have to phagocytose the necrotic cells and cellular debris (Cheng, 1981). This clumping process is easily observed after removing haemolymph for *in vitro* studies (Cummings and Graf, 2015).

The mechanism used by haemocytes for recognition of pathogens or foreign particles is unclear but the process itself is specific and follows an elaborated chain of events: nonself recognition, cell proliferation, locomotion, binding, ingestion and intracellular degradation (Gosling, 2008). The migration of the haemocytes is believed to be chemotaxis activated, peptides or small proteins secreted by the invader particle, that are detected and recognise by these cells as being foreign (Fawcett and Tripp, 1994). Several studies used the haemocytes density, morphology, phagocytic capacity and enzymatic activity associated to immune responses, as a good parameter to quantify an immunological response to pathological or toxic stress especially for marine bivalves (Pipe, 1990b; Canesi et al., 2002; Canesi et al., 2003; Lambert et al., 2003; Canesi et al., 2005; Pruzzo et al., 2005; Bigas et al., 2006; Canesi et al., 2015).

Phagocytosis

The process by which the phagocytic particle is internally eliminated can be mediated by the release of lysosomal enzymes or lysins, reactive oxygen species (ROS) – superoxide, hydrogen peroxide, oxygen or hydrogen radicals (Pipe, 1990b). Haemocytes also process antioxidant enzymes that protect against ROS and antimicrobial peptides. If the cell cannot manage to neutralize the particle an apoptotic chain may be triggered in order to control the invader (Glinski and Jarosz, 1997; Danilova, 2006; Gestal et al., 2008).

Encapsulation and nodulation

Encapsulation is the process by which the haemocytes migrate to parasitic infection places. As these organisms are too big to be phagocytise, a substance is secreted which surrounds the parasite, involving it in what resembles a capsule. An example is encapsulation observed in *T. semidecussatus* parasitized by *Perkinsus* sp. (Montes et al., 1995).

Apoptosis – altruistic death

Cells seem to have developed mechanisms of entrapment of pathogens inside the infected cell and follow a suicidal cascade of reactions to assure the survival of the others. The most common type of programmed cellular death is apoptosis (Danilova, 2006; Canesi et al., 2015).

Nacrezation

The capacity to form pearls or nacrezation (Cheng, 1981) is another amazing feature that these freshwater bivalves have. Whether they are micropearls (1-20 µm diameter) or pearls visible to the naked eye, they are triggered by the immune system. They are formed by deposition of nacre, that is, calcium carbonate, around a foreign particle, that can be a parasite, or toxic compound accumulated or just a sand grain that is irritating the mussel tissue. This process usually occurs in the mantle region, more active in this process, since it is attached to the shell. The depositions are made in layers, intercalating organic and inorganic matter (Cheng, 1981; Gosling, 2008; Cummings and Graf, 2015). The pearl formation is not yet completely understood, but in an early stage can be mediated by haemocytes, as a consequence of an immune response, since many small micropearls can be found inside haemocytes from several freshwater bivalves' species (personal data) (Figure 9) and also in marine mussels, particularly *Mytilidae* species.

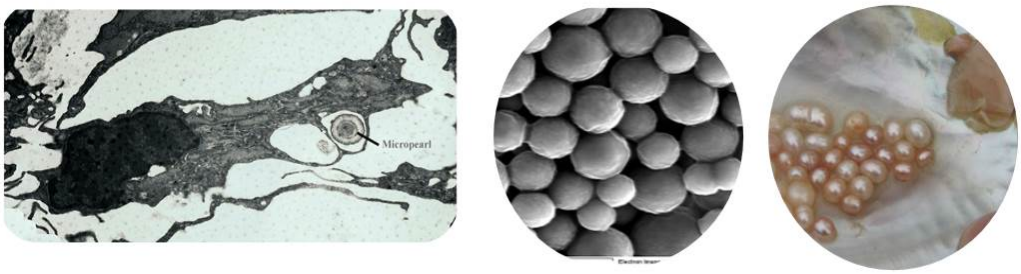


Figure 9 – Micropearls in the unionids can be found inside the haemocytes (first image), deposited in the gills (middle image), or as big as pearls associated to the shell and mantle (image in the right – source: http://molluskconservation.org/MUSSELS/Pearls.html referring to the Mikimoto experiments to culture pearls).

Other haemocytes functions

The haemocytes still have the function of nutrient digestion, linked to their respective transport (Cheng, 1981). Haemocytes also play a digestive role, by cleaving particles that are too big for the digestive gland cells, as indicated by the presence of lipases, esterases, carbohydrases, proteases and peroxidases, which occur, for instance, in the granules and plasma membrane of *Mytilus* sp.(Lopez et al., 1997). Reserve substances (glycogen and lipids) can also be stored inside haemocytes. The digested molecules can then be transported through the haemolymph to the various body parts, according to necessity (Cheng, 1981).

Excretion is also an important function in which haemocytes collaborate, being the brown cells the main intervenient neutralizing toxic compounds in globules that are then excreted though the kidney (Cheng, 1981).

Humoral immunity

All eukaryotic organisms have a diverse array of natural anti-infection agents; together they compose the humoral response that plays along with the cellular defence; assisting or activating it, these mechanisms need to be constantly adapted since new pathogens-host interactions are constantly being made, to safeguard survival (Danilova, 2006). Many substances may be involved: lysins, lectins, opsonins, agglutinins, antimicrobial factors and enzymes (Gosling, 2008).

Marine invertebrates have a rich variety of effective anti-infective molecules and strategies to respond to pathogens (bacteria and virus) that are already well documented. Some

englobe the more conventional antimicrobial molecules (peptides), but there are also other molecules involved like: histone, fatty acids, pigments, lectins, proteins and binding molecules (Smith et al., 2010).

Antimicrobial peptides (AMP)

Peptides with antimicrobial activity are fundamental in the defence against pathogens, targeting specific structures from the pathogens cellular membranes (due to their amphipathic design) or intracellular components (Danilova, 2006).
AMPs are usually peptides composed by small amino acids chains (12-50), located where they are more needed: periphery of the haemocytes or associated with the epithelial surfaces. They can be synthetized as precursors containing the active peptide, which is released by proteolytic cleavage of the anionic portion. Several AMPs from marine bivalves have already been isolated and identified: Big defensins from scallops, *Cg*-prp and *Cg*-Def in *Crassostrea* sp. oyster., defensins in oysters and *Mytilus* spp. and Myticins, Mytilins and Mytimycin from *Mytilus* spp. (Charlet et al., 1996; Mitta et al., 1999; Mitta et al., 2000a; Mitta et al., 2000b; Zhao et al., 2007; Gueguen et al., 2009; Li Cheng-Hua et al., 2009; Smith et al., 2010; Li et al., 2011).
Usually the structure of these AMPs is similar, a peptide chain rich in cysteine and disulphide bonds with strong antimicrobial activity against gram positive bacteria, gram negative bacteria and fungi (Li Cheng-Hua et al., 2009; Smith et al., 2010).

Metallothioneins (MTs)

Metallothionins are intracellular cysteine-rich proteins, with low molecular, associated with detoxification mechanisms in eukaryotic cells, due to its metal-binding proteins properties, can sequester metals dangerous for the cell, like cadmium (Klaassen et al., 1999; Chelomin et al., 2005), copper (Bienengraber et al., 1995), zinc and mercury (Viarengo et al., 1993), guaranteeing the metal homeostasis.

Other Proteins

Histones are nuclear proteins, present in all eukaryotes and are highly conserved among phyla, with other functions besides packing the DNA. Some have antibacterial and anti-infective activity, however the way of action is still poorly understood and little studied in bivalves (Smith et al., 2010).

Other intracellular proteins from marine animals have demonstrated antibacterial activity against pathogens.

Antioxidant Enzymes

Several enzymes participate directly or indirectly in the immune response, in mechanisms of prevention against toxins. The antioxidant system include in the mussels superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH), very efficient in the rapid elimination of reactive oxygen species (ROS) protecting the cell membranes from lipid peroxidation (Antognelli et al., 2006; Bigot et al., 2010).

Prophenoloxidase system (proPo)

The prophenoloxidase (proPo) cascade is responsible for the melanisation of pathogens and damaged tissues. Melanin diminishes or inhibits the pathogen proliferation and narrows the area of damage, crucial mechanisms in the immune response (Cerenius and Söderhäll, 2004; Cerenius et al., 2008). The proPO activating system is activated by the presence of compounds of microbial source, such as β-1,3-glucans, lipopolysaccharides, and peptidoglycans, which guarantee that the system is triggered in the presence of potential pathogens, (Cerenius and Söderhäll, 2004). This signaling pathway also facilitates other types of immune response like phagocytosis and was already identified in haemocytes and plasma of several mussels species like: *C. gigas*, *M. edulis*, *Saccostrea glomerata* and *Perna viridis* (Coles and Pipe, 1994; Asokan et al., 1997; Aladaileh et al., 2007; Hellio et al., 2007).

Lectins

Lectins are glycoproteins (sugar-binding proteins), soluble or membrane-bound factors associated to plasma, which recognize specific carbohydrate and bind reversibly to them, being involved in biological recognition. Lectins have an important role for invertebrates in the labelling foreign organisms, which are covered with different carbohydrate receptors. Lectins play agglutinating and opsonising roles, facilitating interaction of the surface of haemocytes with foreign particles, agglutinating supporting and immune processes like phagocytosis (Donaghy et al., 2009; Smith et al., 2010; Fujii et al., 2012; Fujii et al., 2013; Hasan et al., 2015; Adhya and Singha, 2016). Lectins in freshwater mussels are not known, but in marine mussel several were already identified, like the Mytilec from *Mytilus*

galloprovincialis (Fujii et al., 2012; Fujii et al., 2013; Hasan et al., 2015), a C-type lectin (CvML) in *Crassostrea virginica* (Jing et al., 2011). Other lectin identified was MeML for *M. edulis* mucocyte, which is believed to be involved in the food selection process (Pales Espinosa et al., 2010).

Other molecules

The cell membrane is rich in antimicrobial compounds, especially the free fatty acids (FFAs), bound to other groups. The unsaturated forms are potent inhibitors of several bacteria, inclusive human opportunistic like gram negative bacteria: *Escherichia coli* and *Pseudomonas aeruginosa* and gram positive bacteria: *Staphylococcus aureus* and *S. epidermidis* (Smith et al., 2010).

The immune response cascade

Many of these immune responses are haemolymph dependent: the presence of essential factors serologically active (opsonins, lysins, agglutinins, antimicrobial factors, lysozymes, lectins) and enzymes of lysosomal origin (aminopeptidases, β-glucuronidases, acid phosphatase, alkaline phosphatase, α-mannosidase, esterases, and peroxidases) that assist the haemocytes in the immune response (Wootton et al., 2003a; Gestal et al. 2008).The effect of pollutants on the immune response can be dual, besides trigger its own mechanisms to detoxification, can diminish other function like phagocytosis, depressing even more the immune system, an example is the effect of polycyclic aromatic hydrocarbons (PAHs) in *Mytilus edulis* haemocytes, which interferes with the lysosomes, key elements in the phagocytosis (Wootton et al., 2003b; Canesi et al., 2006). This structural alteration can be used as biomarker in pollutants exposure (Livingstone et al., 2000). Crude oil for instance has a negative effect on the production of ROS, fundamental in to eliminate pathogens and parasites by the haemocytes (Dyrynda et al., 1997), although the opposite effect can also occur in the presence of contaminants, together with the increase of density, rate, locomotion and superoxide formation by haemocytes (Fisher et al., 2000). The damage induced in the chromosomes is also a measure of the carcinogenic potential of toxic compounds (Mitchelmore et al., 1998; Bolognesi and Fenech, 2012), or by detecting nuclear abnormalities (Giannapas et al., 2012) or of immunomodulation detected by gene transcription (Astuya et al., 2015).

Diseases

Pathogens trigger the bivalve's immune system which may not be capable to give an effective response, leading to a disease scenario. In terms of diseases affecting bivalves much is known for the marine, commercial cultivated species, whether considering viruses, bacteria, fungi, protozoa, helminths or other parasitic invertebrates (Gosling, 2008), which can be the cause of mass mortalities. The malignant neoplasia that occurs in *Crassostrea* spp. and several other marine mussels is the focus of many studies due to its interest to the aquaculture industry (Carballal et al., 2015; Pila et al., 2016).

Freshwater mussels have the same vulnerability. The new sources of pollution (pathogens, toxics and nanoparticles) as well as the introduction of invading species which besides the negative impacts of competition with native ones, can also be a source of new diseases, causing severe health problems similar to the ones affecting marine molluscs (Grizzle and Brunner, 2009).

Virus

Viruses, the smallest agents known, yet with the highest infective capacity, multiply inside host cells, destroying them in the end. There are more than 20 virus identified that can cause disease in bivalves, but the link with specific diseases is still empirical, due to technical limitation concerning bivalve cell culturing techniques (Gosling, 2008; Grizzle and Brunner, 2009). The major alterations that can be attributed are at the level of the digestive tract, reproductive system, gills and in some extreme cases massive deaths (Gosling, 2008).

In freshwater bivalves only one viral disease was described, with the Chinese pearl mussel *Hyriopsis cumingii* in aquaculture, believed to be from the *Arenaviridae* family, causing lesions in the digestive tract. Common marine viruses are from the family *Birnaviridae*, which can also affect freshwater mussels, like *Corbicula fluminea*, includes virus known to cause pancreatic infections that also infect fish (Grizzle and Brunner, 2009).

Bacteria

Bacteria are abundant in the aquatic environment and bivalves due to their filter-feeding behaviour, incorporate many and different microorganisms. Although the filtration speed can vary among species according with anatomic differences, the zebra mussel *Dresseina polymorpha* is the more efficient, followed by the Asiatic clam *Corbicula fluminea* and only

then the other unionids (Grizzle and Brunner, 2009). The majority of the filtrated bacteria are harmless and serve as food, others especially when reaching high abundances can become pathogenic, for them or through the food chain, causing diseases to the bivalve's consumers, even humans, acting as carriers. The main stripes causing problems belong to the genus *Vibrio* and *Pseudomonas* (Gosling, 2008). The use of modern genomic methods can provide correct identification of bacteria species, so the real virulent impact can be associated to each species (Grizzle and Brunner, 2009).

Bivalves seem to share the capacity to select bacteria, this capacity can be size related. In terms of algae preference for feeding there is a clear selectivity, according to size and nutritional needs (Lopes-Lima et al., 2014). The particle is filtrated, but if it is undesirable there is a path to expel it, without entering in the digestive tract (Grizzle and Brunner, 2009).

Bivalves also have what is known as natural flora, weather this relation is symbiotic or accidental, is still unestablished. However, no bacteria are usually detected on the body surface. There is a layer of mucus that moist the epithelial surface and avoids the bacteria proliferation, while the organism is alive, on the other hand, inside the organs bacteria can be detect. The flora from healthy and moribund animals is not necessarily different, is more a balance problem (Grizzle and Brunner, 2009).

The capacity to eliminate bacteria like *Escherichia coli* and enterococci from the freshwater mussel *Anodonta cygnea* is undeniable (Antunes et al., 2010).

Bacteria aren't usually the main or sole cause of diseases. The accumulation with other stress factors, which lower the immune system, makes the organism more prone to contract infections. Major complications reside in aquaculture facilities and usually concern the early stages of the bivalve's life cycle. Antibiotics can help in many cases, but in others may favour the proliferations of others pathogens, and reduce bivalve resistance (Gosling, 2008).

Protists

Marine mollusks have several protist parasites: *Perkinsus* spp. (Apicomplexa), *Bonomia ostrae* (haplosporidium protozoa); *Haplosporidium* spp., *Mikrocytos mackini* (new taxonomic order, Mikrocytida) and *Marteilia* spp (Paramyxean organisms), responsible for mass damages to the bivalves and great economic losses in farm species (Gosling, 2008; Grizzle and Brunner, 2009).

Freshwater mussels usually are more affected by ciliates like *Conchophthirus* spp. (family Conchophthiridae), as symbionts present in all unionids, living on the fluids, gills, mantle and palps, or *Trichodina unionis* that is frequently found on the mantle of *Anodonta cygnea* and *Unio* spp., reaching 100% of a population and a density of 10 per host, other species of these genus are also frequent, occurring in the palps and gills, but no lesions are associated (Grizzle and Brunner, 2009; Cummings and Graf, 2015).

In the Trematode there are two subclasses affecting mussels, the Aspidogastrea, which included species that complete their life cycle within the mollusks and the Digenea, that use the mollusks as primary intermediate host and a vertebrate as final host. The local of infection can vary, mainly: the mantle cavity, pericardial cavity, kidney, intestine and gills (Gosling, 2008; Grizzle and Brunner, 2009; Cummings and Graf, 2015).

Digenea includes a higher number of mollusks parasitic species, there is more information about this class, since they have a vertebrate as their final host that can be the man. Although the intermediate stage in mollusks, sporocyst, is hard to identify to the species level without resource of genetic tools. *Bucephalus polymorphus* is a common parasite in *Dreissena* spp., but not frequent in other unionids, where *Rhipidocotyle* spp. occurs (Grizzle and Brunner, 2009). The consequence that this trematode can cause is infertility; the reproductive tissue is substituted by sporocysts and fibrosis, interfering with the life cycle. Other impacts can be in the growth, the higher the infestation, the less the mussels grow and the capacity the resist to other stress factors is diminished (Grizzle and Brunner, 2009).

Many parasites from this class that use unionids remain unidentified due to difficulties to establish their full life cycle and all host species.

Nematodes can be found as parasites in unionids intestines and in zebra mussel (Grizzle and Brunner, 2009). Oligocheta, leeches, mites, copepods also have some species that parasitize freshwater mussels (Cummings and Graf, 2015).

Nevertheless there is a global lack of information concerning the impact of these parasites in freshwater mussels.

Freshwater bivalves as bioindicators for ecotoxicology

Historically bivalves and also freshwater mussels have been largely used in ecotoxicological studies, for pollution monitoring in marine areas using marine species (Livingstone et al., 2000), but also freshwater ecosystems using unionid species (Farris and Van Hassel, 2006).

Bivalve molluscs are used as bioindicator species, working as sentinel organisms, and biomonitors of ecosystem health (Walker et al., 2001). Their worldwide dissemination associated with a sedentary lifestyle, filter-feeding behaviour and ability to accumulate pollutants (Frouin et al., 2007) in their tissues and pseudofaeces makes them important in the maintenance of the water quality and ideal species to use in ecotoxicity assays, though the marine species are more used due to their commercial importance (Wootton et al., 2003a; Bauer and Wächtler, 2012; Hartmann et al., 2015). However, when studding freshwater ecosystems, more affected by anthropogenic sources of pollution, freshwater mussels are preferred (Rocher et al., 2006). Primarily adult animals were used, more recently glochidia and juveniles (Milam et al., 2005; Wang et al., 2010; Fritts et al., 2014; Hartmann et al., 2016), since a pregnant female unionid can offer a great pool of glochidia and juveniles respectively, thus avoiding the handling of adults (Farris and Van Hassel, 2006; Wang et al., 2007; Wang et al., 2008).

The imperilled status of freshwater mussels can limit their usage, however a balanced use can be beneficial if adequate monitoring, surveys and sampling strategies are applied, further life history information are also fundamental to establish their nutritional requirements or even remediation impacts (Farris and Van Hassel, 2006).

Bivalves respond to an array of stressors when exposed to a supposedly simple environmental stressor, like a contaminant, there are always other interaction acting: biological, water, sediment, energetics, hydrodynamics or habitat geomorphology (Farris and Van Hassel, 2006), their impacts in the homeostasis have to be taken into account, otherwise can bias the study. Ecotoxicity studies end up working with biomarkers like mortality, behavioural, enzymatic reactions and contaminated tissue quantification (Farris and Van Hassel, 2006); little attention is given to the immunological impact.

The hypothesis raised by Faria et al. 2010 suggesting that naiades species might be more tolerant to pollution than exotic species is encouraging, though there are contradictory studies giving lower toxic sensitivity to invasion freshwater species (Oliveira et al., 2015).

Aims of this thesis

Freshwater mussels like all bivalves are constantly being exposed to something, either a physical, chemical or biological agent; their immune system must be fit to face all the challenges.

This thesis aimed at studying the immunity of freshwater mussels, assessing the main haemolymph intervenients (haemocytes and plasma), so they can be used as biomarkers in ecological, toxicological, pathological and physiological assays. It also aimed at testing the impact of the exposure organisms to different conditions on the immune system and fitness in relation to other more conventional parameters (behavior like filtration or physiological like quantification of enzymes or glycogen).

The hypothesis is that all stressors have a physiological impact, which may cause a direct immune response and these alterations are the first to manifest, even at low doses or exposure.

In order to achieve this general goal, several tasks focused primarily in the characterization and application of immune system components were carried out in order to be used in future studies.

Chapter one is an overview of naiades anatomy, immune system characterization and an integrative ecological perspective.

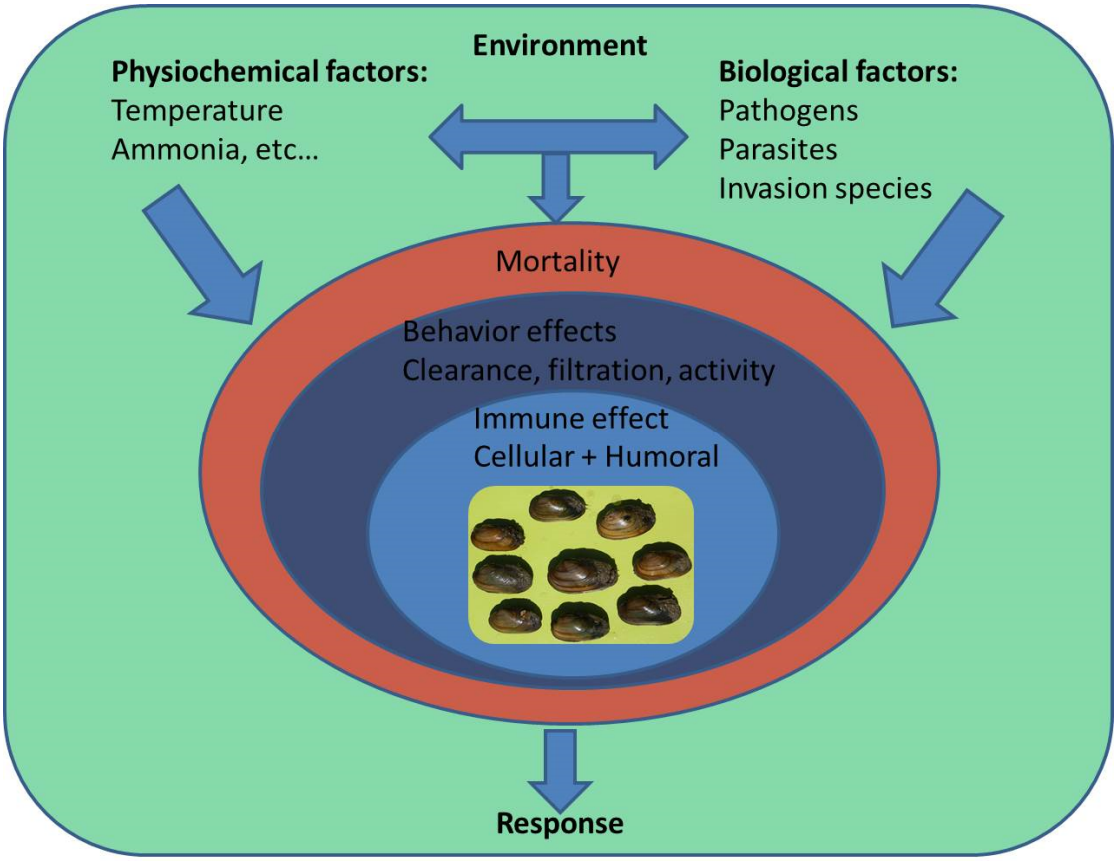
In the follow three chapters, a methodological perspective is given. Chapter two consists on an assessment of the best conditions to maintain the cells, with the selection of an appropriate antiaggregate solution, tested in three different species of freshwater mussels. Chapter three is a detailed characterization and comparative study of the haemocytes of two *Anodonta* species (*A. cygnea* and *A. anatina*), made by microscopy and flow cytometry. Besides cell differentiation and morphology, lectin affinity is determined. In chapter four the micropearls of *A. cygnea* are exhaustively characterized, using different techniques to show their structure, morphology and composition. Micropearls are fundamental structures, being reservoir components, to achieve growth and for the generation of the offspring shell, they can also incorporate all the elements in which the mussels have been exposed, having in this sense an important immunological function.

In the following three chapters different applications of the use of these immune elements are given. Chapter 5 is a combined study of the effect of chemical (ammonia) and physiological (temperature) parameters in the filtration behavior of the mussel *Unio*

pictorum, a common biomarkers used and immune parameters, highlighting this last ones to assess the health status of mussels.

In chapters 6 and 7 the combined effect of bacteria strains to the haemolymph, haemocytes and plasma is presented in two different perspectives. In chapter 6 the action of the bacteria on these elements and how they are affected, functional action of the haemocytes is detected (phagocytosis), besides inhibition of bacteria growth. In chapter 7 the antimicrobial potential of mussel's fluids is explored, the antibiofilm capacity of the plasma is determined, as well as some inhibition associated to the cells fractions, with bacteria known to be opportunistic to man.

Chapter 8 concludes the work with final remarks on all the topics addressed, introducing also some future perspectives presenting new approaches to study the immunity of freshwater mussels.



References

Adhya, M., Singha, B., 2016. Gal/GalNAc specific multiple lectins in marine bivalve Anadara granosa. Fish & Shellfish Immunology 50, 242-246.

Aladaileh, S., Nair, S.V., Birch, D., Raftos, D.A., 2007. Sydney rock oyster (*Saccostrea glomerata*) hemocytes: morphology and function. Journal of Invertebrate Pathology 96, 48-63.

Antognelli, C., Baldracchini, F., Frosini, R., Piazzoli, A., Talesa, V., Giovannini, E., 2006. Effects of exposure to sublethal copper concentration on glyoxalase system enzymes in specimens of *Scapharca inaequivalvis*. Biochemical Systematics and Ecology 34, 275-281.

Antunes, F., Hinzmann, M., Lopes-Lima, M., Machado, J., Martins da Costa, P., 2010. Association between environmental microbiota and indigenous bacteria found in hemolymph, extrapallial fluid and mucus of *Anodonta cygnea* (Linnaeus, 1758). Microbial Ecology 60, 304-309.

Asokan, R., Arumugam, M., Mullainadhan, P., 1997. Activation of prophenoloxidase in the plasma and haemocytes of the marine mussel *Perna viridis* Linnaeus. Developmental and Comparative Immunology 21, 1-12.

Astuya, A., Carrera, C., Ulloa, V., Aballay, A., Nunez-Acuna, G., Hegaret, H., Gallardo-Escarate, C., 2015. Saxitoxin Modulates Immunological Parameters and Gene Transcription in *Mytilus chilensis* Hemocytes. International Journal of Molecular Sciences 16, 15235-15250.

Bauer G., 1987. Reproductive Strategy of the Freshwater Pearl Mussel Margaritifera margaritifera. Journal of Animal Ecology 56, 691-704.

Bauer, G., 2001. Factors Affecting Naiad Occurrence and Abundance, in: Bauer, G., Wächtler, K. (Eds.), Ecology and Evolution of the Freshwater Mussels Unionoida, Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 155-162.

Bauer, G., Wächtler, K., 2001. Ecology and Evolution of the Naiads, in: Bauer, G., Wächtler, K. (Eds.), Ecology and Evolution of the Freshwater Mussels Unionoida, Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 383-388.

Bauer, G., Wächtler, K., 2012. Ecology and Evolution of the Freshwater Mussels Unionoida. Springer Berlin Heidelberg.

Bienengraber, M., Forderkunz, S., Klein, D., Summer, K.H., 1995. Determination of Cu-containing metallothionein: comparison of Ag saturation assay, thiomolybdate assay, and enzyme-linked immunosorbent assay. Analytical Biochemistry 228, 69-73.

Bigas, M., Durfort, M., Poquet, M., 2006. Cytological response of hemocytes in the European flat oyster, *Ostrea edulis*, experimentally exposed to mercury. Biometals 19, 659-673.

Bigot, A., Vasseur, P., Rodius, F., 2010. SOD and CAT cDNA cloning, and expression pattern of detoxification genes in the freshwater bivalve Unio tumidus transplanted into the Moselle river. Ecotoxicology 19, 369-376.

Blaise, C., Trottier, S., Gagne, F., Lallement, C., Hansen, P.D., 2002. Immunocompetence of bivalve hemocytes as evaluated by a miniaturized phagocytosis assay. Environmental Toxicology 17, 160-169.

Bogan, A.E., 1993. Fresh-Water Bivalve Extinctions (Mollusca, Unionoida) - a Search for Causes. American Zoologist 33, 599-609.

Bogan, A.E., 2008. Global diversity of freshwater mussels (Mollusca, Bivalvia) in freshwater. Hydrobiologia 595, 139-147.

Bolognesi, C., Fenech, M., 2012. Mussel micronucleus cytome assay. Nature Protocols 7, 1125-1137.

Brusca, R.C., Brusca, G.J., 2003. Invertebrates. Sinauer Associates.

Burkhard, M.J., Leavell, S., Weiss, R.B., Kuehnl, K., Valentine, H., Watters, G.T., Wolfe, B.A., 2009. Analysis and cytologic characterization of hemocytes from freshwater mussels (*Quadrula* sp.). Veterinary clinical pathology / American Society for Veterinary Clinical Pathology 38, 426-436.

Canesi, L., Betti, M., Ciacci, C., Lorusso, L.C., Gallo, G., Pruzzo, C., 2005. Interactions between Mytilus haemocytes and different strains of *Escherichia coli* and *Vibrio cholerae* O1 El Tor: role of kinase-mediated signalling. Cellular Microbiology 7, 667-674.

Canesi, L., Betti, M., Ciacci, C., Lorusso, L.C., Pruzzo, C., G., G., 2006. Cell signalling in the immune response of mussel hemocytes. Invertebrate Survival Journal 3.

Canesi, L., Ciacci, C., Balbi, T., 2016a. Invertebrate Models for Investigating the Impact of Nanomaterials on Innate Immunity: The Example of the Marine Mussel *Mytilus* spp. Current Bionanotechnology 02, 1-7.

Canesi, L., Ciacci, C., Bergami, E., Monopoli, M.P., Dawson, K.A., Papa, S., Canonico, B., Corsi, I., 2015. Evidence for immunomodulation and apoptotic processes induced by cationic polystyrene nanoparticles in the hemocytes of the marine bivalve *Mytilus*. Marine Environmental Research 111, 34-40.

Canesi, L., Ciacci, C., Betti, M., Scarpato, A., Citterio, B., Pruzzo, C., Gallo, G., 2003. Effects of PCB congeners on the immune function of *Mytilus* hemocytes: alterations of tyrosine kinase-mediated cell signaling. Aquatic Toxicology 63, 293-306.

Canesi, L., Gallo, G., Gavioli, M., Pruzzo, C., 2002. Bacteria-hemocyte interactions and phagocytosis in marine bivalves. Microscopy Research and Technique 57, 469-476.

Canesi, L., Grande, C., Pezzati, E., Balbi, T., Vezzulli, L., Pruzzo, C., 2016c. Killing of *Vibrio cholerae* and *Escherichia coli* Strains Carrying D-mannose-sensitive Ligands by *Mytilus* Hemocytes is Promoted by a Multifunctional Hemolymph Serum Protein. Microbial ecology. DOI 10.1007/s00248-016-0757-1.

Canesi, L., Procházková, P., 2014. The Invertebrate Immune System as a Model for Investigating the Environmental Impact of Nanoparticles. 91-112.

Carballal, M.J., Barber, B.J., Iglesias, D., Villalba, A., 2015. Neoplastic diseases of marine bivalves. Journal of Invertebrate Pathology 131, 83-106.

Cerenius, L., Lee, B.L., Soderhall, K., 2008. The proPO-system: pros and cons for its role in invertebrate immunity. Trends in Immunology 29, 263-271.

Cerenius, L., Söderhäll, K., 2004. The prophenoloxidase-activating system in invertebrates. Immunological Reviews 198, 116-126.

Charlet, M., Chernysh, S., Philippe, H., Hetru, C., Hoffmann, J.A., Bulet, P., 1996. Innate immunity - Isolation of several cysteine-rich antimicrobial peptides from the blood of a mollusc, *Mytilus edulis.* Journal of Biological Chemistry 271, 21808-21813.

Chelomin, V.P., Zakhartsev, M.V., Kurilenko, A.V., Belcheva, N.N., 2005. An *in vitro* study of the effect of reactive oxygen species on subcellular distribution of deposited cadmium in digestive gland of mussel *Crenomytilus grayanus*. Aquatic Toxicology (Amsterdam, Netherlands) 73, 181-189.

Cheng, T.C., 1981. Bivalves, in: Ratcliffe, N.A., A. F. Rowley (Eds.), Invertebrate Blood Cells Academic Press, New York., pp. 233-300.

Ciacci, C., Citterio, B., Betti, M., Canonico, B., Roch, P., Canesi, L., 2009. Functional differential immune responses of *Mytilus galloprovincialis* to bacterial challenge. Comparative biochemistry and physiology. Part B, Biochemistry & Molecular Biology 153, 365-371.

Coimbra, J., Machado, J., Fernandes, P.L., Ferreira, H.G., Ferreira, K.G., 1988. Electrophysiology of the mantle of *Anodonta cygnea*. Journal of Experimental Biology 140, 65-88.

Coles, J.A., Pipe, R.K., 1994. Phenoloxidase activity in the haemolymph and haemocytes of the marine mussel *Mytilus edulis*. Fish & shellfish immunology 4, 337-352.

Cummings, K.S., Graf, D.L., 2015. Chapter 19 - Class Bivalvia1 A2 - Rogers, James H. ThorpD. Christopher, Thorp and Covich's Freshwater Invertebrates (Fourth Edition), Academic Press, Boston, pp. 423-506.

Danilova, N., 2006. The evolution of immune mechanisms. Journal of experimental zoology. Part B, Molecular and Developmental Evolution 306, 496-520.

Donaghy, L., Lambert, C., Choi, K.S., Soudant, P., 2009. Hemocytes of the carpet shell clam (*Ruditapes decussatus*) and the Manila clam (Ruditapes philippinarum): Current knowledge and future prospects. Aquaculture 297, 10-24.

Dyrynda, E.A., Pipe, R.K., Ratcliffe, N.A., 1997. Sub-populations of haemocytes in the adult and developing marine mussel, *Mytilus edulis*, identified by use of monoclonal antibodies. Cell and Tissue Research 289, 527-536.

Faria, M., Lopez, M.A., Diez, S., Barata, C., 2010. Are native naiads more tolerant to pollution than exotic freshwater bivalve species? An hypothesis tested using physiological responses of three species transplanted to mercury contaminated sites in the Ebro River (NE, Spain). Chemosphere 81, 1218-1226.

Farris, J.L., Van Hassel, J.H., 2006. Freshwater Bivalve Ecotoxicology. Taylor & Francis.

Fawcett, L.B., Tripp, M.R., 1994. Chemotaxis of *Mercenaria mercenaria* Hemocytes to Bacteria *in vitro*. Journal of Invertebrate Pathology 63, 275-284.

Fisher, W.S., Oliver, L.M., Winstead, J.T., Long, E.R., 2000. A survey of oysters *Crassostrea virginica* from Tampa Bay, Florida: associations of internal defense measurements with contaminant burdens. Aquatic Toxicology 51, 115-138.

Frances, E.L., Lyubov, E.B., Alexander, Y.K., Sergey, E.M., David, T.Z., 2013. Zebra Mussel Impacts on Unionids, Quagga and Zebra Mussels, CRC Press, pp. 623-646.

Fritts, A.K., Barnhart, M.C., Bradley, M., Liu, N., Cope, W.G., Hammer, E., Bringolf, R.B., 2014. Assessment of toxicity test endpoints for freshwater mussel larvae (glochidia). Environmental Toxicology and Chemistry / SETAC 33, 199-207.

Fritts, A.K., Peterson, J.T., Hazelton, P.D., Bringolf, R.B., MacLatchey, D., 2015. Evaluation of methods for assessing physiological biomarkers of stress in freshwater mussels. Canadian Journal of Fisheries and Aquatic Sciences 72, 1450-1459.

Frouin, H., Pellerin, J., Fournier, M., Pelletier, E., Richard, P., Pichaud, N., Rouleau, C., Garnerot, F., 2007. Physiological effects of polycyclic aromatic hydrocarbons on soft-shell clam *Mya arenaria*. Aquatic Toxicology (Amsterdam, Netherlands) 82, 120-134.

Fujii, Y., Dohmae, N., Takio, K., Kawsar, S.M.A., Matsumoto, R., Hasan, I., Koide, Y., Kanaly, R.A., Yasumitsu, H., Ogawa, Y., Sugawara, S., Hosono, M., Nitta, K., Hamako, J., Matsui, T., Ozeki, Y., 2013. A lectin from the mussel *Mytilus galloprovincialis* has a highly novel primary structure and induces glycan-mediated cytotoxicity of globotriaosylceramide-expressing lymphoma cells. Journal of Biological Chemistry 288, 6588-6588.

Geist, J., 2010. Strategies for the conservation of endangered freshwater pearl mussels (*Margaritifera margaritifera* L.): a synthesis of Conservation Genetics and Ecology. Hydrobiologia 644, 69-88.

Geist, J., 2015. Seven steps towards improving freshwater conservation. Aquatic Conservation: Marine and Freshwater Ecosystems 25, 447-453.

Gestal, C., Roch, P., Renault, T., Pallavicini, A., Paillard, C., Novoa, B., Oubella, R., Venier, P., Figueras, A., 2008. Study of Diseases and the Immune System of Bivalves Using Molecular Biology and Genomics. Reviews in Fisheries Science 16, 133-156.

Giannapas, M., Karnis, L., Dailianis, S., 2012. Generation of free radicals in haemocytes of mussels after exposure to low molecular weight PAH components: immune activation, oxidative and genotoxic effects. Comparative biochemistry and physiology. Toxicology & Pharmacology 155, 182-189.

Glinski, Z., Jarosz, J., 1997. Molluscan immune defenses. Archivum Immunologiae et Therapiae Experimentalis 45, 149-155.

Gosling, E., 2008. Bivalve Molluscs: Biology, Ecology and Culture. Wiley.

Grande, C., Araujo, R., Ramos, M.A., 2001. The gonads of *Margaritifera auricularia* (Spengler, 1793) and *M. margaritifera* (Linnaeus, 1758) (Bivalvia : Unionoidae). Journal of Molluscan Studies 67, 27-35.

Grizzle, J.M., Brunner, C.J., 2009. Infectious Diseases of Freshwater Mussels and Other Freshwater Bivalve Mollusks. Reviews in Fisheries Science 17, 425-467.

Gueguen, Y., Bernard, R., Julie, F., Paulina, S., Delphine, D.-G., Franck, V., Philippe, B., Evelyne, B., 2009. Oyster hemocytes express a proline-rich peptide displaying synergistic antimicrobial activity with a defensin. Molecular Immunology 46, 516-522.

Hartmann, J.T., Beggel, S., Auerswald, K., Geist, J., 2016. Determination of the most suitable adhesive for tagging freshwater mussels and its use in an experimental study of filtration behaviour and biological rhythm. Journal of Molluscan Studies, 1-7.

Hartmann, J.T., Beggel, S., Auerswald, K., Stoeckle, B.C., Geist, J., 2015. Establishing mussel behavior as a biomarker in ecotoxicology. Aquatic Toxicology 70, 279-288.

Hasan, I., Sugawara, S., Fujii, Y., Koide, Y., Terada, D., Iimura, N., Fujiwara, T., Takahashi, K.G., Kojima, N., Rajia, S., Kawsar, S.M., Kanaly, R.A., Uchiyama, H., Hosono, M., Ogawa, Y., Fujita, H., Hamako, J., Matsui, T., Ozeki, Y., 2015. MytiLec, a Mussel R-Type Lectin, Interacts with Surface Glycan Gb3 on Burkitt's Lymphoma Cells to Trigger Apoptosis through Multiple Pathways. Marine Drugs 13, 7377-7389.

Heard, W., 1975. Sexuality and other aspects of reproduction in *Anodonta* (Pelecypoda: Unionidae). Malacologia 15, 81-103.

Hellio, C., Bado-Nilles, A., Gagnaire, B., Renault, T., Thomas-Guyon, H., 2007. Demonstration of a true phenoloxidase activity and activation of a ProPO cascade in Pacific oyster, *Crassostrea gigas* (Thunberg) *in vitro*. Fish & Shellfish Immunology 22, 433-440.

Hine, P.M., 1999. The inter-relationships of bivalve haemocytes. Fish & Shellfish Immunology 9, 367-385.

Hinzmann, M., Lopes-Lima, M., Teixeira, A., Varandas, S., Sousa, R., Lopes, A., Froufe, E., Machado, J., 2013a. Reproductive cycle and strategy of *Anodonta anatina* (L., 1758): notes on hermaphroditism. Journal of Experimental Zoology Part A, 319, 378-390.

Jing, X., Espinosa, E.P., Perrigault, M., Allam, B., 2011. Identification, molecular characterization and expression analysis of a mucosal C-type lectin in the eastern oyster, *Crassostrea virginica*. Fish & Shellfish Immunology 30, 851-858.

Jiravanichpaisal, P., Lee, B.L., Soderhall, K., 2006. Cell-mediated immunity in arthropods: hematopoiesis, coagulation, melanization and opsonization. Immunobiology 211, 213-236.

Jones, H.D., 1983. 4 - The Circulatory Systems of Gastropods and Bivalves A2 - Wilbur, A.S.M. Saleuddinkarl M, The Mollusca, Academic Press, San Diego, pp. 189-238.

Kat, P.W., 1984. Parasitism And The Unionacea (Bivalvia). Biological Reviews 59, 189-207.

Klaassen, C.D., Liu, J., Choudhuri, S., 1999. Metallothionein: an intracellular protein to protect against cadmium toxicity. Annual Review of Pharmacology and Toxicology 39, 267-294.

Kopij, G., 2011. The endangered swan mussel (*Anodonta cygnea*) (Linnaeus, 1758) is threatened by the common otter (*Lutra lutra*). Folia Malacologica 19, 191-192.

Kovitvadhi, S., Kovitvadhi, U., Sawangwong, P., Machado, J., 2007. Morphological development of the juvenile through to the adult in the freshwater pearl mussel, *Hyriopsis* (Limnoscapha) myersiana, under artificial culture. Invertebrate Reproduction & Development 50, 207-218.

Lambert, C., Soudant, P., Choquet, G., Paillard, C., 2003. Measurement of *Crassostrea gigas* hemocyte oxidative metabolism by flow cytometry and the inhibiting capacity of pathogenic vibrios. Fish & Shellfish Immunology 15, 225-240.

Li Cheng-Hua, Jian-Min, Z., Lin-Sheng, S., 2009. review of advances in research on marine molluscan antimicrobial peptides and their potential application in aquaculture. Molluscan Research 29, 17-26.

Li, H., Parisi, M.G., Parrinello, N., Cammarata, M., Roch, P., 2011. Molluscan antimicrobial peptides, a review from activity-based evidences to computer-assisted sequences. Invertebrate Survival Journal 8, 85-97.

Livingstone, D.R., Chipman, J.K., Lowe, D.M., Minier, C., Pipe, R.K., 2000. Development of biomarkers to detect the effects of organic pollution on aquatic invertebrates: recent molecular, genotoxic, cellular and immunological studies on the common mussel (*Mytilus edulis* L.) and other mytilids. International Journal of Environment and Pollution 13, 56-91.

Lopes-Lima, M., Freitas, S., Pereira, L., Gouveia, E., Hinzmann, M., Checa, A., Machado, J., 2012. Ionic regulation and shell mineralization in the bivalve *Anodonta cygnea* (swan mussel) following heavy-metal exposure. Canadian Journal of Zoology 90, 267-283.

Lopes-Lima, M., Lima, P., Hinzmann, M., Rocha, A., Machado, J., 2014. Selective feeding by *Anodonta cygnea* (Linnaeus, 1771): The effects of seasonal changes and nutritional demands. Limnologica - Ecology and Management of Inland Waters 44, 18-22.

Lopes-Lima, M., Sousa, R., Geist, J., Aldridge, D.C., Araujo, R., Bergengren, J., Bespalaya, Y., Bódis, E., Burlakova, L., Van Damme, D., Douda, K., Froufe, E., Georgiev, D., Gumpinger, C., Karatayev, A., Kebapçi, Ü., Killeen, I., Lajtner, J., Larsen, B.M., Lauceri, R., Legakis, A., Lois, S., Lundberg, S., Moorkens, E., Motte, G., Nagel, K.-O., Ondina, P., Outeiro, A., Paunovic, M., Prié, V., von Proschwitz, T., Riccardi, N., Rudzīte, M., Rudzītis, M., Scheder, C., Seddon, M., Şereflişan, H., Simić, V., Sokolova, S., Stoeckl, K., Taskinen, J., Teixeira, A., Thielen, F., Trichkova, T., Varandas, S., Vicentini, H., Zajac, K., Zajac, T., Zogaris, S., 2016. Conservation status of freshwater mussels in Europe: state of the art and future challenges. Biological Reviews, n/a-n/a.

Lopez, C., Carballal, M.J., Azevedo, C., Villalba, A., 1997. Morphological characterization of the hemocytes of the clam, *Ruditapes decussatus* (Mollusca: Bivalvia). Journal of Invertebrate Pathology 69, 51-57.

Machado, J., Castilho, F., Coimbra, J., Monteiro, E., Sa, C., Reis, M., 1988. Ultrastructural and Cytochemical Studies in the Mantle of *Anodonta cygnea*. Tissue & Cell 20, 797-807.

McMahon, R., Bogan, A.E., 2001. Mollusca: Bivalvia.

Milam, C.D., Farris, J.L., Dwyer, F.J., Hardesty, D.K., 2005. Acute toxicity of six freshwater mussel species (Glochidia) to six chemicals: implications for Daphnids and *Utterbackia imbecillis* as surrogates for protection of freshwater mussels (Unionidae). Archives of Environmental Contamination and Toxicology 48, 166-173.

Mitchelmore, C.L., Birmelin, C., Livingstone, D.R., Chipman, J.K., 1998. Detection of DNA Strand Breaks in Isolated Mussel (*Mytilus edulis* L.) Digestive Gland Cells Using the “Comet” Assay. Ecotoxicology and Environmental Safety 41, 51-58.

Mitta, G., Vandenbulcke, F., Hubert, F., Roch, P., 1999. Mussel defensins are synthesised and processed in granulocytes then released into the plasma after bacterial challenge. Journal of Cell Science 112, 4233-4242.

Mitta, G., Vandenbulcke, F., Hubert, F., Salzet, M., Roch, P., 2000a. Involvement of mytilins in mussel antimicrobial defense. Journal of Biological Chemistry 275, 12954-12962.

Mitta, G., Vandenbulcke, F., Roch, P., 2000b. Original involvement of antimicrobial peptides in mussel innate immunity. FEBS Letters 486, 185-190.

Montes, J., Durfort, M., García-Valero, J., 1995. Cellular defence mechanism of the clam *Tapes semidecussatus* against infection by the protozoan *Perkinsus* sp. Cell and Tissue Research 279, 529-538.

Mount, A.S., Wheeler, A.P., Paradkar, R.P., Snider, D., 2004. Hemocyte-mediated shell mineralization in the eastern oyster. Science 304, 297-300.

Muta, T., Iwanaga, S., 1996. The role of hemolymph coagulation in innate immunity. Current Opinion in Immunology 8, 41-47.

Neves, R.J., Odom, M.C., 1989. Muskrat Predation on Endangered Freshwater Mussels in Virginia. The Journal of Wildlife Management 53, 934-941.

Oliveira, P., Lopes-Lima, M., Machado, J., Guilhermino, L., 2015. Comparative sensitivity of European native (*Anodonta anatina)* and exotic (*Corbicula fluminea*) bivalves to mercury. Estuarine, Coastal and Shelf Science 167, 191-198.

Pales Espinosa, E., Perrigault, M., Allam, B., 2010. Identification and molecular characterization of a mucosal lectin (MeML) from the blue mussel *Mytilus edulis* and its potential role in particle capture. Comparative biochemistry and physiology. Part A, Molecular & Integrative Physiology 156, 495-501.

Parry, H.E., Pipe, R.K., 2004. Interactive effects of temperature and copper on immunocompetence and disease susceptibility in mussels (*Mytilus edulis*). Aquatic Toxicology (Amsterdam, Netherlands) 69, 311-325.

Pila, E.A., Sullivan, J.T., Wu, X.Z., Fang, J., Rudko, S.P., Gordy, M.A., Hanington, P.C., 2016. Haematopoiesis in molluscs: A review of haemocyte development and function in gastropods, cephalopods and bivalves. Developmental & Comparative Immunology 58, 119-128.

Pipe, R.K., 1990b. Hydrolytic enzymes associated with the granular haemocytes of the marine mussel *Mytilus edulis*. Histochemestry Journal 22, 595-603.

Pruzzo, C., Gallo, G., Canesi, L., 2005. Persistence of vibrios in marine bivalves: the role of interactions with haemolymph components. Environmental Microbiology 7, 761-772.

Reis, J., 2006. Atlas dos Bivalves de Água Doce de Portugal Continental. Lisboa ed. Instituto da Conservação da Natureza.

Rocher, B., Le Goff, J., Peluhet, L., Briand, M., Manduzio, H., Gallois, J., Devier, M.H., Geffard, O., Gricourt, L., Augagneur, S., Budzinski, H., Pottier, D., Andre, V., Lebailly, P., Cachot, J., 2006. Genotoxicant accumulation and cellular defence activation in bivalves chronically exposed to waterborne contaminants from the Seine River. Aquatic Toxicology 79, 65-77.

Sindermann, C.J., 1990. Principal Diseases of Marine Fish and Shellfish: Diseases of marine fish. Academic Press.

Smith, D.R., McRae, S.E., Augspurger, T., Ratcliffe, J.A., Nichols, R.B., Eads, C.B., Savidge, T., Bogan, A.E., 2015. Developing a conservation strategy to maximize persistence of an endangered freshwater mussel species while considering management effectiveness and cost. Freshwater Science, 000-000.

Smith, V.J., Desbois, A.P., Dyrynda, E.A., 2010. Conventional and unconventional antimicrobials from fish, marine invertebrates and micro-algae. Marine Drugs 8, 1213-1262.

Soares-da-Silva, I.M., Ribeiro, J., Valongo, C., Pinto, R., Vilanova, M., Bleher, R., Machado, J., 2002. Cytometric, morphologic and enzymatic characterisation of haemocytes in *Anodonta cygnea*. Comparative Biochemistry and Physiology A 132, 541-553.

Söderhäll, I., Bangyeekhun, E., Mayo, S., Söderhäll, K., 2003. Hemocyte production and maturation in an invertebrate animal; proliferation and gene expression in hematopoietic stem cells of Pacifastacus leniusculus. Developmental & Comparative Immunology 27, 661-672.

Song, L., Wang, L., Qiu, L., Zhang, H., 2010. Bivalve Immunity, in: Söderhäll, K. (Ed.), Invertebrate Immunity, Springer US, Boston, MA, pp. 44-65.

Sousa, R., Nogueira, A.J.A., Gaspar, M.B., Antunes, C., Guilhermino, L., 2008. Growth and extremely high production of the non-indigenous invasive species *Corbicula fluminea* (Müller, 1774): Possible implications for ecosystem functioning. Estuarine, Coastal and Shelf Science 80, 289-295.

Sousa, R., Varandas, S., Cortes, R., Teixeira, A., Lopes-Lima, M., Machado, J., Guilhermino, L., 2012. Massive die-offs of freshwater bivalves as resource pulses. Annales de Limnologie - International Journal of Limnology 48, 105-112.

Strayer, D.L., 2008. Freshwater Mussel Ecology: A Multifactor Approach to Distribution and Abundance. University of California Press.

Strayer, D.L., Eviner, V.T., Jeschke, J.M., Pace, M.L., 2006. Understanding the long-term effects of species invasions. Trends in Ecology & Evolution 21, 645-651.

Strayer, D.L., Malcom, H.M., 2007a. Effects of zebra mussels *(Dreissena polymorpha*) on native bivalves: the beginning of the end or the end of the beginning? Journal of the North American Benthological Society 26, 111-122.

Strayer, D.L., Malcom, H.M., 2007b. Shell decay rates of native and alien freshwater bivalves and implications for habitat engineering. Freshwater Biology 52, 1611-1617.

Tame, A., Yoshida, T., Ohishi, K., Maruyama, T., 2015. Phagocytic activities of hemocytes from the deep-sea symbiotic mussels *Bathymodiolus japonicus*, *B. platifrons*, and *B. septemdierum*. Fish & Shellfish Immunology 45, 146-156.

Viarengo, A. , L. Canesil, A. Mazzucotelli, Ponzano', E., 1993. Cu, Zn and Cd content in different tissues of the Antarctic scallop *Adamussium colbecki*: role of metallothionein in heavy metal homeostasis and detoxication Marine Ecology Progress Series 95, 163-168

Walker, K.F., Byrne, M., Hickey, C.W., Roper, D.S., 2001. Freshwater Mussels (Hyriidae) of Australasia, in: Bauer, G., Wächtler, K. (Eds.), Ecology and Evolution of the Freshwater Mussels Unionoida, Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 5-31.

Wang, N., Augspurger, T., Barnhart, M.C., Bidwell, J.R., Cope, W.G., Dwyer, F.J., Geis, S., Greer, I.E., Ingersoll, C.G., Kane, C.M., May, T.W., Neves, R.J., Newton, T.J., Roberts, A.D., Whites, D.W., 2007. Intra- and interlaboratory variability in acute toxicity tests with glochidia and juveniles of freshwater mussels (Unionidae). Environmental Toxicology and Chemistry / SETAC 26, 2029-2035.

Wang, N., Erickson, R.J., Ingersoll, C.G., Ivey, C.D., Brunson, E.L., Augspurger, T., Barnhart, M.C., 2008. Influence of pH on the acute toxicity of ammonia to juvenile freshwater mussels (fatmucket, Lampsilis siliquoidea). Environmental Toxicology and Chemistry. SETAC 27, 1141-1146.

Wang, N., Ingersoll, C.G., Ivey, C.D., Hardesty, D.K., May, T.W., Augspurger, T., Roberts, A.D., van Genderen, E., Barnhart, M.C., 2010. Sensitivity of early life stages of freshwater mussels (Unionidae) to acute and chronic toxicity of lead, cadmium, and zinc in water. Environmental Toxicology and Chemistry / SETAC 29, 2053-2063.

Wootton, E.C., Dyrynda, E.A., Pipe, R.K., Ratcliffe, N.A., 2003a. Comparisons of PAH-induced immunomodulation in three bivalve molluscs. Aquatic Toxicology 65, 13-25.

Wootton, E.C., Dyrynda, E.A., Ratcliffe, N.A., 2003b. Bivalve immunity: comparisons between the marine mussel (*Mytilus edulis*), the edible cockle (*Cerastoderma edule*) and the razor-shell (*Ensis siliqua*). Fish & Shellfish Immunology 15, 195-210.

Young, M.R., Cosgrove, P.J., Hastie, L.C., 2001. The Extent of, and Causes for, the Decline of a Highly Threatened Naiad: *Margaritifera margaritifera*, in: Bauer, G., Wächtler, K. (Eds.), Ecology and Evolution of the Freshwater Mussels Unionoida, Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 337-357.

Zhao, J., Song, L., Li, C., Ni, D., Wu, L., Zhu, L., Wang, H., Xu, W., 2007. Molecular cloning, expression of a big defensin gene from bay scallop *Argopecten irradians* and the antimicrobial activity of its recombinant protein. Molecular Immunology 44, 360-368.

https://cmsdata.iucn.org/downloads/portugal_s_biodiversity_at_risk_fact_sheet_may_2013.pdf

http://molluskconservation.org/MUSSELS/Pearls.html

CHAPTER 2

Antiaggregant and toxic properties of different solutions on haemocytes of three freshwater bivalves

Antiaggregant and toxic properties of different solutions on haemocytes of three freshwater bivalves

Hinzmann, M. F.[a,b]; Lopes-Lima, M.[a,b]; Machado, J.[a,b]

[a]ICBAS - Instituto de Ciências Biomédicas de Abel Salazar, Departamento de Produção Aquática, Universidade do Porto, Porto, Portugal.

[b]CIIMAR - Centro Interdisciplinar de Investigação Marítima e Ambiental, Universidade do Porto, Porto, Portugal

Adapted from: Hinzmann, M.F., Lopes-Lima, M., Gonçalves, J., Machado, J., 2013. Antiaggregant and toxic properties of different solutions on haemocytes of three freshwater bivalves. Toxicological & Environmental Chemistry 95 (5), 790-805.

Abstract

Haemocytes are main blood components of bivalves playing important roles in toxicological and immune responses. Consequently, a study on these cells may be useful to understand the invertebrate immunological systems. The aim of the present study was to find the best antiaggregant solution, based on its capacity of preserving the natural morphology and viability of haemocytes from the freshwater bivalves: *Anodonta cygnea, Unio delphinus* and *Corbicula fluminea.* Haemocytes from the three species were collected and maintained in different antiaggregant solutions, i.e., ethylenediaminetetraacetic acid (EDTA), EDTA-citrate, modified Alsever solution (MAS), N-ethylmaleimide (NEM), NEM-NaCl and heparin. The cell morphology and viability were analysed periodically with light microscopy techniques. Between these assays with various antiaggregant solutions, the best results were obtained with NEM at a concentration of 0.05 mol L^{-1}. It is also shown that, as expected, the osmolarity of the antiaggregant solutions plays a very important role in the cell viability. Based on this study, NEM at 0.05 mol L^{-1} was considered an adequate antiaggregant solution for future research on immunological and toxicological responses and other physiological studies of freshwater bivalve haemocytes.

Introduction

Anodonta cygnea (Linnaeus 1758) and *Unio delphinus* (Spengler 1793) are two freshwater bivalve species native in Europe belonging to the Unionidae family. The unionids have been suffering a substantial decrease in their populations mainly in the last 50 years (Bauer, 1988; Bogan, 1993; Neves et al., 1997; Graf and Cummings, 2007). The reasons for this decline are mainly anthropogenic and are due to habitat degradation, pollution and river system compartmentalization. On the other hand, *Corbicula fluminea* (Muller 1774) is an Asiatic invasive species of which populations have been increasing and spreading all over Europe with a high impact on natural habitats (Vaughn and Spooner, 2006; Sousa et al., 2008). The research on freshwater bivalve immunology seems relevant in order to identify the causes for their decline or expansion, having in mind that the immune systems may be differentially affected by several factors. The main components of the bivalve immunological system are the haemocytes (Glinski and Jarosz, 1997; Danilova, 2006). These cells can be found mainly in the circulating haemolymph and play different roles, not only in the immune system but also in detoxification, oxygen transport, and biomineralization (Cajaraville and Pal, 1995; Soares-da-Silva et al., 2002). In fact, *A.*

cygnea granulocytes are thought to be involved in immunoresponses to foreign particles with the digestion of small residues with the formation of calcareous deposits, while the hyalinocytes may be more responsible for detoxification and elimination of soluble cytotoxic compounds (Moura et al., 1999; Soares-da-Silva et al., 2002). In laboratory studies haemolymph collection is often used, under *in vitro* conditions, to reduce the number of sacrificed animals. The conditions for maintaining the cells in laboratory are not always optimal, interfering with the results obtained in the assays since the viability and integrity of the cells are not guaranteed. Handling invertebrate haemocytes *in vitro* is often difficult because when sampled in physiological saline buffer, the haemocytes tend to clump irreversibly, compromising experimental designs (Adena et al., 1994; Silva et al. 2000). Most studies on invertebrate haemocytes use fixed cells, although at such conditions it is hard to evaluate their physiological and immune capacities.

The vertebrate and invertebrate animals have developed efficient cellular and molecular mechanisms to prevent blood loss in case of injury through agglutination of blood components in a process designated as coagulation in vertebrates. This mechanism is well known in vertebrates that share a similar coagulation system, based on the induced aggregation by proteolyses of fibrinogen into insoluble fibrin. Among invertebrates, a more diversified group, distinct aggregation mechanisms can be found, although detailed information on this process is only known for a few species. The cell aggregation in bivalves with the formation of clumps is mainly associated with homeostasis and wound healing. This aggregation differs from the blood clotting in vertebrates, since no extracellular fibres are formed, being a reversible process with dispersion and re-entry in the circulatory system of most aggregated cells after some time (Chen and Bayne, 1995). *In vitro*, the bivalve haemocytes tend to spontaneously aggregate when the haemolymph is removed from the organisms. An adequate way to avoid this adhesion and clumping process *in vitro* is to identify an efficient inhibitor of haemocyte aggregation or adhesion, usually affected by antiaggregation or antiaggregant solution (Chen and Bayne, 1995).

The most commonly used antiaggregants are composed by ethylenediaminetetraacetic acid (EDTA), heparin, or citrate, with antiaggregation properties that help in the preservation of cell morphology. EDTA and citrate exert their antiaggregant effects by decreasing the Ca^{2+} concentration in plasma (Macey et al., 2003). Most antiaggregant solutions have a similar composition, using an antiaggregation component (EDTA or citrate, as a metal cation chelator), sodium chloride (to give ionic strength) and glucose (as a radical scavenger) (Torreilles et al., 1999). Heparin is the most often used antiaggregant

in vertebrates, although its use is rare in invertebrates. Heparin works by inhibiting or inactivating the three major blood clotting factors - thrombin, thromboplastin, and prothrombin. It slows the process of thromboplastin synthesis, decelerates the conversion of prothrombin to thrombin, and inhibits the effects of thrombin on fibrinogen, blocking its conversion to fibrin (Björk and Lindahl, 1982; McNeely and Griffith, 1985). N-ethylmaleimide (NEM) is a sulfhydril inhibitor and under certain conditions it is shown to prevent vertebrate platelets to agglutinate (Bryan et al., 1964). This effect has also been observed with other invertebrate haemocytes, namely in *Limulus polyphemus* (Bryan et al., 1964) and *Mytilus californianus* (Chen and Bayne, 1995). From these studies, it is inferred that sulfhydril groups play a role in the agglutination of vertebrate and invertebrate blood cells.

The literature about antiaggregants for bivalve haemocytes is scarce, so a selection of antiaggregants generally used for studies of vertebrate and invertebrate blood cells was accomplished. It should be emphasized that relative to marine molluscs the antiaggregants are successfully applied under hyperosmotic conditions. So, several antiaggregant solutions were also tested at slightly hyperosmotic conditions on haemocytes from *A. cygnea*, *U. delphinus* and *C. fluminea* to assess the anti-aggregation properties. To evaluate the osmotic resistance and to find the adequate cell viability conditions without cellular aggregation, in order to use them in short-term laboratory assays such as to measure morpho-physiological parameters in the haemocytes, were the main goals.

Methods

Animal collection

The three species of freshwater bivalves used in this study, were collected from northern Portugal (see Figure 1); *A. cygnea* was collected from the Mira Lagoon, *U. delphinus* from the Douro River near S. João da Pesqueira and *C. fluminea* from the Minho River near Caminha. They were kept in aerated tanks with dechlorinated water and acclimatized in these conditions for two weeks. The animals were fed daily with a microalgae diet. The organisms were considered healthy if the surface of the shell was smooth and shiny and if they closed the valves when disturbed.

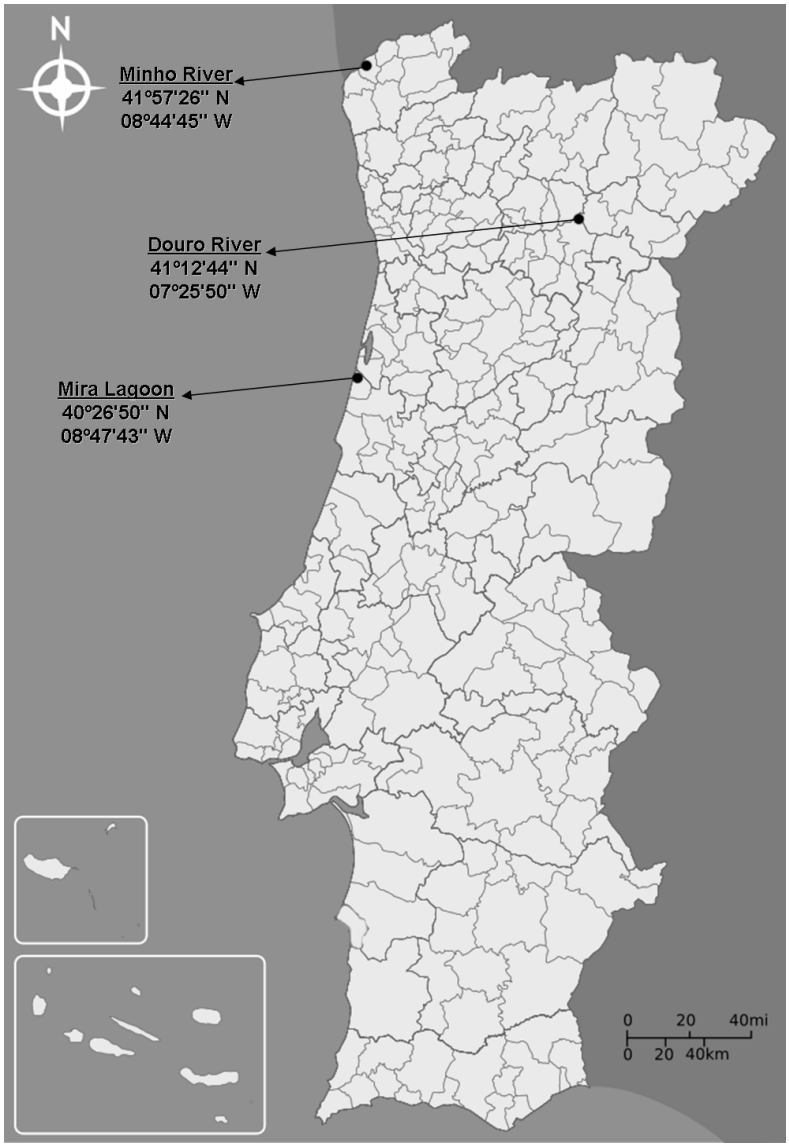


Figure 1 – Map showing the three sampling sites from the northern Portugal, used to collect the study species.

Haemolymph collection

Haemolymph from *A. cygnea* and *U. delphinus* was carefully extracted from the organisms using a 21G needle (Braun, Melsungen, Germany) attached to a 2 mL sterile syringe (Braun) by insertion between the valves across the inner layer of the mantle into the interepithelial space. In *C. fluminea,* a smaller bivalve, the haemolymph collection had to be carried out by disrupting the adductor muscle and opening the valves slowly above a Falcon tube, the mantles were cut to guarantee that most fluid was collected. A pool of

haemolymph from three organisms was made for each species to reduce individual variation, and the haemolymph was maintained on ice to avoid aggregation (Silva et al., 2000; Gagnaire et al., 2004). To teste each antiaggregant solution, from each pool three haemolymph aliquots were sampled: one was maintained on ice as control, to the second was added the concentrated antiaggregant solutions at a 1:10 ratio, and to the third was added the same antiaggregant but previously diluted (Table 1). Then, a total of four replicates were sampled and analysed for each of the three aliquots. Haemocytes viability and morphology were evaluated at 0, 60, 120 and 180 minutes.

Table 1 - Osmolarity of the antiaggregant solutions.

Antiaggregant solutions	Mother solution osmolarity (mOsm L^{-1})	Antiaggregant + Hem. *A. cygnea* 1:10 final osmolarity (mOsm L^{-1})	Antiaggregant + Hem. *U. delphinus* 1:10 final osmolarity (mOsm L^{-1})	Antiaggregant + Hem. *C. fluminea* 1:10 final osmolarity (mOsm L^{-1})
EDTA buffer	1663.7 ± 8.62	193.7 ± 1.31	194.88 ± 1.20	202.43 ± 0.94
EDTA buffer diluted	81.7 ± 0.58	49.88 ± 1.05	51.06 ± 0.91	58.61 ± 0.53
MAS	1003.7 ± 3.79	133.70 ± 1.10	134.88 ± 0.97	142.43 ± 0.63
MAS diluted	100.7 ± 1.15	51.61 ± 1.05	52.79 ± 0.92	60.34 ± 0.54
EDTA/citrate	448.0 ± 5.20	83.18 ± 1.15	84.36 ± 1.02	91.91 ± 0.71
EDTA/citrate diluted	89.3 ± 3.51	50.57 ± 1.09	51.75 ±0.96	59.30 ± 0.62
NEM +NaCl	1481.0 ± 4.00	177.10 ± 1.11	178.27 ± 0.98	185.82 ± 0.64
NEM+ NaCl diluted	101.7 ± 0.58	51.7 ± 1.05	52.88 ± 0.91	60.43 ± 0.53
NEM	353.0 ± 1.00	74.54 ± 1.05	75.72 ± 0.91	83.27 ± 0.54
NEM diluted	97.0 ± 1.00	51.27 ±1.05	52.45 ± 0.91	60.00 ± 0.54
Heparin	252.7 ± 1.15	65.43 ± 1.05	66.61 ± 0.92	74.15 ± 0.54
Heparin diluted	87.7 ± 0.58	50.43 ±1.05	51.61 ± 0.91	59.15 ± 0.53

Antiaggregant solutions

For each species six antiaggregant solutions were prepared as follows:

Antiaggregant solution 1 (EDTA buffer): 500 mmol L^{-1}Tris-HCl, 111 mmol L^{-1} glucose, 342 mmol L^{-1} NaCl, 13.4 mmol L^{-1} EDTA, pH 7.3 (Bigas et al., 2006).

Antiaggregant solution 2 ((Modified Alsever Solution (MAS)): 115 mmol L^{-1} Glucose, 27 mmol L^{-1} sodium citrate, 11.5 mmol L^{-1} EDTA, 382 mmol L^{-1} NaCl pH 7.4 (Bachère et al., 1988; Mitta et al., 1999; Serpentini et al., 2000).

Antiaggregant solution 3 (EDTA/citrate): 98 mmol L^{-1} NaOH, 146 mmol L^{-1} NaCl, 10 mmol L^{-1} EDTA, 41 mmol L^{-1} citric acid pH 7.0 (Silva et al., 2000).

Antiaggregant solution 4 (NEM+NaCl): 200 mmol L^{-1} NEM, 513 mmol L^{-1} NaCl (Soares da Silva et al., 2002).
Antiaggregant solution 5 (NEM): 200 mmol L^{-1} NEM.
Antiaggregant solution 6 - Sodium Heparin (5000 IU mL^{-1}) (Braun).

The osmolarity of each solution was measured using an automatic micro-osmometer (Löser, Berlin, Germany). The preliminary experiments were accomplished with final concentration around 50 mOsm L^{-1}, equivalent to that of bivalve fluids but presenting weak antiaggregation capacity. So, slightly hyperosmotic versions of the different antiaggregants were used to obtain osmolarity values around twice that of the haemolymph. The osmolarities of these solutions could not be the same as in the bivalve's fluids but had to be higher, as a compromise between a concentration of the active compound necessary to be effective and to have the osmotic power to maintain the viability of the cells. The diluted versions of the different antiaggregant solutions were adjust using the following dilution factors:

Antiaggregant solution 1 (EDTA) diluted by a factor of 1:20; antiaggregant solution 2 (MAS) diluted by a factor of 1:10; antiaggregant solution 3 (EDTA-citrate) diluted by a factor of 1:5; antiaggregant solution 4 (NEM-NaCl) diluted by a factor of 1:15; antiaggregant solution 5 (NEM) diluted by a factor of 1:4; antiaggregant solution 6 (heparin) diluted by a factor of 1:3.

Light Microscopy

The cells were observed under light microscopy (BX 41 with digital camera DP70, Olympus, Tokyo, Japan) to evaluate the effects of each antiaggregant, its antiaggregation properties, and its impact on cell morphology.

Haemocytes Viability

The viability of the cells was checked immediately after haemolymph collection and after 60, 120 and 180 minutes. The cell viability was estimated by the trypan blue exclusion assay (Ford and Haskins, 1988, Tirard et al., 1997) for each established period. The cells were observed under the light microscope and counted using an improved Neubauer haemocytometer (Boeco, Hamburg, Germany).

Statistics

The data obtained were compared by one-way analysis of variance followed by Scheffé's post-hoc tests in all statistical analysis (Analysis ToolPak for Microsoft Excel 2002 SP3, Microsoft Corporation, USA). All tests were performed at a significance level of 5%.

Results

Osmolarity

The determination of the osmolarity values of the experimental antiaggregant solutions is presented in Table 1, being the lower values of the diluted solutions, close to those in normal haemolynph. The values of osmolarity in both *A. cygnea* and *U. delphinus* are very similar, i.e., 46.7 ± 1.2 mOsm L^{-1} and 48.0 ± 1.0 mOsm L^{-1} respectively, being higher (56.5 ± 0.6 mOsm L^{-1}) in *C. fluminea*.

Quantitative analysis

For *A. cygnea* haemocyte viability (see Figure 2), the antiaggregants that are cation-binding agents, as EDTA, MAS, and EDTA/citrate buffers, presented a significant variability. In the case of EDTA, there was only a slight negative impact on cell viability in comparison to the control. Relative to MAS, there was an even stronger negative impact on the haemocytes of these species, decreasing cell viability significantly ($p < 0.05$), i.e., either by 83% with the concentrated form or by 37% with the diluted form of this antiaggregant. On the other hand, the antiaggregant EDTA/citrate, also with the same cation-sequestering ability but lower osmolarity, after 120 minutes effected an increase in cell viability of 6% and 8% relative to the control in their concentrated and diluted form, respectively.

The other antiaggregants tested in *A. cygnea* (Figure 3) showed that NEM, a sulfhydril inhibitor, with and without NaCl, had a low impact on cell viability in the diluted form. Only the concentrated form of NEM with NaCl showed a statistically significant decrease ($p < 0.05$) of 12% on haemocyte viability.

Heparin, both concentrated and diluted, revealed a dual behaviour when added to the cells of *A. cygnea*, presenting a decrease in the first 120 minutes. However, after this time, there was a slight increase of 5% ($p < 0.05$) in the cells viability.

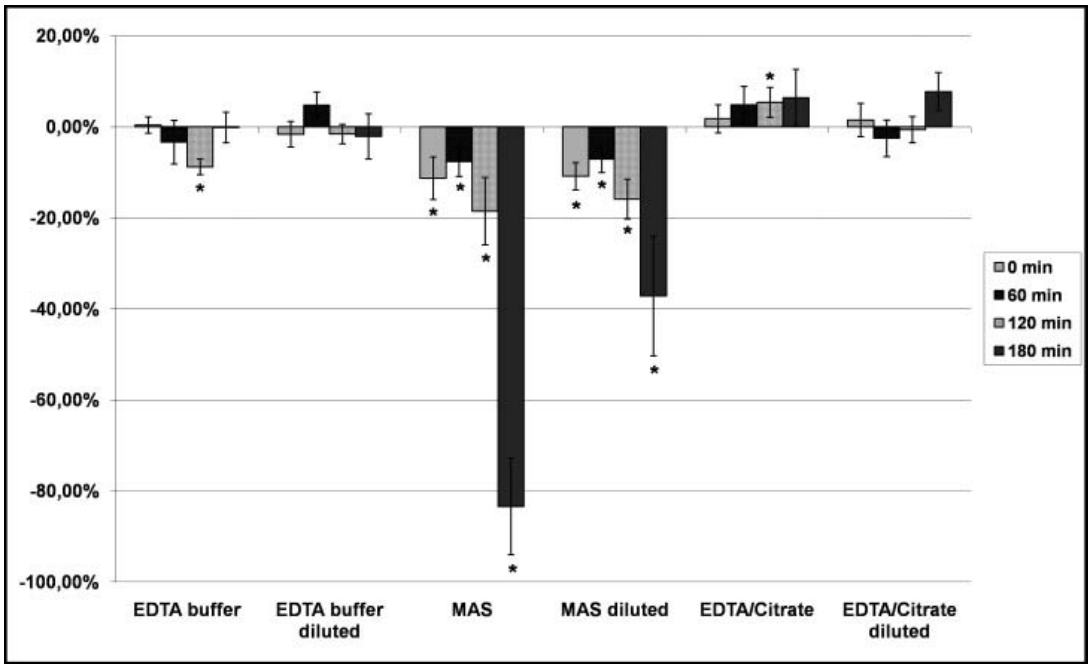


Figure 2 - Percentage ratio of the EDTA based antiaggregants on *A. cygnea* haemocytes viability to the control situation haemocytes viability. Asterisks over the bars represent a significant difference ($p < 0.05$) relative to the control.

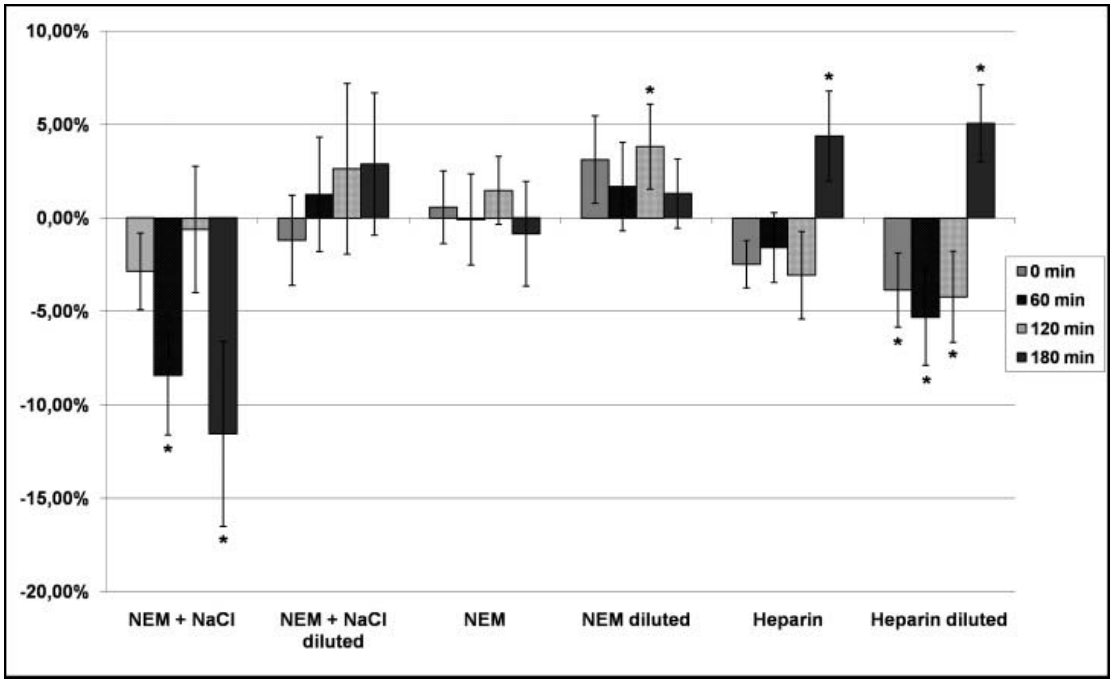


Figure 3 - Percentage ratio of the NEM based antiaggregants on *A. cygnea* haemocytes viability to the control situation haemocytes viability. Asterisks over the bars represent a significant difference ($p < 0.05$) relative to the control.

For *U. delphinus*, an unionid, the results were slightly different (Figure 4) with the antiaggregants that act as cation-binding agents, showing in general, a positive effect on the haemocytes of these species. In fact, after 180 minutes, the undiluted form of EDTA buffer revealed a significant ($p < 0.05$) increase of 6% in viability of these cells, while the undiluted and diluted form of EDTA/citrate showed an increase of 5 and 6%, respectively. The only exception occurred with a negative impact of MAS under the diluted form in comparison to the control.

For the second group of antiaggregants, the best results were obtained with all NEM solutions (Figure 5), increasing the haemocytes viability of *U. delphinus*, especially the diluted NEM without NaCl. In fact, after 180 minutes, the NEM-NaCl revealed an increase of 3% and 8% in the undiluted and diluted forms, respectively. The NEM without NaCl showed, after 180 minutes, significant ($p < 0.05$) increases on cell viability of 6 and 14% for the undiluted and diluted forms, respectively. Heparin had little impact on the cells of *U. delphinus.*

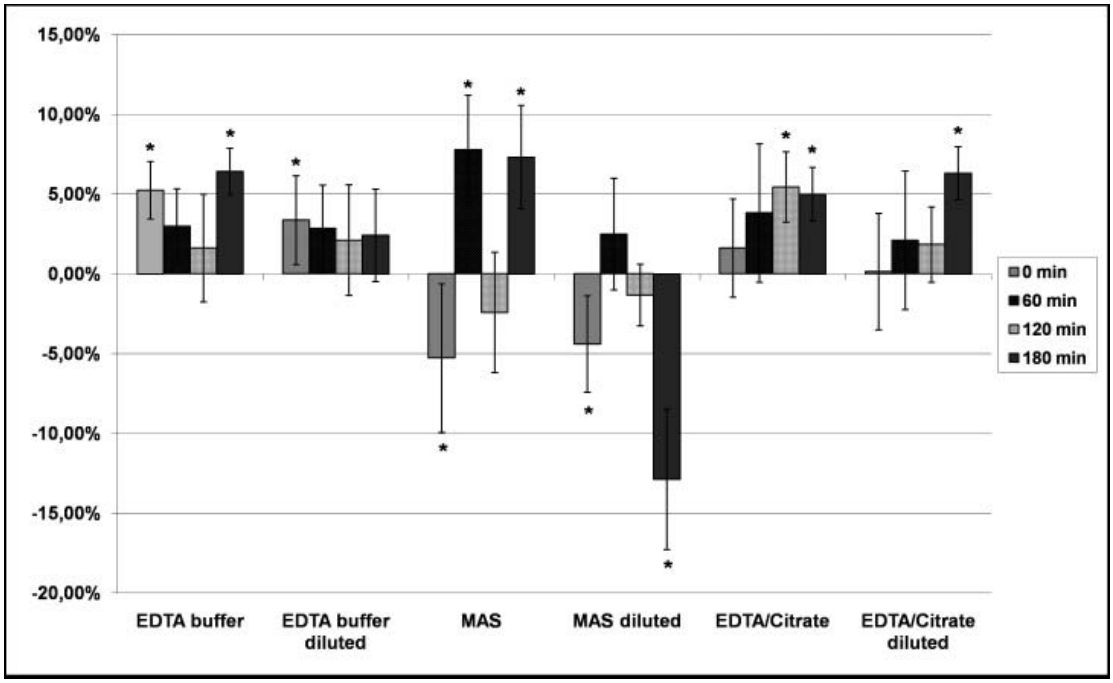


Figure 4 - Percentage ratio of the EDTA based antiaggregants on *U. delphinus* haemocytes viability to the control situation haemocytes viability. Asterisks over the bars represent a significant difference ($p < 0.05$) relative to the control.

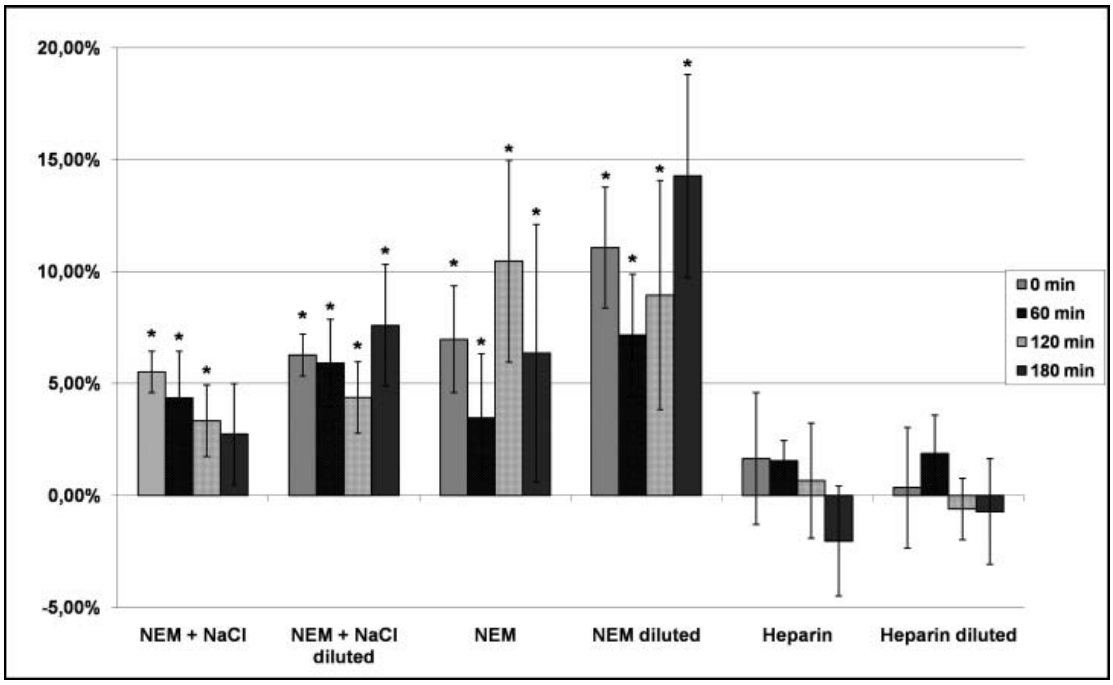


Figure 5 - Percentage ratio of the NEM based antiaggregants on *U. delphinus* haemocytes viability to the control situation haemocytes viability. Asterisks over the bars represent a significant difference ($p < 0.05$) relative to the control.

Concerning the haemocytes from *C. fluminea*, it seems to be more tolerant to the variety of antiaggregants with a neutral or positive effect on the cells viability (Figures 6 and 7). The haemocytes of *C. fluminea* in the presence of antiaggregants containing a cation-sequestering agent increased in viability, especially with the antiaggregants in the undiluted form. In fact, the exposure of the cells to undiluted EDTA solution revealed a significant ($p < 0.05$) increase in cell viability of 25% after 120 minutes and of 10% after 180 minutes. Also, with MAS, there were significant ($p < 0.05$) increases in cell viability of 15% and 11% with the undiluted and diluted forms, respectively. The antiaggregant EDTA/citrate also showed an increase but with small variations, the highest increase being of 6% at 120 min ($p < 0.05$) in comparison to the control.

The antiaggregants based on NEM showed uniform increases in cell viability over time. In fact, the NEM-NaCl induced an increase in cell viability of 3% with the undiluted form. The NEM without NaCl induced significant ($p < 0.05$) increases of 6% and 9% in their undiluted or diluted form, respectively. Finally, heparin also had a positive effect on cell viability mainly in its undiluted form, increasing significantly ($p < 0.05$) by 7% after 180 minutes.

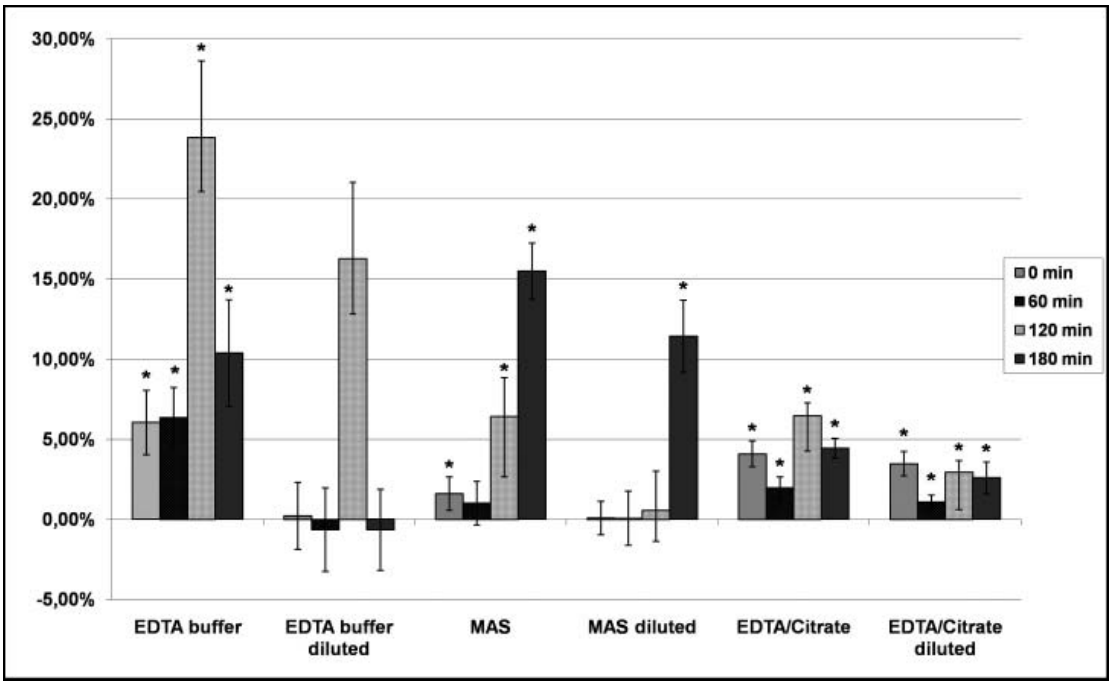


Figure 6 - Percentage ratio of the EDTA based antiaggregants on *C. fluminea* haemocytes viability to the control situation haemocytes viability. Asterisks over the bars represent a significant difference ($p < 0.05$) relative to the control.

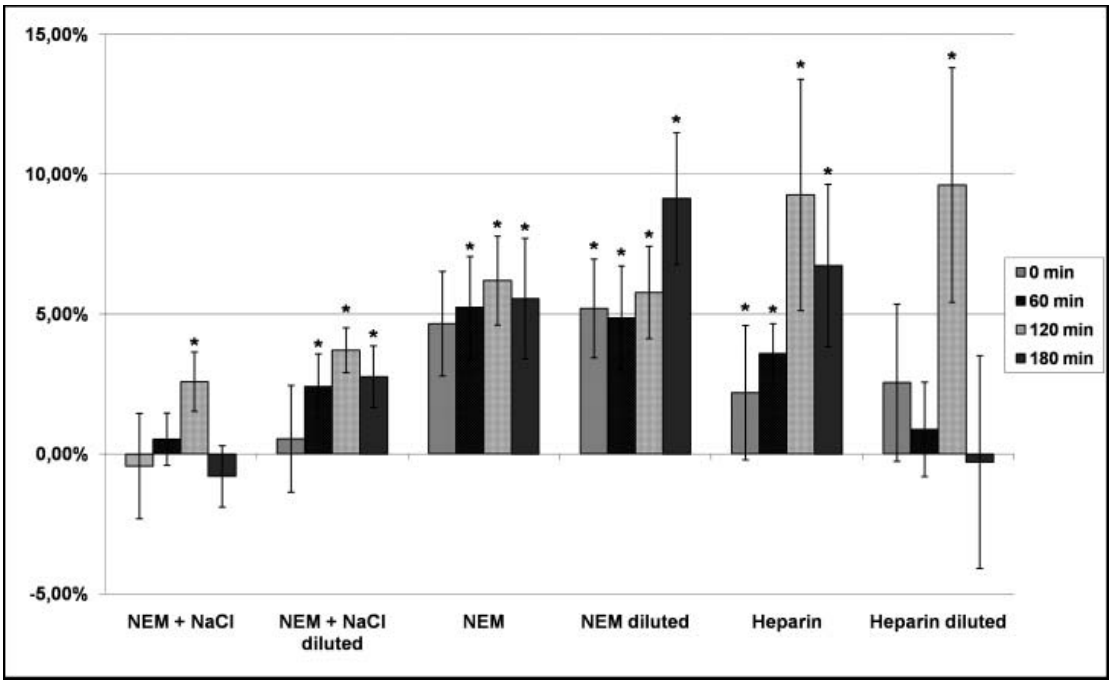


Figure 7 - Percentage ratio of the NEM based antiaggregants on *C. fluminea* haemocytes viability to the control situation haemocytes viability. Asterisks over the bars represent a significant difference ($p < 0.05$) relative to the control.

Qualitative analysis

The microscopic imaging revealed that the control granulocytes and hyalinocytes showed a high level of aggregation and had a small number of long pseudopods and a good visibility of the main cellular features as nucleus, vesicles, membranes and granules (Figures 8-10). The visibility is compromised with several antiaggregant solutions showing a mirror-like shiny silver look. With all the undiluted antiaggregants, the level of aggregation is fairly reduced but with different effects on the cell viability. In fact, when exposed to undiluted EDTA antiaggregant solution, the cells of all the three different bivalve species exhibited considerable shrinkage, increasing the number of smaller and thinner pseudopods, and shinier cytoplasm. In addition, in the groups with diluted EDTA, the cells from the different species presented similar feature changes: the cells showed a stretched look and had a small number of bigger and prolonged pseudopods, with a tendency to increase the formation of small aggregates. The haemocytes of Unionidae bivalves, when exposed to undiluted MAS antiaggregant, suffered cell shrinkage and there was an increase in the number of pseudopods when compared with the control. On the other hand, the haemocytes of *C. fluminea* only showed an increase in the number of smaller pseudopods. For the diluted MAS antiaggregant, the Unionidae cells presented a smaller shrinkage and an increase in the number of pseudopod but with a smaller size. The *Corbicula* species exhibited a similar pattern but with longer pseudopods. In the experiments with undiluted EDTA-citrate, the cells of *A. cygnea* presented cell shrinkage with a shinier appearance of the cytoplasm. In *U. delphinus*, the cells were stretched showing longer pseudopods. The *C. fluminea* cells presented an increased number of pseudopod. With diluted EDTA-citrate, the cells of the three bivalve species presented features very similar to control, but with strong tendency to aggregate.

Effectively, the most positive results were obtained using the diluted NEM with or without NaCl, where the decrease on aggregation level is also much reduced, but the cells maintaining a healthier look with good visibility compared to the control. In fact, in the undiluted NEM-NaCl exposure experiments the cells of the three species showed shrinkage, very shiny look, and considerable increase of small pseudopods. In diluted form, NEM-NaCl also induced, although less extensively, cell shrinkage and shinier look of the cytoplasm. The *C. fluminea* showed similar features for control cells but without any pseudopod in the cells. The undiluted NEM without NaCl still induced shinier aspect of the cells of all species, although in comparison lightly, as well as an increase in pseudopods

with smaller size. Finally, it was evident that with the diluted NEM without NaCl, the cells appeared healthy, with small numbers of pseudopods having similar shape as in the control and with a decreased level of aggregation.

In the experiments using undiluted heparin solution, the cells of the three bivalve species presented high aggregation levels, mild shrinkage of cells, and increased number of pseudopods. For the diluted solution, the cells of all the three species showed higher aggregation, with the increase in the number of pseudopods being lower than that of the undiluted version.

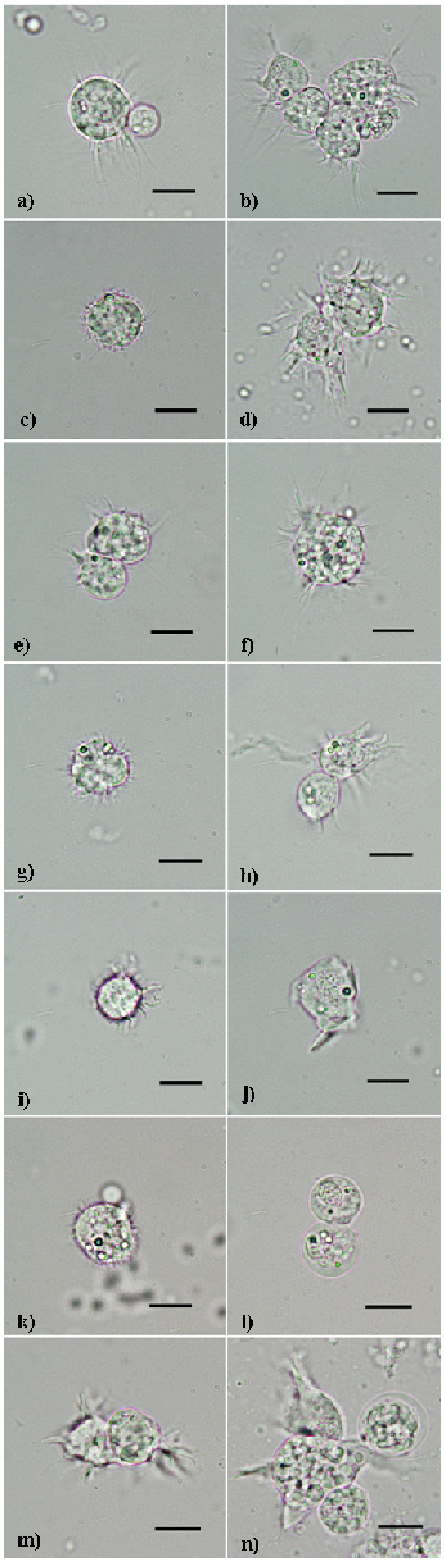


Figure 8 - Images of light microscopy from haemocytes of *A. cygnea* where different antiaggregant solutions were added: a) and b) control, cells maintained on ice, c) cells with EDTA buffer antiaggregant, d) cells with diluted EDTA buffer, e) cells with MAS, f) cells with diluted MAS, g) cells with EDTA/citrate, h) cells with diluted EDTA/citrate, i) cells with NEM-NaCl, j) cells with diluted NEM-NaCl, k) cells with NEM, l) cells with diluted NEM, m) cells with heparin and n) cells with diluted heparin; scale bars 10 μm.

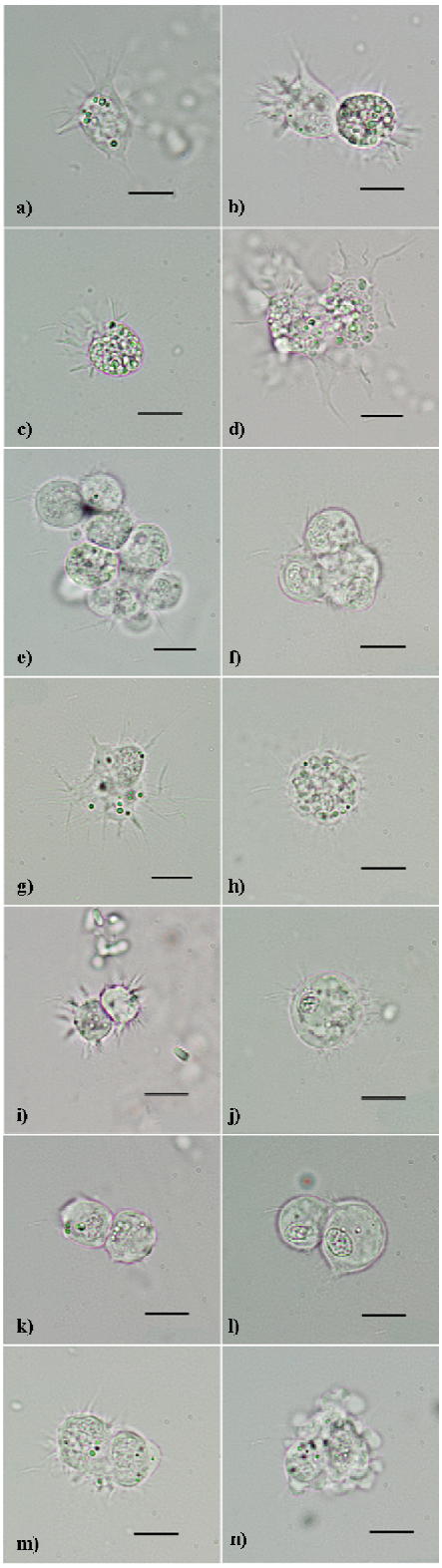


Figure 9 - Images of light microscopy from haemocytes of U. delphinus where different antiaggregant solutions were added: a) and b) control, cells maintained on ice, c) cells with EDTA buffer antiaggregant, d) cells with diluted EDTA buffer, e) cells with MAS, f) cells with diluted MAS, g) cells with EDTA/citrate, h) cells with diluted EDTA/citrate, i) cells with NEM-NaCl, j) cells with diluted NEM-NaCl, k) cells with NEM, l) cells with diluted NEM, m) cells with heparin and n) cells with diluted heparin; scale bars 10 µm.

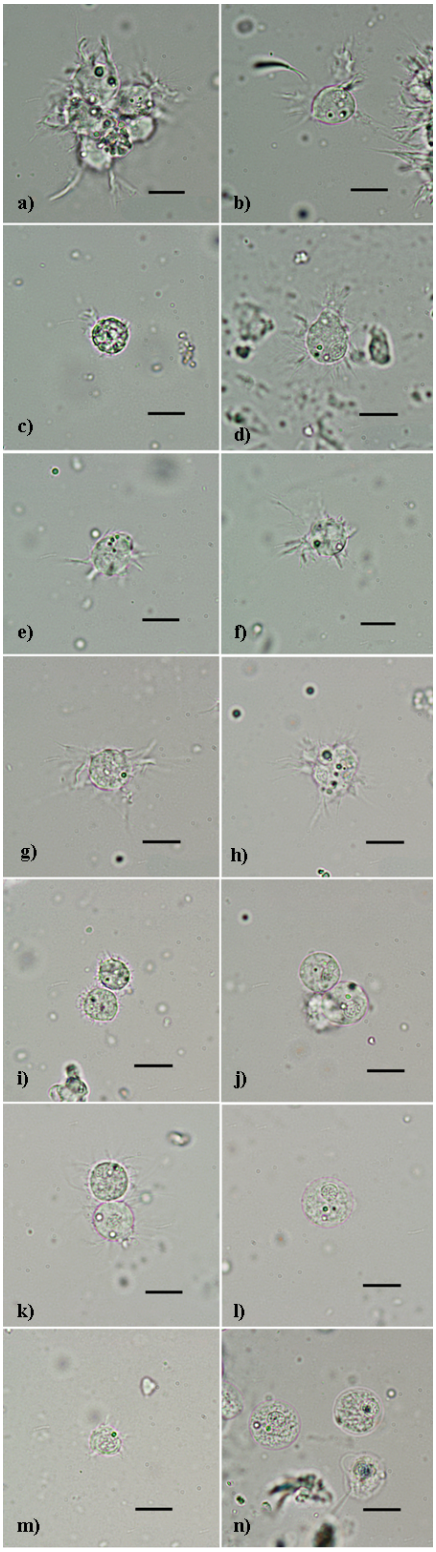


Figure 10 - Images of light microscopy from haemocytes of *C. fluminea* where different antiaggregant solutions were added: a) and b) control, cells maintained on ice, c) cells with EDTA buffer antiaggregant, d) cells with diluted EDTA buffer, e) cells with MAS, f) cells with diluted MAS, g) cells with EDTA/citrate, h) cells with diluted EDTA/citrate, i) cells with NEM-NaCl, j) cells with diluted NEM-NaCl, k) cells with NEM, l) cells with diluted NEM, m) cells with heparin and n) cells with diluted heparin; scale bars 10 μm.

Discussion

The morphological data are generally used in order to distinguish among groups of bivalve haemocytes. However, the nomenclature of haemocyte types may vary considerably depending on the techniques. In *A. cygnea*, a species used in the present study, some of these features were already described (Soares-da-Silva et al., 2002; Salimi et al., 2009). In addition, for functional aspects and other research objectives, it is sometimes relevant to preserve the normal cell morphology and physiology using appropriate buffer solutions.

Antiaggregants are complex natural or synthetic organic compounds, often being carbohydrates that help prevent blood clotting or coagulation. Of these, the most widely used is heparin. Unfortunately, it is not very efficient on invertebrate haemocytes.

The invertebrates, which lack an adaptive immune system, have developed defence systems that respond to common antigens on the surface of potential pathogens. Haemolymph aggregation is one of such defence systems, which has only been studied on very few species like the horseshoe crab (Muta and Iwanga, 1996), in which lipopolysaccharide-sensitive and (1→3)-*β*-D-glucan-sensitive factors were discovered to trigger the aggregation cascade.

The haemocytes also play an important role in the immune system. The ideal conditions to maintain invertebrate haemocytes *in vitro* vary between species and are not always easy to establish. In contrast, the blood cells of vertebrates are easier to keep under similar conditions for all groups.

To study the physiologic mechanisms, first it is important to prevent aggregation by choosing the appropriate antiaggregant; secondly, such antiaggregant must maintain the cell morphology and viability. The freshwater bivalves have low blood-solute concentrations to reduce the concentration gradients between their body and the external medium and to minimize passive ion movements (Coimbra et al., 1988; Machado et al., 1991; Lopes-Lima et al., 2009). The members of the family Corbiculidae have higher rates of sodium transport than members of the family Unionidae, approaching the rates of brackish water species (McCorkle and Dietz, 1980). This might be due to the fact that *C. fluminea* is more tolerant to salinity, having been reported in both estuarine (brackish water up to 5‰ salinity) and freshwater habitats. Probably, this is the main reason why haemocytes of these species are better preserved by antiaggregants with higher osmolarity normally used for marine species.

Previous studies have shown that low temperature, non-wettable surfaces, and heparin were largely ineffective in preventing haemocyte agglutination. The cation-binding agents (oxalate, citrate and EDTA) were effective only in some species (Bryan et al., 1964). In the present study, the antiaggregant solutions with these types of component (EDTA, MAS, and EDTA/citrate) in their undiluted forms were efficient in avoiding aggregation of haemocytes of all the three species, but clearly failed in maintaining cell viability. In fact, these solutions caused alterations of the morphological features of the haemocytes, inducing cell shrinkage and a shinier cytoplasm together with an increased number of pseudopods. These changes were not so relevant for haemocytes of *C. fluminea,* probably due to a better tolerance to high osmolarity typical for these antiaggregant solutions. So, although these antiaggregants are very effective in some marine invertebrates, they are not suitable for haemocyte maintenance of these freshwater bivalves, due to the fact that the latter have low osmolarity in their internal fluids. In addition, when the same compounds were used in more diluted forms, they showed low capacity in avoiding cell aggregation; the morphological alterations, although still negative, were not so dramatic.

Same as the undiluted NEM antiaggregant with or without NaCl, a similar pattern was noticed with the undiluted compounds that have EDTA in its composition. In fact, the undiluted NEM antiaggregant solutions have a good antiaggregation capacity but, once again, compromised cell viability affected the cellular structure and morphology. Still, the same effect was noticed in the diluted NEM solution with NaCl, but to a lower extent. In contrast, the diluted NEM solution without NaCl clearly presented positive results. In fact, this antiaggregant showed very good antiaggregation property, increased cellular viability, and did not affect the normal cellular morphology. In general, the NEM may be suggested as the best antiaggregant for the haemocytes of freshwater bivalves, since it maintains the cellular integrity over time in all the three species.

The last type of antiaggregant solutions tested, heparin, exhibited very poor results. It promoted aggregation, induced strong alterations of the haemocyte features, and affected cell viability.

In conclusion, this study, revealed that haemocyte viability and morphology of *A. cygnea*, *U. delphinus* and *C. fluminea* were highly affected by the osmolarity of the antiaggregants solution tested, although to a lesser extent in the case of *C. fluminea*. The highest viability of haemocytes of *C. fluminea* was obtained with undiluted EDTA buffer but it has a higher toxicity and alters cell morphology and physiology. The best results were achieved with the NEM solution at a concentration of 50 mmol L^{-1} that significantly increased the viability of

the haemocytes of all the three species, without aggregation and maintaining the natural cell morphology.

Acknowledgments

This work was supported by “Fundação para a Ciência e Tecnologia” (FCT) under projects PTDC/MAR/098066/2008, PTDC/AAC-AMB/117688/2010 and the PhD scholarship SFRH / BD / 76265 / 2011. We would also like to thank the António Rocha for the laboratorial expertise.

References

Adena, C.M., van Deutekon-Mulder, E.C., van der Knaap, W.P.W., Sminia, T., 1994. Schistosomicidal activities of *Lymnaea stagnalis* haemocytes: the role of oxygen radicals. Parasitology 109, 479-485.

Bachère, E., Chagot, D., Grizel, H., 1988. Separation of *Crassostrea gigas* hemocytes by density gradient centrifugation and counterflow centrifugal elutriation. Develomental and Comparative Immunology 12, 549-559.

Bauer, G., 1988. Threats to the freshwater pearl mussel *Margaritifera margaritifera* in central Europe. Conservation Biology 45, 239-253.

Bigas, M., Durfort, M., Poquet, M., 2006. Cytological response of hemocytes in the European flat oyster, *Ostrea edulis*, experimentally exposed to mercury. Biometals 19, 659-673.

Björk, I., Lindahl, U., 1982. Mechanism of the anticoagulant action of heparin. Molecular and Cellular Biochemistry 48, 161-182.

Bogan, A.E., 1993. Fresh-Water Bivalve Extinctions (Mollusca, Unionoida) - a Search for Causes. Am. Zool. 33, 599-609.

Bryan, F.T., Robinson Jr., C.W., Gilbert, C.F., Langdell, R.D., 1964. N-ethylmaleimide inhibition of horshoe crab hemocyte agglutination. Science 144, 1147-1148.

Cajaraville, M.P., Pal, S.G., 1995. Morphofunctional study of the hemocytes of the bivalve mollusk *Mytilus galloprovincialis* with emphasis on the endolysosomal compartment. Cell Structure and Function 20, 355-367.

Chen, J.H., Bayne, C.J., 1995. Bivalve Mollusc hemocyte behaviors: characterization of hemocyte aggregation and adhesion and their inhibition in the California mussel (*Mytilus californianus*). Biological Bulletin 188, 255-266.

Coimbra, J., Machado, J., Fernandes, P.L., Ferreira, H.G., Ferreira, K.G., 1988. Electrophysiology of the mantle of *Anodonta cygnea*. Journal of Experimental Biology 140, 65-88.

Danilova, N., 2006. The evolution of immune mechanisms. Journal of Experimental Zoology. Part B, Molecular and Developmental Evolution 306, 496-520.

Ford, S.E., Haskin, H.H., 1988. Comparison of *in vitro* salinity tolerance of oyster parasite, *Haplosporidium nelsoni* (MSX) and hemocytes from host, *Crassostrea virginica*. Comparative Biochemistry Physiology, Part A 90, 183-187.

Gagnaire, B., Thomas-Guyon, H., Renault, T., 2004. *In vitro* effects of cadmium and mercury on Pacific oyster, *Crassostrea gigas* (Thunberg), haemocytes. Fish Shellfish Immunology 16, 501-512.

Glinski, Z., Jarosz, J., 1997. Molluscan Immune Defenses. Archivum Immunologiae et Therapiae Experimentalis 45, 149-155.

Graf, D.L., Cummings, K.S., 2007. Review of the systematics and global diversity of freshwater mussel species (Bivalvia: Unionoida). Journal Molluscan Studies 73, 291-314.

Lopes-Lima, M., Lopes, A., Casaca, P., Nogueira, I., Checa, A., Machado, J., 2009. Seasonal variations of pH, pCO2, pO2, HCO3- and Ca2+ in the haemolymph: implications on the calcification physiology in *Anodonta cygnea*. Journal of Comparative Physiology, Part B 179(3), 279-286.

Macey, M., McCarthy, D., Azam, U., Milne, T., Golledge, P. Newland, A., 2003. Ethylenediamanetetraacetic acid plus Citrate-Theophylline-Adenosine-Dipyridamole Cytometric assessment of platelet and neutrophil activation Ex Vivo in Whole Blood. Cytometry, Part B Clinical Cytometry 52, 30-40.

Machado, J., Reis, M.L., Coimbra, J. and Sá, C., 1991. Studies on chitin and calcification in the inner layers of the shell of *Anodonta cygnea*. Journal of Comparative Physiology, Part B 161, 413-418.

McCorkle, S., Dietz, T., 1980. Sodium transport in the freshwater Asiatic clam *Corbicula fluminea*. Biological. Bulletin 159, 325-336.

McNeely, T.B., Griffith, M.J., 1985. The anticoagulant mechanism of action of heparin in contact-activated plasma: inhibition of factor X activation. Blood 65, 1226-1231.

Mitta, G., Vandenbulcke, F., Hubert, F., Roch, P., 1999. Mussel defensins are synthesised and processed in granulocytes then released into the plasma after bacterial challenge. Journal of cell science 112, 4233-4242.

Moura, M., Guedes, R., Machado, J., 1999. The extracellular mineral concretions in *Anodonta cygnea* (L.): different types and manganese exposure-caused changes. Journal Shellfish Research 18, 645–650.

Muta, T., Iwanaga, S., 1996. The role of hemolymph coagulation in innate immunity. Current opinion in immunology 8, 41-47.

Neves, R.J., Bogan, A.E., Williams, J.D., Ahlstedt, S.A., Hartfield, P.W., 1997. Status of aquatic mollusks in the southeastern United States: a downward spiral of diversity. In: Aquatic Fauna in Peril: The Southeastern Perspective, ed. G.W. Benz and D.E. Collins, pp. 45–86. Southeast Aquatic Research Institute, Decatur, GA.

Salimi, L., Jamili, S., Motalebi, A., Eghtesadi-Araghi, P., Rabbani, M., Rostami-Beshman, M., 2009. Morphological Characterization and Size of Hemocytes in *Anodonta Cygnea*. Journal of Invertebrate Pathology 101(2), 81–85.

Serpentini, A., Ghayor, C., Poncet, J.M., Hebert, V., Galera, P., Pujol, J.P., Boucaud-Camou, E., Lebel, J.M., 2000. Collagen study and regulation of the de novo synthesis by IGF-I in hemocytes from the gastropod mollusc, *Haliotis tuberculata*. Journal Experimental. Zoology 287, 275-284.

Silva, C., Dunphy, G.B., Rau, M.E., 2000. Interaction of hemocytes and prophenoloxidase system of fifth instar nymphs of *Acheta domesticus* with bacteria. Developmental & Comparative Immunology 24, 367-379.

Soares-da-Silva, I.M., Ribeiro, J., Valongo, C., Pinto, R., Vilanova, M., Bleher, R., Machado, J., 2002. Cytometric, morphologic and enzymatic characterisation of haemocytes in *Anodonta cygnea*. Comparative Biochemistry and Physiology, A 132, 541-553.

Sousa, R., Nogueira, A.J.A., Gaspar, M.B., Antunes, C., Guilhermino, L., 2008. Growth and extremely high production of the non-indigenous invasive species *Corbicula fluminea* (Müller, 1774): Possible implications for ecosystem functioning. Estuarine, Coastal and Shelf Science 80, 289-295.

Tirard, C.T., Grossfeld, R. M., Levine, J.F., Kennedy-Stoskopf, S., 1997. Effect of Osmotic Shock on Protein Synthesis of Oyster Hemocytes *In Vitro*. Comparative Biochemistry and Physiology, Part A: Physiology 116, 43–49.

Torreilles, J., Guérin, M.C., Roch, P., 1999. Modified Alsever's solution is not a good medium for reactive oxygen metabolite study in bivalves. Fish & Shellfish Immunology 8, 65-69.

Vaughn, C.C., Spooner, D.E., 2006. Scale-dependent associations between native freshwater mussels and invasive *Corbicula*. Hydrobiologia 568, 331–339.

Chapter 3

Identification of distinct haemocyte populations from freshwater bivalves *Anodonta cygnea* and *Anodonta anatina* using wheat-germ agglutinin (WGA)

Identification of distinct haemocyte populations from freshwater bivalves *Anodonta cygnea* and *Anodonta anatina* using wheat-germ agglutinin

M. Hinzmann[ab*], M. Lopes-Lima[b], F. Cerca[a], A. Correia[c], J. Machado[ab] & M. Vilanova[acd]

[a] ICBAS - Abel Salazar Institute for the Biomedical Sciences, Departament of Aquatic Production, University of Porto, Rua de Jorge Viterbo Ferreira n.º 228, 4050-313 Porto, Portugal

[b] CIIMAR - Interdisciplinary Centre of Marine and Environmental Research, University of Porto, Rua dos Bragas, n.289, 4050-123 Porto, Portugal

[c] I3S - Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Portugal

[d] IBMC - Instituto de Biologia Molecular e Celular, Universidade do Porto, Porto, Portugal.

Hinzmann, M., Lopes-Lima, M., Cerca, F., Correia, A., Machado, J. Vilanova, M., 2016. Identification of distinct haemocyte populations from freshwater bivalves *Anodonta cygnea* and *Anodonta anatina* using wheat-germ agglutinin. (In preparation to be submitted to J. Developmental & Comparative Immunology)

Abstract

Haemocytes play a major role in the immune response of molluscs. Nevertheless little is known about their function, especially in freshwater bivalves from the Unionidae family, one of the most endangered faunistic groups. The constant aggressions made on the natural habitats of these organisms not only caused a drastic decrease in their populations and individuals, but also compromised their physiological fitness and immunity. The present work presents a comparative microscopic and cytometric characterization of *Anodonta anatina* and *Anodonta cygnea* circulating haemocytes, using nonlethal methods. Based on the classical classification of cell morphology and cytochemistry, two types of haemocytes were distinguished: granulocytes and hyalinocytes. Cytological features were visualized by using transmission electron microscopy and different staining techniques. Size, granularity, viability and surface staining using lectins as specific probes were analysed by flow cytometry and fluorescence microscopy. The proportion of granulocytes and hyalinocytes determined by microscopy, were significantly distinct between species, being of 70% and 30% for *A. cygnea* and of 85% and 15% for *A. anatina*, respectively. Two haemocyte populations were sorted by flow cytometry based on respective size and granularity that were identified as granulocytes and hyalinocytes. Interestingly, two different granulocyte populations and hyalinocytes could be further discriminated in *A. cygnea* according to their binding affinity to the lectin wheat germ agglutinin. Contrastingly, the granulocytes of *A. anatina* were all similarly stained with this lectin, as assessed by flow cytometry and fluorescence microscopy. Similarly tested peanut agglutinin and phytohaemaglutinnin lectins, showed low affinity for the granulocytes of both species, and did not bound to hyalinocytes.

Introduction

Anodonta cygnea (Linnaeus 1758) and *Anodonta anatina* (Linnaeus 1758) are two freshwater bivalve species native in Europe belonging to the Unionoida order. The unionoids have been suffering a substantial decrease in their populations mainly in the last 50 years (Bauer, 1988; Bogan, 1993; Neves et al., 1997; Graf & Cummings, 2007). The reasons for this decline are mainly anthropogenic and are essentially due to habitat degradation including siltation, pollution and river system compartmentalization. In this context, research on freshwater bivalve immunology acquires particular relevance in order to better understand the causes for their decline or expansion, having in mind that the

immune system of these animals may be differentially affected by several factors (Pipe and Coles, 1995; Girón-Pérez, 2010).

As most bivalves, these species have an open circulatory system, the haemolymph is pumped from the heart to the arteries and sinuses and from these spaces and tissues the haemolymph enters into the veins and eventually is carried back to the heart (Brand, 1972; Cheng, 1981). The main components of the bivalve immune system are the haemocytes (Danilova, 2006; Glinski and Jarosz, 1997). These cells can be mainly found in the circulating haemolymph and play several roles, not only those more closely associated with immune function, like wound and shell repair and phagocytosis, but also in coagulation, oxygen transport, nutrient digestion and biomineralization (Cajaraville and Pal, 1995; Soares-da-Silva et al., 2002; Cheng, 1981).

In the last years few researchers have addressed the different roles played by haemocytes in the immune response. Haemocytes classification is still not consensual, but most researchers use the terminology proposed by Cheng in 1981. This classification is based only on morphological parameters without the use of biochemical markers. Several other authors have distinguished two haemocyte types in bivalves, based on morphological, cytochemical and functional characteristics (Hine, 1999; Chang et al., 2005). In these studies hyalinocytes are characterised as cells with fewer granules in their cytoplasm and a higher nucleus to cell ratio, when compared with the granulocytes, which have abundant granules. In other studies, density gradient centrifugation (Carballal et al., 1997; Friebel and Renwrantz, 1995; Bachère et al., 1988), flow cytometry (Alcox and Ford, 1998; Hégaret et al., 2003a; Hégaret et al., 2003b; Xue et al., 2001; Allam et al., 2002) and functional assays have been also used to separate and identify haemocyte populations, from which more haemocyte types have been proposed (Pipe et al., 1997; Nakayama et al., 1997; Carballal et al., 1997; Lopez et al., 1997). However, morphological characteristics are difficult to analyse since they often vary with taxa, life stage, and with the different haemocytes sampling and culture techniques (Ittoop et al., 2001; Carballal et al., 1997). So, an ambiguity surrounding cell classification still persists and a more functional classification is needed, being one of the missing links associated with the lack of knowledge on the origin of haemocytes in bivalves.

Immunity in freshwater mussels is not only assured by haemocytes as it is complemented by the humoral components of the haemolymph such as agglutinins (lectins), antibacterial peptides and enzymes, although these elements are more extensively characterized in

marine species, where several antibacterial peptides have already been identified (Mydlarz et al., 2006).

Nowadays it is fundamental to avoid lethal methods to study the freshwater bivalve species. It is thus essential to assure the health status of the organisms by using appropriate sampling techniques to collect small samples of haemolymph avoiding damaging their integrity (Gustafson et al., 2005). The new technologies used to study vertebrate blood cells, such as flow cytometry, which allow the analysis of large numbers of cells and can be coupled with other techniques such as fluorescence labelling, may contribute to haemocytes characterization and fill some of the gaps in the knowledge on cells differentiation, function and respective classification. In the present study, nonlethal methods and a multi-technique approach were used to characterize *Anodonta anatina* and *Anodonta cygnea* circulating haemocytes, and establish an easier and more accurate classification of haemocyte populations.

Materials and Methods

Animals collection

The two species of freshwater bivalves used in this study, were collected in northern Portugal. *Anodonta cygnea* was collected from the "Barrinha" lagoon (Mira-Portugal - 40° 27′ 22″ N, 8° 48′ 7″ W) and *A. anatina* from the Tâmega River near Mondim de Basto (41º 24′ 52″ N, 7° 57′ 51″ W). They were kept in aerated tanks with dechlorinated water and were acclimatised in these conditions for two weeks. The animals were fed daily with a microalgae diet. The organisms were considered healthy if the surface of the shell was smooth and shiny and if they closed the valves when disturbed.

Haemolymph collection

Haemolymph from ten organisms of each species (*A. cygnea* and *A. anatina*), was carefully extracted using a 21G needle (Braun) attached to a 2 mL sterile syringe (Braun), by insertion between the valves across the inner layer of the mantle into the intraepithelial space. Each haemolymph tube was maintained on ice, immediately after collection, to avoid aggregation (Gagnaire et al., 2004; Silva et al*.,* 2000) and an anticoagulant solution of N-ethylmaleimide 0,05 M was added in a proportion of 1/10 of total volume (Hinzmann et al., 2013).

Light Microscopy

Haemocytes morphology and cell counts

Haemocytes were immediately analysed after collection. Briefly, fresh cell preparations of each of the previously collected samples were made and observed under a light microscope (Olympus BX 41) coupled with a digital camera (Olympus DP70). General differentiation of cell types was done and respective measurements of cell and nuclei diameters were taken. For cell counting an improved Neubauer haemocytometer (Marienfeld) was used. The relative percentage of the different haemocyte populations was calculated based on the results obtained for 10 animals, and the cell measurements were made on a minimum of 100 cells.

Light microscopy coloration

Haematoxylin-eosin and May-Grünwald Giemsa

Haemolymph was collected as described above and an aliquot was set onto a glass slide for adhesion. The coloration technique was conducted as described in the procedure of Hemacolor® (Merck, Kenilworth, NJ, USA) and May-Grünwald's eosin methylene blue for microscopy (Merck) for air dried smears fixed with methanol 100%. In the end, slides were washed and let to dry. Prior to observation in the light microscope, slides were mounted with DPX (Merck) for long term preservation.

Transmission Electron Microscopy

A haemolymph pool from three organisms was assessed for each species, to reduce individual variation. The cells were fixed in 2.5% glutaraldehyde (Merck) in 0.1 M cacodylate (Sigma, St Louis, MO, USA) buffer, pH 7.2 for 2 h at 4 ºC. The cells were then centrifuged at 3000 rpm for 10 min at 4 ºC and washed twice in cacodylate buffer. The pellet was fixed in 1% osmium tetroxide (TAAB, Sigma) in 0.1 M cacodylate (Sigma) buffer for 1 h and then washed in cacodylate buffer and dehydrated in crescent ethanol (Merck) solutions (30%, 50%, 70%, 90%, 95%, 100%), 15 min for each, at 4 ºC until the 95% ethanol solution and then two washes at room temperature for the final concentration). Then propylene oxide 100% (Sigma) was added to the cells prior to impregnation in epoxy resin (Fluka, St Louis, MO, USA). Semi-thin sections were stained with methylene blue

and observed under a light microscope. Thin sections were stained with uranyl acetate (Sigma) and lead citrate (Sigma) and observed in a Jeol 100 CXII (JEOL, Inc, Peabody, MA, USA) transmission electron microscope.

Flow cytometry analysis

Flow cytometry analysis was performed using a EPICS XL flow cytometer equipped with a 488 nm laser and the standard filter setup using the EXPO32ADC software (Beckman Coulter, >Miami, FL, USA). PBS was used as sheath liquid. Data were analysed using the Flowjo 10.1 software (Tree Star, Ashland, OR, USA).

Haemolymph was collected as described above and filtered to remove aggregates and detritus. Samples were incubated with propidium iodide (PI, Sigma), a marker of membrane integrity, at 5 µg/ ml for 5 min at room temperature prior to acquisition to determine cell viability. Fresh haemocyte samples from 24 individuals of each species were analysed based on forward Scatter (FSC), Side Scatter (SSC) parameters and, when suitable, on green channel fluorescence.

Anodonta cygnea Haemocyte Sorting

Haemolymph from two *Anodonta cygnea* individuals was collected as described above, and filtered through 100 µm pore filter units. Haemocytes were sorted by flow cytometry in a FACSAria II cell sorter equipped with the FACSDiva software (Becton Dickinson, San Jose, CA, USA) based on FSC and SSC parameters. One thousand sorted cells of each population were spoted onto microscope slides, fixed with methanol 100%, stained with Haematoxylin-eosin, washed and let to dry. At least 10 spots were collected per population, per animal. Prior to observation in the light microscope, slides were mounted with DPX (Merck) for long term preservation.

Lectin labelling

To assess lectin binding to haemocytes, 2×10^5 freshly collected haemocytes were incubated for 15 min in the dark on ice with each of the following fluorescein conjugated-lectins: wheat germ agglutinin (WGA) at 5 µg/mL (Sigma), peanut agglutinin (PNA) at 2 µg/mL (Sigma) and phytohemagglutinin-L (PHA-L) at 2 µg/mL (Vector Laboratories, Burlingame, CA, USA). Fluorescein-conjugated streptavidin at 2 µg/mL (Vector) was as a negative control of fluorescence. Propidium iodide (PI) was added at the end of the

incubation period to exclude dead cells. Cells were analysed by flow cytometry based on FSC, SSC and green channel fluorescence parameters.

Fluorescence Microscopy of lectin-labbeled cells

Haemocytes were extracted as mentioned previously, aliquots were incubated for 15 min with fluorescein-conjugated WGA, PNA and PHA-L at the concentrations mentioned above. After this incubation, cells were centrifuged for 5 min at 1100 rpm in a Cytospin 3 (Thermo Fisher Scientific Inc.;USA), cytospins were air dried for 10 min, fixed with paraformaldehyde 4% for 10 min, washed with PBS 0.5 mM, and mounted with 15 µl Vectorshield mounting medium with DAPI and phalloidin. Cytospins were kept at 4ºC for 30 min prior observation under the fluorescence microscope Olympus BX 40 with the digital photocamera DP70, and DAPI, FITC and TRITC filters (Olympus). Combined images from DAPI, FITC, and TRITC were made to assess lectin labelling of the cells.

Statistics

Statistical analysis was performed using GraphPad software (Version 6.0, GraphPad Software Inc, La Jolla, CA, USA). Unless otherwise indicated, statistical analysis between populations was performed using unpaired two-way ANOVA followed by Sidak's multiple comparisons test. Column graphs are represented showing means plus one SD. *P*-values lower than 0.05 were considered statistically significant.

Results

Haemocyte analysis

Microscopy - Haemocyte morphology and relative abundance

Live cells were observed under light microscopy for measurement and counting, so that their characteristics were kept as intact as possible. Two main cell types were distinguished, in both studied species (Table 1, Figures 1A, 1B1 and 1B2, 2A and 2B1 and 2B2). After adhesion haemocytes comprising the larger and more abundant cell type, tended to form thin extensions (pseudopods) and to aggregate (Figures 1A and 2A). These cells also presented an eccentric nucleus, few to several refringent vacuoles in the cytoplasm and granules that were not always visible, and were designated here as

granulocytes. The other visible cell type was composed by smaller and less abundant cells, with a rounder shape, a centric nucleus, low cytoplasm content, less refringent vacuoles and absence of granules. These cells were designated as hyalinocytes.

In *A. cygnea* the population of granulocytes comprised a mean of 70% of total cells found in the haemolymph. The remaining cells were hyalinocytes (30.3±17.93 cells/ml). In *A. anatina* the predominance of granulocytes was more marked, around 86%, but these values varied between organisms. A high individual variability among biochemical and cellular parameters is common when working with wild mussels, and was more evident in *A. cygnea*, which presents a larger deviation from the mean values. In terms of abundance variability also occurred. The number of cells varied substantially among organisms, but in average, the haemolymph of *A. cygnea* had more cells, around 8×10^5 cells/ ml, while in *A. anatina* the number was around $5{,}5 \times 10^5$ cells/ ml.

The granulocytes in both species varied considerably in size. These cells presented a diameter in the range 10-20 µm, low nucleus/cytoplasm ratio and a more constant nucleus diameter; some cells also presented extensions that varied in size and shape, from small and thin to long and thicker projections, designated as pseudopods. Among the hyalinocytes, the dimensions were more constant, the cells were smaller, rounder and with a higher nucleus/cytoplasm ratio. The majority of the cytoplasm was occupied by the nucleus. Granulocytes were slightly smaller in *A. anatina* when compared with *A. cygnea* and an inverse pattern occurred with hyalinocytes (Table 1).

The morphometric measures and abundances data supported the classification adopted to characterize the haemolymph haemocytes from both *A. cygnea* and *A. anatina*. The established groups of cells were significantly different for both species (Table 1).

Table 1 – Comparison of haemocytes from *Anodonta anatina* and *Anodonta cygnea,* by microscopy.

			A. anatina		***A. cygnea***	
		Haemocyte type	**Granulocytes**	**Hyalinocytes**	**Granulocytes**	**Hyalinocytes**
Light Microscopy	**Proportion**	abundances (cells/ml x 10^5)	4,72 ± 1,18	0,74 ± 0,19	5,88 ± 1,74	2,78 ± 1,82
		percentage (%)	86,5 ± 0,93	13,5 ± 0,93	69,97 ± 17,93	30,3 ± 17,93
	Cell	diameter (µm)	13,61 ± 1,55***	10,47 ± 0,73	15,24 ± 2,46***	8,92 ± 1,14
	Nucleus	shape	oval to spherical	spherical	oval to spherical	spherical
		position	eccentric	centric	eccentric	centric
		diameter (µm)	5,57 ± 0,50*	5,70 ± 0,43	5,84 ± 1,06***	6,35 ± 1,05
	Cytoplasm	pseudopod	prominent	few or none	long and thin	none
	N/C ratio		0,41 ± 0,05***	0,55 ± 0,06	0,39 ± 0,07***	0,71 ± 0,09
	n		164	22	100	85
	Other		mobile, with tendency to aggregate	rare, no mobile, without tendency to aggregate	mobile, with tendency to aggregate	no mobile, without tendency to aggregate
	Staining	Hemacolor	basophilic and acidophilic	basophilic	basophilic	basophilic
		May_Grunwald's eosin methylen	basophilic with purple granules	basophilic	basophilic with pink granules	basophilic
		Blue methylene in semi-thin sections	many large granules	centric nucleus	many granules	centric nucleus
Electron Microscopy	**Granules**	size	many large	few to none	several	few to none
		shape	variable	round	variable	round
	Nucleus	Chromatin	dense	dense	dense	dense
Statistics	Granulocytes vs Hyalinocytes where * p<0.05, **p<0.01 and *** p<0.001					

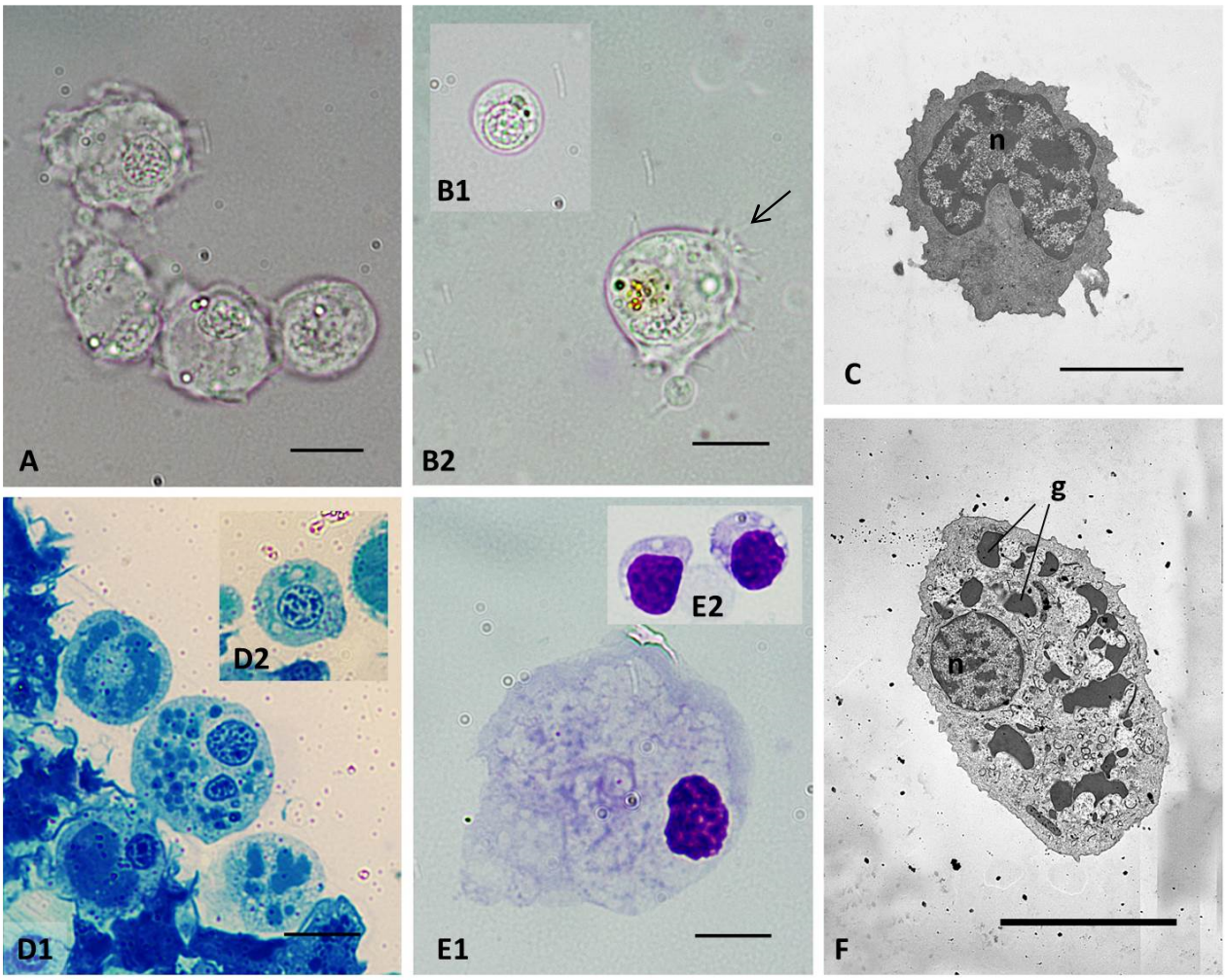


Figure 1 - Microscopy analysis of *Anodonta cygnea* haemocytes. Images **A**, **B**, **D** and **E** were obtained using light microscopy. **A**. Aggregate of live granulocytes, **B**. Live haemocytes: B1 hyalinocytes and B2 granulocyte (arrow marking the presence of pseudopods). **D**. Semi-thin section stained with methylene blue, D1 granulocytes and D2 hyalinocyte; **E**. Cells stained with hemacolor, E1 granulocyte and E2 hyalinocytes; In light microscopy images scale bars correspond to 10 µm. Transmission electron microscopy images: **C**. hyalinocyte, scale bar corresponds to 5µm and **F**. granulocyte, scale bar correspond to 10 µm; n – nucleus and gr – granules.

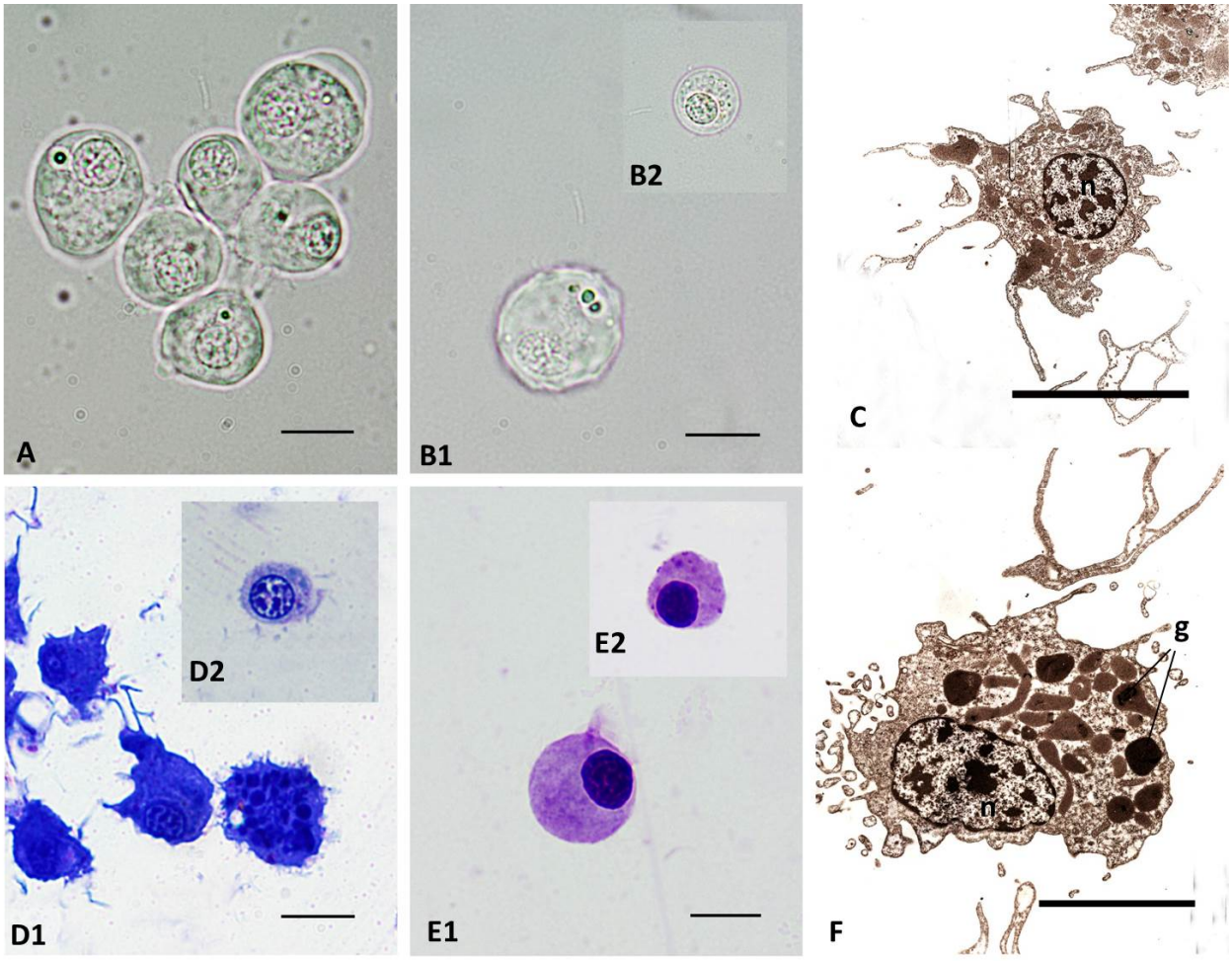


Figure 2 - Microscopy analysis of *Anodonta anatina* haemocytes. Images **A**, **B**, **D** and **E** were obtained using light microscopy. **A**. Aggregate of live granulocytes, **B**. Live haemocytes: B1 granulocyte and B2 hyalinocyte. **D**. Semi-thin section stained with methylene blue, D1 granulocytes and D2 hyalinocyte; in **E**. Cells stained with hemacolor, E1 – granulocyte and E2 – hyalinocyte. Transmission electron microscopy images: **C**. hyalinocyte and **F**. granulocyte, n – nucleus and gr – granules. Scale bars correspond to 10 µm.

Light microscopy observation of stained cells

Staining with May-Grünwald blue methylene or Hemacolor® on spontaneously adhering cells also revealed the two main types of haemocytes: granulocytes and hyalinocytes. The cytoplasmatic granules were not always visible by light microscopy, probably because the staining procedure favours degranulation, being only possible to infer about the acidophilic or basophilic properties of the cells, given by the coloration of the cytoplasm. In *A. cygnea*

most cells from the haemolymph presented a violet coloration, indicating that most of the granulocytes and hyalinocytes have a basophilic content (Figures 1D1 and 1D2). In these species, few acidophilic granulocytes and hyalinocytes were detected. Similar results were obtained for *A. anatina*. However, using the May Grunwald's eosin methylene coloration technique the granules of *A. anatina* were in some cases slightly more evident (data not shown). In fact, with light microscopy the granules of *A. cygnea* were only visible in the thin sections stained with methylene blue (Figure 1F); this coloration technique was also suitable to visualize the granules of *A. anatina* granulocytes (Figure 2F).

Transmission electron microscopy

Under transmission electron microscopy, the same cell types were found for both species and their organelles could be analysed in more detail (Figures 1A, 1B, 2A and 2B). Granulocytes (Figures 1B and 2B) display eccentric nuclei with large clumps of chromatin, a few thin cytoplasmic projections, and a low nucleus/cytoplasm ratio. Variable numbers of mitochondria, Golgi complexes, vesicles and cytoplasmic granules were simultaneously contained in the cytoplasm. The granules were electron-dense and electron-lucent and the shape and size varied. Granulocytes had more-prominent pseudopods, and residual bodies were occasionally detected in the cytoplasm. The hyalinocytes of both species examined (Figures 1A and 2A), contrarily to granulocytes, had few to no cytoplasmic granules. The nuclei were larger than in granulocytes, with stippled chromatin, surrounded by long profiles of rough endoplasmic reticula.

Flow cytometry

Flow cytometric analysis of fresh haemocytes also revealed two viable populations differing in size and granulosity/internal complexity according to FCS and SSC parameters, respectively (Figures 3A and 4A). Since these parameters correlated to morphological characteristics of these two distinct cell populations previously observed by light microscopy techniques, the two populations of haemocytes collected from *A. cygnea* were sorted by flow cytometry and observed under light microscopy upon hemacolor staining. This allowed identifying the cell population with the smallest size and lowest granulosity gated in R1 as hyalinocytes and of the other cell population with higher size and granulosity, gated in R2, as granulocytes (Figure 3B).

The identification of the two populations detected by flow cytometry also allowed the evaluation of the relative abundance of viable hyalinocytes and granulocytes. Although wide inter- and intra-mussel variability could be observed, a significantly higher percentage of hyalinocytes was found in *A. cygnea* comparatively to *A. anatina* (Figures 4A and 4B). These relative abundances were consistent with the ones found by light microscopy analysis. The relative percentage of hyalinocytes in *A. anatina* was nevertheless slightly lower when evaluated by flow cytometry.

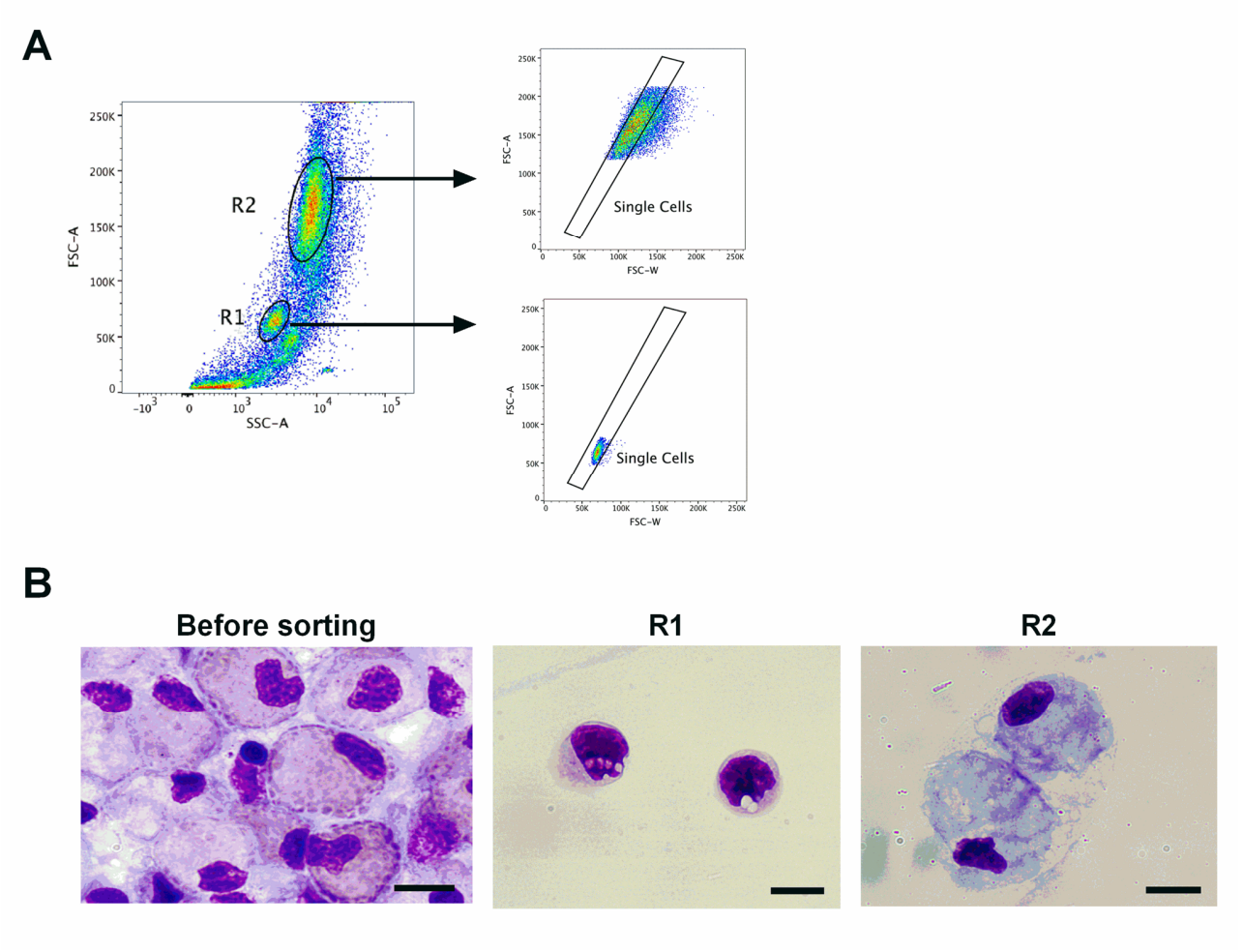


Figure 3 - Flow cytometry sorting of haemolymph cells from *A. cygnea*. **A.** Representative dot plots showing the gating strategy used to sort the two distinct cell populations, as assessed by flow cytometry in Region 1 (R1) and Region 2 (R2), respectively. **B.** Representative pictures of unsorted and sorted cells, as indicated, stained with hemacolor and observed in a bright-field microscope. Bar = 10 µm.

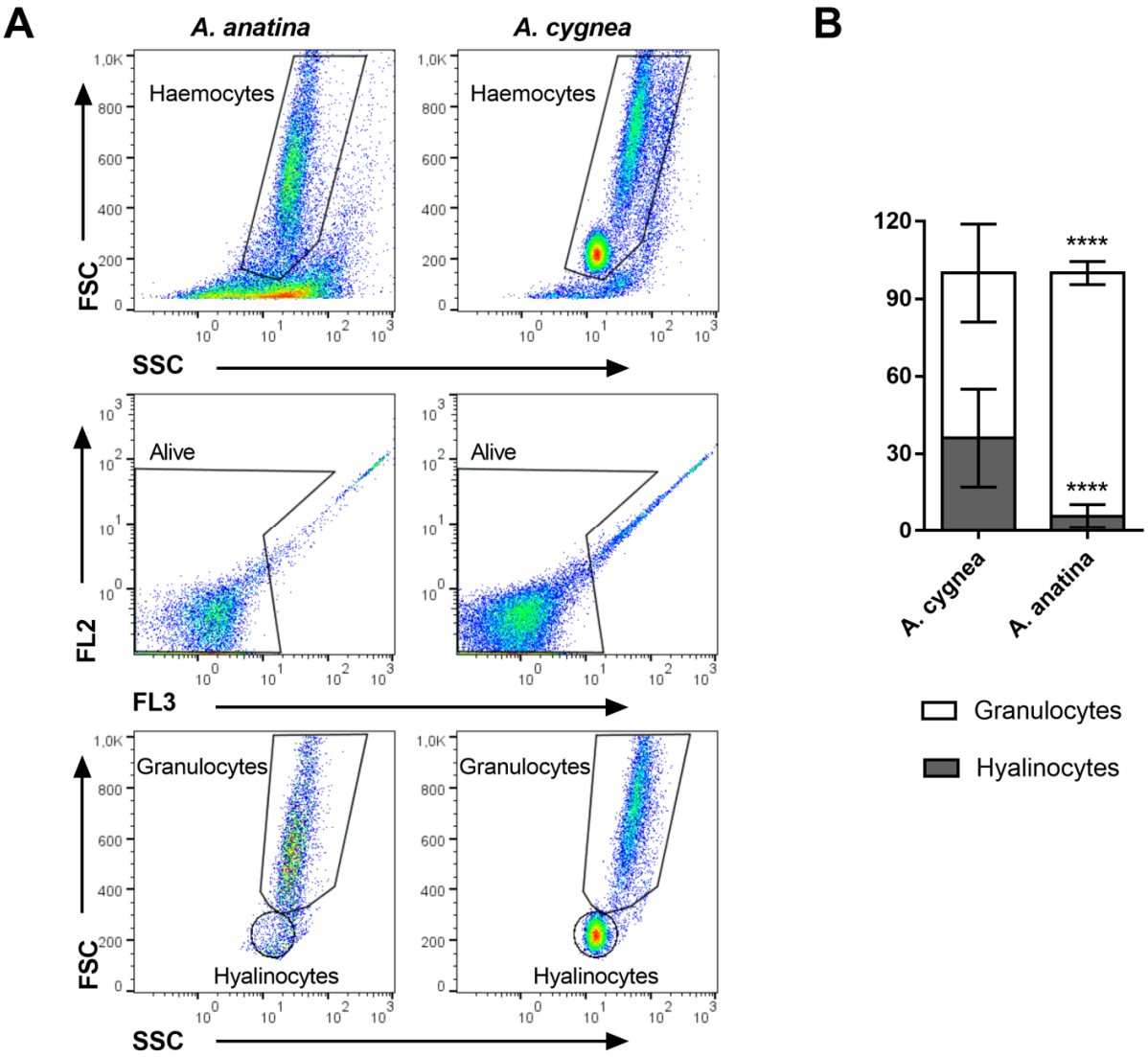


Figure 4 A - Representative example showing the gating strategy used to discriminate by flow cytometry live hyalinocytes and granulocytes from the haemolymph of *A. anatina* and *A. cygnea*. **B.** Percentage of hyalinocytes and granulocytes assessed by flow cytometry in the haemolymph of *A. anatina* and *A. cygnea*, as indicated. Bars represent means ± SD. **** *P*<0.0001, Two-way ANOVA followed by Sidak's multiple comparisons test. (n=24/group).

Lectin binding analysis

Lectin binding to surface or intracellular polysaccharides has been previously used to characterize bivalve haemocytes, since different types of haemocytes frequently display distinct lectin binding patterns or levels. Here, the extent to which WGA, PHA-L and PNA bound to *A. anatina* and *A. cygnea* haemocytes was evaluated by flow cytometry and confirmed by fluorescence microscopy. Granulocytes from live *A. cygnea* exhibited two distinct WGA binding levels, while granulocytes from live *A. anatina* had roughly the same fluorescence intensity due to WGA labelling. Contrastingly, WGA bound live hyalinocytes from both species to a much lower extent (Figure 5A). In general, WGA total staining intensity was higher in *A. cygnea* than in *A. anatina* (Figure 5C). The disparate binding of WGA to hyalinocytes and granulocytes was confirmed by fluorescence microscopy (Figure 5B). PNA and most notably PHA-L bound haemocytes to a lower extent than WGA (Figure 5C). No differences were observed between the fluorescence intensity detected in granulocytes and hyalinocytes due to PNA or PHA-L binding. Also, no notorious differences were found in the staining due to PNA binding between *Anodonta* species, although the mean fluorescence intensity (MFI) of PHA-L was slightly higher in *A. cygnea* than in *A. anatina* (Figure 5C).

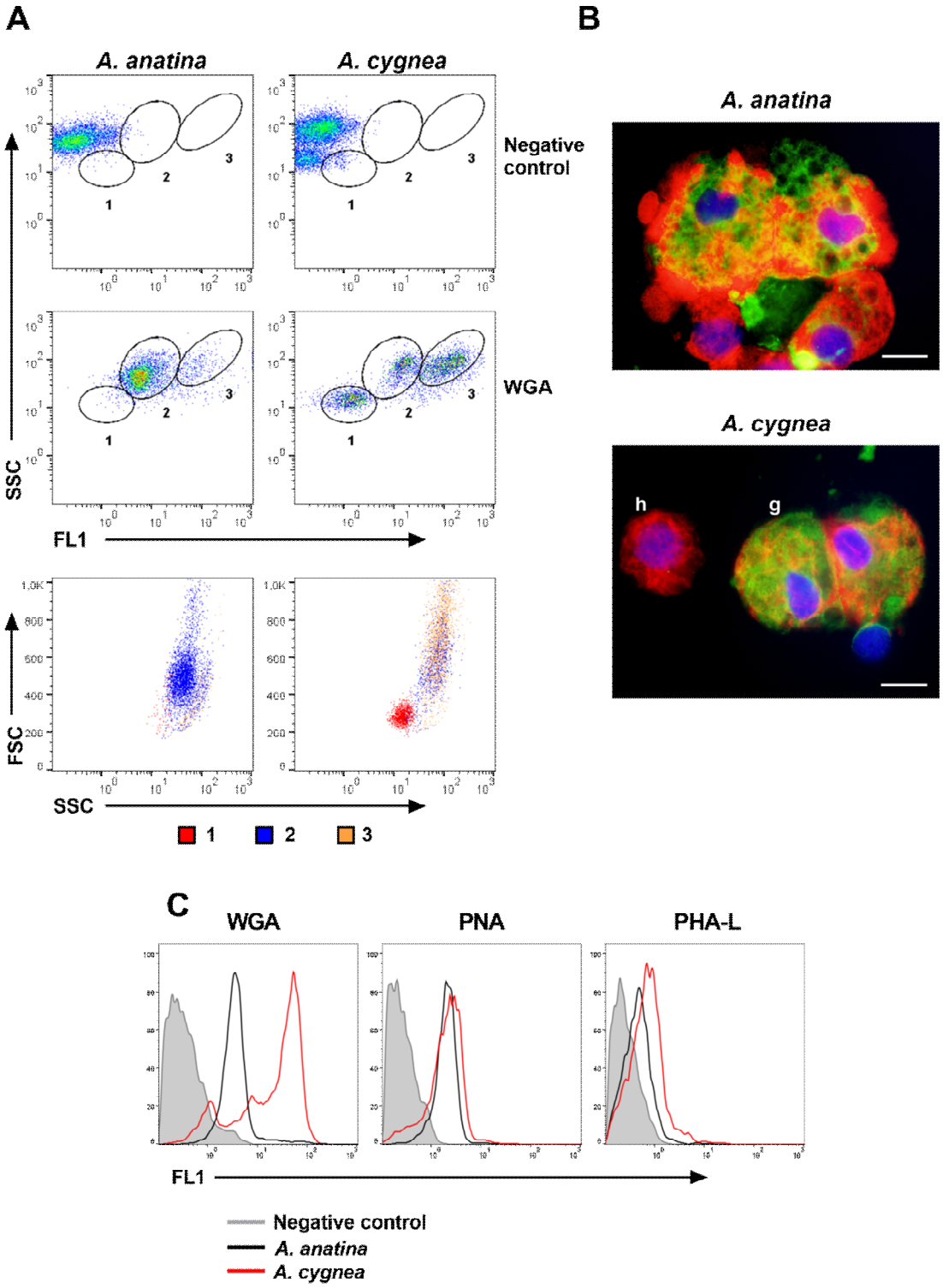


Figure 5A - Representative example of flow cytometry analysis showing WGA staining of live cells from the haemolymph of *A. anatina* and *A. cygnea*. Gate 1 corresponds to cells with FSC and SSC parameters characteristic of hyalinocytes while gates 2 and 3 consist of cells displaying granulocyte-type parameters. **B.** Fluorescence microscopy image representative of haemocytes collected from *A. anatina* and *A. cygnea* after staining with FITC-conjugated WGA. DAPI was used

to stain nuclei and phalloidin-TRITC was used to stain F-actin filaments. Bar = 10 µm; h-hyalinocyte and g-granulocyte. **C.** Histogram overlays showing the fluorescence resulting from the staining of *A. anatina* and *A. cygnea* haemocytes, as indicated, with fluorescein-conjugated WGA, PNA and PHA-L and analysed by flow cytometry. Negative controls correspond to cells incubated with FITC-conjugated streptavidin.

Discussion

Invertebrate animals, which lack an adaptive immune system, have developed a defence system that responds to common antigens on the surface of potential pathogens (Mitta et al., 1999). One of the main components of their immune system is composed by the cells circulating in their body fluids, the haemocytes. These are involved in several immune functions such as phagocytosis, detoxification processes or even in the formation of microspheres (Hinzmann et al., 2014). In the present work, cells from the haemolymph were analysed in detail and their main morphologic features were described.

To distinguish among groups of bivalve haemocytes, morphological data are generally used. However, the nomenclature of haemocyte types may vary considerably depending on the used techniques. Different techniques focus on different parameters resulting in the establishment of different groups, what difficult reaching a consensus terminology. Morphological criteria are the basis of the majority of studies addressing the characterisation of haemocytes. However, the nomenclature adopted is ambiguous and inconsistent among researchers, varying according to the researcher and the used technique (Cheng, 1981; Salimi et al., 2009). Based on morphological data, Cheng, 1981, already proposed the classification of invertebrate haemocytes dividing these cells in two main types: granulocytes and hyalinocytes. However, Hine (1999) pointed out that classifying haemocytes into granular and agranular forms was too simplistic, posing great constraints in our understanding of haemocytes functions.

Here a detailed microscopic and flow cytometric analysis was presented of haemolymph haemocytes from *A. anatina* which complements previous reported data on *A. cygnea* haemolymph haemocytes (Soares-da-Silva, 2002; Salimi et al., 2009). Both species presented similar haemocyte types – granulocytes and hyalinocytes – easily differentiated by microscopy, although with abundances high variability among specimens, influenced by natural, anthropogenic or stress non controllable factors (Wootton et al., 2003; Pipe and Coles, 1995). Soares-da-Silva et al., 2002, had already reported the presence of these same types of haemocytes in *A. cygnea*. Under light microscopy the granules were not

always observed, this effect might be attributed to the anticoagulant solution used that, as most anticoagulants, favour degranulation (Burkhard et al., 2009). Nevertheless, its use was fundamental to disaggregate the cells. Smith and Soderhall, 1983, found that the haemocytes of the freshwater crayfish *Astacus astacus* easily showed profound degranulation and lysis under stress conditions. Therefore, a similar process may occur in the haemocytes of *Anodonta* species. Consistent with this hypothesis, granules were easily observed by electron microscopy in these species. Most of the cells of *A. cygnea* showed basophilic properties, although some acidophilic cells were also found. The presence of granules a characteristic of the granulocytes was not always visible in these species what we assume to be caused by some of the components of the coloration technique that may favour the degranulation process. Nonetheless, their presence was proved using blue methylene in semi-thin sections. *A. anatina* haemocytes showed basophilic properties, and the granules stained well with May-Grünwald blue methylene and blue methylene coloration. Cheng, 1981, suggested that acidophilic granulocytes are the more mature cells and are probably originated from basophilic granulocytes which are partially in line with the results from Lin et al., 2013, showing that eosinophilic granulocytes are more active phagocytes than basophilic granulocytes. Similar two distinct cell populations were also identified in other bivalve species, although some authors also include an intermediate group of granulocytes, usually more heterogeneous, comprised of medium-sized cells with few granules. The reports cover species as different as the clams *Mya arenaria* (Huffman et al., 1982), *Mercenaria mercenaria* (Pipe et al., 1997; Allam et al., 2002), *Ruditapes philippinarum* (Allam et al., 2002), the mussels *Mytillus edulis* (Pipe et al., 1997) and *Mytillus galloprovincialis* (Cajaraville and Pal, 1995), the oysters *Ostrea edulis* (Bigas et al., 2006; Cochennec-Laureau et al., 2003) and *Crassostrea virginica* (Allam et al., 2002; Goedken and Guise, 2004) and *Crassostrea rhizophorae* (Rebelo et al., 2013), and the deep sea mussels of the *Bathymodiolus* genus (Bettencourt et al., 2010; Tame et al., 2015).

A constraint of microscopy is that it is very time consuming and limits the study to a small number of cells (Goedken and Guise, 2004) when compared with flow cytometry analysis. Through flow cytometry more populations of cells could be analysed and sub-populations could be discriminated, namely profiting from distinct lectin binding profiles. Moreover, the granularity of the cells is more accurately assessed by using this technique, since different degrees of granularity can be discriminated (Alcox and Ford, 1998). In the present study the two cell types identified for both *Anodonta* species by using light microscopy were also

detected by flow cytometry. Flow cytometric assisted cell sorting of the two distinct haemocyte populations further confirmed the identification of hyalinocytes and granulocytes in *A. cygnea*. This technique has been widely used to confirm the identification of the haemocyte populations distinguished by FSC and SSC parameters (Alcox and Ford, 1998; Allam et al., 2002; Goedken and Guise, 2004; Rebelo et al., 2013). The correlation between microscope and flow cytometry estimates for cell population's relative percentages was high, which is in accordance with previous reports (Alcox and Ford, 1998; Allam et al., 2002). Thus, characterisation of the haemocytes by flow cytometry will enable a quicker, simpler and less subjective quantification of these cells. This technique also allows the characterization of the glycoconjugates composing the surface membranes of haemocytes by using fluorescence-labelled lectins with defined sugar-binding specificities. Here we have shown that different lectins bound the surface of *Anodonta* haemocytes at different extents and even to distinct cell types. While WGA only stained granulocytes, PNA indistinctly labelled the two cell types with lower staining intensity. In contrast, PHA-L bound very weakly to both cell types. The disparate ability of different lectins to bind bivalve haemocytes has already been noticed in previous reports. WGA was shown to bind the plasma membrane of granulocytes from the clam *Tapes semidecussata* (Montes et al., 1995), the mussel *Mytillus edulis* and species of the genus *Bathymodiolus* (Bettencourt et al., 2010; Tame et al., 2015) but not to hyalinocytes. Although we have shown some fluorescence intensity of *Anodonta* haemocytes due to PNA binding, it was not found in *Mytillus edulis* (Pipe, 1990; Wootton et al., 2003), *Cerastoderma edule* and *Ensis silique* (Wootton et al., 2003). The majority of the studies also indicate that PHA-L had no affinity with the membrane of bivalve haemocytes (Tame et al., 2015). Consistent with these results, Kannaley and Ford reported in 1990 that WGA was able to agglutinate oyster haemocytes while PHA was not. Curiously, here we observed that WGA labelled *A. cygnea* granulocytes with two distinct fluorescence intensities, which contrasts the uniform staining of WGA on *A. anatina* that was also reported for *Mytillus edulis* granulocytes (Pipe, 1990). Tame et al. (2015) in a study with *Bathymodiolus* species reported that WGA bound to two distinct types of granulocytes but made no reference to differences in intensity staining between them. It could be hypothesized that lectin binding strength could reflect physiological states of haemocytes, since binding affinities of *Drosophila* lamellocytes to WGA were correlated with intracellular Ca^{2+} concentrations (Tirouvanzian et al., 2004). Whether the relative abundance of N-acetylglucosamine and/or N-acetylneuraminic acid, the ligands of WGA, on the surface of

A. cygnea granulocytes has a physiological role remains unknown and would be worth exploring.

In conclusion, we characterized *A. anatina* and *A. cygnea* haemocytes in detail and have shown that two major cell populations, hyalinocytes and granulocytes, could be identified by microscopy and also by flow cytometry. The last technique proved a reliable and much easier and faster technique than microscopy for the characterization of cell relative abundance. We have also shown that haemolymph cell populations exhibited distinct interspecific lectin binding, as well as differences in binding among cells of a single individual. Further studies exploring the glycoconjugates composing the membranes of the haemocytes and their potential physiological consequences could extend our knowledge on these important cells of the innate defence of bivalves.

Acknowledgments

This work was supported by the projects PTDC/AAC-AMB/117688/2010 CONBI and PTDC/MAR/098066/2010 SYNERG and the research grant (SFRH / BD / 76265 / 2011) from FCT - Portuguese Foundation for Science and Technology. We would also like to thank to Elsa Oliveira from Cellular Biology Laboratory from ICBAS and Catarina Leitão from I3S for microscopy and cytometry expertise respectively.

References

Allam, B., Ashton-Alcox, K.A., Ford S.E., 2002. Flow cytometry comparison of haemocytes from three species of bivalve molluscs. Fish & Shellfish Immunology 13, 141-158.

Alcox, K.A., Ford, S.E., 1998. Variability in molluscan hemocytes a flow cytometry study. Tissue & Cell 30(2), 195-204.

Bachère, E., Chagot, D., Grizel, H., 1988. Separation of *Crassostrea gigas* hemocytes by density gradient centrifugation and counterflow centrifugal elutriation. Developmental & Comparative Immunology 12, 549-559.

Bauer, G., 1988. Threats to the freshwater pearl mussel *Margaritifera margaritifera* in central. Europe. Biological Conservation 45, 239-253.

Bettencourt R., Pinheiro, M., Egas, C., Gomes, P., Afonso, M., Shank, T., Santos, R.S., 2010. High-throughput sequencing and analysis of the gill tissue transcriptome from the deep-sea hydrothermal vent mussel *Bathymodiolus azoricus*. BMC Genomics 11.

Bigas, M., Durfort, M., Poquet, M., 2006. Cytological response of hemocytes in the European flat oyster, *Ostrea edulis*, experimentally exposed to mercury. Biometals 19, 659-673.

Bogan, A.E., 1993. Freshwater bivalve extinctions (Mollusca: Unionoida): a search for causes. American Zoologist 33, 599–609.

Brand, A.R., 1972. The mechanisms of blood circulation in *A. anatina* (L) (Bivalvia, Unionidae). Journal of Experimental Biology 56, 361-379.

Burkhard, M., Leavell, S., Weiss, R., Kuehnl, K., Valentine, H., Watters, G., Wolfe, B., 2009. Analysis and cytological characterization of hemocytes from freshwater mussels (*Quadrula* sp.). Veterinary Clinical Pathology 38 (4), 426-436.

Cajaraville, M., Pal, S., 1995. Morphofunctional study of the hemocytes of the bivalve mollusk *Mytilus galloprovincialis* with emphasis on the endolysosomal compartment. Cell Structure and Function 20, 355-367.

Carballal, M., Carmen, L., Carlos, A., Villalbal, A., 1997. Hemolymph cell types of the mussel *Mytilus galloprovincialis*. Diseases of aquatic organisms 29, 127-135.

Chang, S.J., Tseng, S.M., Choi, H.Y., 2005. Morphological characterization via light and electron microscopy of the hemocytes of two cultured bivalves: a comparison study between the hard calm (*Meretrix lusoria*) and Pacific oyster (*Crassostrea gigas*). Zoological Studies 44, 144–153.

Cheng, T.C., 1981. Bivalves. In: Invertebrate Blood Cells. Vol. I. Ratcliffe NA, Rowley AF, editors. Academic Press. London, pp 231-300.

Cochennec-Laureau, N., Auffret, M., Renault, T., Langlade, A., 2003. Changes in circulating and tissue infiltration hemocyte parameters of European flat oysters, *Ostrea edulis*, naturally infected with *Bonamia ostreae*. Journal of Invertebrate Pathology 83, 23-30.

Danilova, N., 2006. The evolution of immune mechanisms. Journal of Experimental Zoology, Part B Molecular and developmental evolution 306, 496-520.

Friebel, B., Renwrantz, L., 1995. Application of density gradient centrifugation for separation of eosinophilic and basophilic hemocytes from *Mytilus edulis* and characterization of both cell groups. Comparative Biochemistry and Physiology 112A, 81-90.

Gagnaire, B., Thomas-Guyon, H., Renault, T., 2004. *In vitro* effects of cadmium and mercury on Pacific oyster, *Crassostrea gigas* (Thunberg), haemocytes. Fish & Shellfish Immunology 16, 501-512.

Girón-Pérez, M.I., 2010. Relationships between innate immunity in bivalve molluscs and environmental pollution. Invertebrate Survival Journal 7, 149-156.

Glinski, Z., Jarosz, J., 1997. Molluscan Immune Defenses Archivum immunologiae et therapiae experimentalis 45, 149-155.

Goedken, M., Guise, S., 2004. Flow cytometry as a tool to quantify oyster defense mechanisms. Fish & Shellfish Immunology 16, 539-552.

Ittoop, G., George, K., Sanil, N., Sobhana, K., Nisha, P., 2001. Maintenance of haemocytes of edible oyster*, Crassostrea madrasensis* (preston) in artificial media. Indian Journal of Fisheries 48(2), 177-182.

Graf, D., Cummings, K., 2007. Review of the systematics and global diversity of freshwater mussel species (Bivalvia: Unionoida). Journal of Molluscan Studies 73, 291-314.

Gustafson, L., Stoskopf, M., Bogan, A., Showers, W., Kwak, T., Hanlon, S., Levine, J., 2005. Evaluation of a nonlethal technique for hemolymph collection in *Elliptio complanata*, a freshwater bivalve (Mollusca: Unionidae). Diseases of Aquatic Organisms 65, 159-165.

Hégaret, H., Wikfors, G.H., Soudant, P., 2003a. Flow cytometric analysis of haemocytes from eastern oysters*, Crassostrea virginica*, subjected to a sudden temperature elevation: I. Haemocyte types and morphology. Journal of Experimental Marine Biology and Ecology 293(2), 237–248.

Hégaret, H., Wikfors, G.H., Soudant, P., 2003b. Flow cytometric analysis of haemocytes from eastern oysters*, Crassostrea virginica*, subjected to a sudden temperature elevation: II. Haemocyte functions: aggregation, viability, phagocytosis, and respiratory burst. Journal of Experimental Marine Biology and Ecology 293(2), 249–265.

Hine, P.M., 1999. The inter-relationships of bivalve haemocytes. Fish & Shellfish Immunology 9, 367-385.

Hinzmann, M., Lopes-Lima, M., Gonçalves, J., Machado, J., 2013. Antiaggregant and toxic properties of different solutions on haemocytes of three freshwater bivalves. Toxicological & Environmental Chemistry 95(5), 790-805.

Hinzmann, M., Lopes-Lima, M., Bobos, I., Ferreira, J., Domingues, B., Machado, J., 2014. Morphological and chemical characterization of mineral concretions in the freshwater bivalve *Anodonta cygnea* (Unionidae). Journal of Morphology 276 (1), 65–76.

Huffman, J., Tripp, M., 1982. Cell types and hydrolytic enzymes of soft shell clam (*Mya arenaria*) haemocytes. Journal of Invertebrate Pathology 40, 68–74.

Lin, T., Yao, Z., Hao, Z., Lai, Q., Zhou, K., 2013. Classification of Granulocytes in Three Bivalve Species Using Neutral Red Staining. Journal of Shellfish Research 32(3), 861-865.

Lopez, C., Carballal, M.J., Azevedo, C., Villalba, A., 1997. Morphological characterization of the hemocytes of the clam, *Ruditapes decussatus* (Mollusca: Bivalvia). Journal of Invertebrate Pathology 69(1), 51-57.

Mitta, G., Vandenbulcke, F., Hubert, F., Roch, P., 1999. Mussel defensins are synthesised and processed in granulocytes then released into the plasma after bacterial challenge. Journal of Cell Science 112, 4233-4242.

Montes, J., Durfort, M., García-Valero, J., 1995. Cellular defence mechanism of the clam Tapes semidecussatus against infection by the protozoan *Perkinsus* sp. Cell and Tissue Research 279 (3), 529-538.

Mydlarz, L.D., Jones, L. E., Harvell, C. D., 2006. Innate Immunity, Environmental Drivers, and Disease Ecology of Marine and Freshwater Invertebrates. Annual Review of Ecology, Evolution, and Systematics 37(1), 251-288.

Nakayama, K., Nomotto, A.M., Nishijima, M., Maruyama, T., 1997. Morphological and functional characterization of hemocytes in the giant clam *Tridacna crocea*. Journal of Invertebrate Pathology 69, 105-111.

Neves, R.J., Bogan, A.E., Williams, J.D., Ahlstedt, S.A., Hartfield, P.W., 1997. Status of aquatic mollusks in the southeastern United States: a downward spiral of diversity. In: Aquatic Fauna in Peril: The Southeastern Perspective. Benz GW, Collins DE, editors. Southeast Aquatic Research Institute, Decatur, GA. p 45–86.

Pipe, R.K., 1990. Differential binding of lectins to haemocytes of the mussel *Mytilus edulis*. Cell and Tissue Research 261(2), 261-268.

Pipe, R.K., Coles, J.A.,1995. Environmental contaminants influencing immunefunction in marine bivalve molluscs. Fish & Shellfish Immunology 5 (8), 581–595.

Pipe, R.K., Farley, S.R., Coles, J.Á., 1997. The separation and characterization of haemocytes from the mussel *Mytilus edulis*. Cell Tissue Res. 289, 537-545.

Rebelo, F., Figueiredo, S., Mariante, R., Nobrega, A., Barros, C.M., Allodi, S., 2013. New insights from the oyster *Crassostrea rhizophorae* on bivalve circulating hemocytes. PLoS One 8(2), e57384.

Renwrantz, L., Siegmund, E., Woldmann, M., 2013. Variations in hemocyte counts in the mussel, *Mytilus edulis*: Similar reaction patterns occur in disappearance and return of molluscan hemocytes and vertebrate leukocytes. Comparative Biochemistry and Physiology, Part A 164, 629–637.

Salimi, L., Jamili, S., Motalebi, A., Eghtesadi-Araghi, P., Rabbani, M., Rostami-Beshman, M., 2009. Morphological characterization and size of hemocytes in *Anodonta cygnea*. J. Invertebrate Pathology 101 (2), pp. 81-85.

Silva, C., Dunphy, G., Rau, M.E., 2000. Interaction of hemocytes and prophenoloxidase system of fifth instar nymphs of *Acheta domesticus* with bacteria. Developmental & Comparative Immunology 24, 367-379.

Smith, V.J., Soderhall, K., 1983. Induction of degranulation and lysis of haemocytes in the freshwater crayfish, *Astacus astacus* by components of the prophenoloxidase activating system *in vitro*. Cell and Tissue Research 233(2), 295-303.

Soares-da-Silva, I., Ribeiro, J., Valongo, C., Pinto, R., Vilanova, M., Bleher, R., Machado, J., 2002. Cytometric, morphologic and enzymatic characterization of haemocytes in *Anodonta cygnea*. Comparative Biochemistry and Physiology A 132, 541-553.

Tame, A., Yoshida, T., Ohishi, K., Maruyama, T., 2015. Phagocytic activities of hemocytes from the deep-sea symbiotic mussels *Bathymodiolus japonicus*, *B. platifrons*, and *B. septemdierum*. Fish & Shellfish Immunology 45(1), 146-156.

Tirouvanziam, R., Davidson, C.J., Lipsick, J.S., Herzenberg, L.A., 2004. Fluorescence-activated cell sorting (FACS) of *Drosophila* hemocytes reveals important functional similarities to mammalian leukocytes. Proceedings of the National Academy of Sciences of the United States of America 101(9), 2912-2917.

Wootton, E.C., Dyrynda, E.A., Ratcliffe, N.A., 2003. Bivalve immunity: comparisons between the marine mussel (*Mytilus edulis*), the edible cockle (*Cerastoderma edule*) and the razor-shell (*Ensis siliqua*). Fish & Shellfish Immunology 15(3), 195-210.

Xue, Q., Renault, T., Chilmonczyk, S., 2001. Flow cytometric assessment of haemocyte sub-populations in the European flat oyster, *Ostrea edulis*, haemolymph. Fish & Shellfish Immunology 11, 557–567.

Chapter 4

Morphological and chemical characterization of mineral concretions in the freshwater bivalve*Anodonta cygnea* (Family Unionidae*)*

Morphological and chemical characterization of mineral concretions in the freshwater bivalve *Anodonta cygnea (*Family Unionidae*)*

M. F. Hinzmann[ab], M. Lopes-Lima[b], Iulius Bobos[c], J. Ferreira[d], B. Domingues[ab] & J. Machado[ab]

[a] ICBAS - Instituto de Ciências Biomédicas de Abel Salazar, Departamento de Produção Aquática, Universidade do Porto, Rua de Jorge Viterbo Ferreira n.º 228, 4050-313 Porto, Portugal

[b] CIIMAR - Centro Interdisciplinar de Investigação Marítima e Ambiental, Universidade do Porto, Rua dos Bragas, n.289, 4050-123 Porto, Portugal

[c] FCUP - Faculdade de Ciências da Universidade do Porto, Departamento Geociências,

Rua Campo Alegre 687 / 4169 - 007 Porto, Portugal

[d] INETI - Instituto Nacional de Engenharia, Tecnologia e Inovação, I.P., Rua da

Amieira 4466-956 S. Mamede de Infesta, Portugal

Adapted from: Hinzmann, M. F., Lopes-Lima, M., Bobos, I., Ferreira, J., Domingues, B. Machado, J., 2014. Morphological and chemical characterization of mineral concretions in the freshwater bivalve *Anodonta cygnea* (Unionidae). Journal of Morphology 276 (1), 65-76.

Abstract

The freshwater mussel *Anodonta cygnea* is commonly used as model for biomineralization studies, its peculiar morphofunctional properties also make it an excellent environmental biomonitor. The first detailed study on the calcareous concretions from gill and mantle tissues, as well as fluids of the freshwater bivalve *A. cygnea,* supported by histological, scanning, spectrometry and spectroscopy analyses**.** Through these analyses, the morphology, structure and chemical characterization of these biomineral concretions were accomplished. The concretions represent a high percentage of the dry weight of these organisms. In gill tissue, it can reach up to 50% of dry weight prior to reproductive maturity. Analysis of elemental composition of the tissue concretions showed the presence of calcium and phosphate, as main components, associated with other residual elements like iron, manganese, magnesium, and zinc. Concretions are arranged in concentric alternated layers of organic and inorganic matrix. The shape and size of the concretions vary substantially, from very small, less than 1 µm diameter with very regular round structure, found mainly in the mantle tissue, to more than 50 µm length with irregular globular clusters, found predominantly in the gills. The microstructural organization is of a hydroxyapatite polymorphism in the mantle, in contrast to the gills, which exhibit irregular structure and carbonated hydroxyapatite polymorphism. These differences are supported by higher contents of dinitrogen pentoxide, magnesium, and iron in the mantle concretions, but higher contents of manganese and zinc in the gills. Furthermore, the results indicate that the mineral concretion formation in *A. cygnea* is a haemocytes reaction to particle or toxic invasions. A second relevant role, concerns close involvement of these microspherules on the adult and larval shell calcification.

Introduction

The mechanisms of solid inorganic mineral formation in living organisms are usually designated as biomineralization phenomenons. A large number of biogenic minerals are known; the most widespread biominerals are: calcium carbonate, calcium phosphate, and silicon dioxide (Lowenstam and Weiner, 1989; Epple, 2003; Bäuerlein, 2004). The highest diversity of functions for calcium carbonate and phosphate deposits are found in invertebrates, such as molluscs and crustaceans (Istin and Kirschner, 1968; Coimbra et al., 1988; Pratoomchat et al., 2002; Ziegler et al., 2007; Lopes-Lima et al., 2010). The first major function of this process is the hardening of a skeleton, as a structure to support

muscles and protect against environmental pressures (shell, cuticle) (Lowenstam and Weiner, 1989, Simkiss and Wilbur, 1989). Some other skeletal functions are associated with sensing (otoliths, auditory ossicles), and grinding of the food (teeth, grinding apparatus), and storage and detoxification (in granules) (Pynnönen et al., 1987).

The primary source of calcium used for exoskeleton calcification is from the exogenous environment, specifically the water in which most molluscs and crustaceans live (Ziegler, 2002, Lopes-Lima et al., 2008). In fact, calcium concentration in many species living in freshwater or terrestrial environments is very low and its availability may be low or even absent for different organic functions. Therefore, molluscs and crustaceans have developed different strategies to solve the problem of calcification, in particular by storing calcium during the exoskeleton calcification period (Istin and Girard, 1970; Greenaway, 1985; Pekkarinen and Valovirta, 1997; Fabritius et al., 2005; Ziegler et al., 2005; Graf and Cummings, 2007).

Freshwater mussels have several characteristics that make them ideal organisms as bioindicators (Adams and Shorey, 1998). They are widespread, abundant, large, and long-lived. As sedentary filter-feeders, they are thus very dependent on habitat conditions. The species *Anodonta cygnea* (L. 1758) is a large freshwater bivalve with an obligate parasitic larval stage on native fishes in Europe (Lima et al., 2004). These species belongs to the Unionidae, which has suffered a severe decline over the last decades (Bogan, 1993).

In general in freshwater bivalves, the embryonic and the larval shell stages occur in the gills after internal fertilization. The larval shell is composed of mainly of an organic layer of protein and chitin covered by internal calcareous layer, which is dense and quickly formed from a large calcium storage and diffusion “in loco” (Eyster, 1986; Castilho et al., 1988; Kovitvadhi et al., 2007; Lopes-Lima et al., 2009). The present species of *A. cygnea* exhibit internal mineral concretions, microspheres or external granules as common structures found in many body tissues where they usually function as a mineral reservoir essential for shell formation (Moura et al., 1999). The origin, formation process, shapes, and function of these structures may vary widely among organisms (Ziegler et al., 2002; Lopes-Lima et al., 2008). These concretions can be found in extracellular compartments, mainly within connective tissue in muscle, mantle, gills, kidney, and hepatopancreas and sometimes in the digestive gland (Marigómez et al., 2002). The concretions in *A. cygnea* are mainly of calcium carbonate or calcium phosphate composition (Moura et al., 1999), associated mainly with gills and mantle tissues, and are involved in shell calcification and regeneration of larvae and adult specimens of *A. cygnea* (Silverman et al., 1983, 1985; Machado et al.,

1988a; Neves et al., 1997; Vesk and Byrne, 1999; Moura et al., 1999, 2000; Lopes-Lima et al., 2008; Machado and Lopes-Lima, 2011).

The amount of accumulated ions and metals depends upon abiotic (metal solubility, metal speciation, and complexion) and biotic factors (growth, biochemical composition, reproductive condition, metabolism, and excretion) (Marigómez et al., 2002; Sunda and Huntsman, 1998). Calcareous concretions may also function as a detoxification pathway; toxic metals can be incorporated in the concretions, by specific molecules, as methalothioneins (Pynnönen et al., 1987). It is relevant to emphasize that the stimulus for initiating the formation process of mineral concretions are related with the response of distinct immunological defences in response to different stimuli (particulate matter or toxins). Another functional pathway is closely linked to regulation of internal pH and consequently triggers the shell or carapace constructions (Ziegler et al., 2002, Lopes-Lima et al., 2008).

Previous studies with *A. cygnea* (Lopes-Lima et al., 2008, 2009) showed that shell biomineralization depends on the body fluids, with particular focus on the mantle and gills compartments. During spring and summer, respiratory acidosis due to CO_2 and metabolic organic acids content increase induce calcium and HCO_3^- releases from the calcareous spherules in the mantle and gills (Lopes-Lima et al., 2008, 2009). High calcium and HCO_3^- gradient associated with increased permeability of the outer mantle epithelium (OME), favours the HCO_3^- and Ca_2^+ movements towards the adult shell compartment. At the end of summer (until October) the OME permeability decreases, although with the same ion gradients. Thus, this condition promotes the HCO_3^- and Ca_2^+ diffusion for the larval shell formation in gills (Lopes-Lima et al., 2008, 2009). This process reverses during autumn/winter through a reduction of $CaCO_3$ precipitation in the adult shell due to a high proton pump activity and an increase of $CaCO_3$ spherules counts in the mantle supported by respiratory alkalosis added by HCO_3^- secretion from OME. This process is a biomineralization phenomenon that appears to prepare calcium microspherules for shell calcification in larval and adult stages (Lopes-Lima et al., 2008, 2009).

Despite the fact that freshwater mussels (*Anodonta*, *Unio*, *Ligumia*) contain remarkably high levels of calcium concretions in different tissues, the marine bivalves have not been found to contain inorganic calcium external granules (Pynnönen et al., 1987; Marigómez, et al., 2002). Reports concerning the histology and fine structure of the mineral concretions in the gill and mantle of marine bivalves are almost lacking. Therefore, marine conditions

may support a different mechanism for larval and adult shell calcification than in freshwater.

It is commonly accepted that these mineral concretions are vital to the organism and some studies concerning their origin, location or composition were already completed for molluscs. However, relevant data about structure-formation mechanisms and regulation conditions are still relatively unknown. The aim was to better understand calcareous concretions in *A. cygnea,* aspects of their morphology, ultrastructure and crystal composition.

Material and Methods

Animal collection

A. cygnea used in this study were collected in the northern Portugal, in the "Barrinha" lagoon (Mira-Portugal - 40° 27′ 22″ N, 8° 48′ 7″ W) from benthic mud at 1m depth. They were kept in aerated tanks with dechlorinated water for 2 weeks, the necessary time to perform the sampling. Animals were fed daily with a microalgae diet. Under these conditions, the organisms stayed healthy as indicated by a smooth and shiny shell surface and their ability to tightly close when disturbed. Authorization to collect the bivalves was conceded by the Portuguese Public Institute – Instituto de Conservação da Natureza e das Florestas (ICNF, I.P.).

Haemolymph and Tissue collection

Sample size was always the same for all methods. Haemolymph from three organisms of *A. cygnea* was carefully extracted using a 21G needle (Braun), attached to a 2 mL sterile syringe (Braun), inserted between the valves across the inner layer of the mantle into the interepithelial space. Each haemolymph sample was maintained on ice (Soares-da-Silva et al., 2000; Gagnaire et al., 2004) and a solution of N-ethylmaleimide 0.05 M was added in a proportion of 1/10 of total volume (Hinzmann et al., 2013), to avoid aggregation. The haemolymph samples from the three organisms were prepared separately for light microscopy (LM) and mixed to obtain a significant concretion pool for electron microscopy.

To collect tissue, three *A. cygnea* specimens were anesthetized with chloral hydrate 2%, and then, rinsed with distilled water to remove extraneous material. Tissue samples of 1 cm^2 were removed from the gills and mantle with forceps and scalpel and rinsed with

distilled water to remove sediment particles. Tissue samples were then transferred to sterile dishes. Some subsamples were dried until a constant weight was reached, to be used in further analyses; some were kept at -20 ºC for posterior pooled concretion extraction, and others tissues were processed for *in loco* concretion observation by electron microscopy.

Concretions were prepared using two techniques (pooled samples and *in loco* tissue samples), as required by the specific analyses used. Samples from three individuals were to reduce individual variation and to facilitate the observations mainly from mantle tissues.

Concretions extraction

Concretions were isolated from the tissue samples according to Silverman et al. (1983). The tissues were washed, homogenized in a small amount of water, and sonicated. The homogenate was heated for 2 min at 100 ºC. An equal volume of NaOH 1 N was then added and the mixture was incubated at 60-70 ºC for 1 h. The calcareous material was separated by centrifugation (5 min. at 4,000g) and the pellet obtained suspended in NaOH 1 N, followed by another centrifugation. This procedure was repeated twice with NaOH followed by at least three washes with distilled water. The final pellet obtained was dried until constant dry weight. The mineral pooled concretions were kept for further analysis.

Light Microscopy

Preparations of cells from fresh haemolymph and semithin sections stained with methylene blue figures were observed under a light microscope (Olympus BX41) coupled with a digital camera (Olympus DP70), for morphological (size and shape) characterization of concretions.

Transmission Electron Microscopy study on haemolymph, gill, and mantle concretions

The haemolymph and tissue samples were fixed to 2.5% glutaraldehyde (Merck) in 0.1 cacodylate (Sigma) buffer, pH 7.2 for 2 h at 4º C. To isolate the cells, the haemolymph was then centrifuged at 4,000g for 10 min at 4º C, and washed twice in cacodylate buffer. The tissue samples were rinsed twice with buffer solution. The haemolymph pellet and tissues were both fixed in 1% osmium tetroxide (TAAB, Sigma) in 0.1 cacodylate (Sigma) buffer for 1 h and then washed in cacodylate buffer and dehydrated in increasing concentration

of an ethanol solution (30%, 50%, 70%, 90%, 95%, 100%), 15 min in each at 4º C until 95% ethanol. Two last changes were then carried with the final concentration (100%), at room temperature. The samples were then transferred to propylene oxide 100% (Sigma) prior to impregnation in epoxy resin (Fluka).

Ultrathin sections from haemolymph extracts were stained with uranyl acetate (Sigma) and lead citrate (Sigma) and observed in a Jeol 100 CXII (JEOL, Peabody, MA) transmission electron microscope, to characterise morphology of concretions. The tissue samples could not be observed under TEM, due to the high abundance of concretions.

Scanning Electron Microscopy (SEM) with Energy Dispersive X-ray Spectroscopy (EDS) and Electron Microprobe Analysis (EMPA)

Dehydrated tissue samples of mantle and gill were carbon-coated (FINE-COAT Ion sputter JFC-1100) and glued to aluminium stubs for observation using a JEOL JSM-6301F scanning microscope with INCA software, operated at 10kV, and coupled with a detector. Observations and element analysis were completed only for concretions from tissue samples, since the free concretions in the haemolymph correspond to the mantle tissue.

Small pieces of dehydrated tissue samples from gill and mantle were incorporated in a hard resin, cold-mounting epoxy resin, in a 2.54-cm-diameter mould, and polished for observation in a JEOL JXA-8500F Electron Microprobe Analyzer with Oxford-INCA X-act, operated at 10Kv. Observations and microanalysis of concretions from tissue samples were subsequently made.

Fourier transform infrared spectroscopy and X-Ray diffraction

Concretions isolated from six mantle and gill samples of different bivalves were studied. Infrared spectra were recorded in the 400-4,000 cm^{-1} frequency region using a Bruker FTIR spectrometer. The measurements of the absorption bands integrated intensity were made using OMNIC software supplied by the Bruker Instrument. The pellet discs of 1.5 cm diameter were prepared by mixing 1 mg sample with 200 mg potassium bromide (KBr) and pressing at 14 Kg/cm^2. Prior to analysis, the pellets were heated overnight at 150º C to remove any adsorbed water. Isolated fractions of concretions from six mantle and gill samples of different bivalves were analysed on a Panalytical Xpert MDP with Cu vial to obtain spectra. For both six XRD and FTIR measurements, it was selected the most clear and representative.

Chemical analysis of the calcareous concretions and control tissues by Spectrometry of Atomic Absorption

Isolated fractions of concretions (three from the mantle and three from the gill) were analysed for calcium, cadmium, copper, manganese, zinc, and iron using a VarianSpectra AA 220 FS atomic absorption spectrometer (Varian, Australia) equipped with a deuterium background correction. Samples were compared with control tissue (foot) from three *A. cygnea* mussels. The samples were digested according to Reis et al. (2009), using a 50 mg sample for each replicate. The procedure was validated calculating each sample's percentage of return.

Results

Concretions characterization by LM and Transmission microscopy

Concretions extracted from haemolymph were easily observed in fresh smears LM. The morphology, size and structural arrangement of the concretions may differ. In the haemolymph, these structures appeared isolated, with a rounded shape, although some irregular forms were also observed. The individual size varied from 5 to 20 µm (Figure 1). The number of concretions observed in the haemolymph also varied considerably from 1,000 to 5,000 per mL of haemolymph (mean 3,000 ± 1,404 concretions / mL haemolymph). In Figure 1d, a concretion formation is represented, showing central structures that are surrounded by crystalline and refractive biomineral.

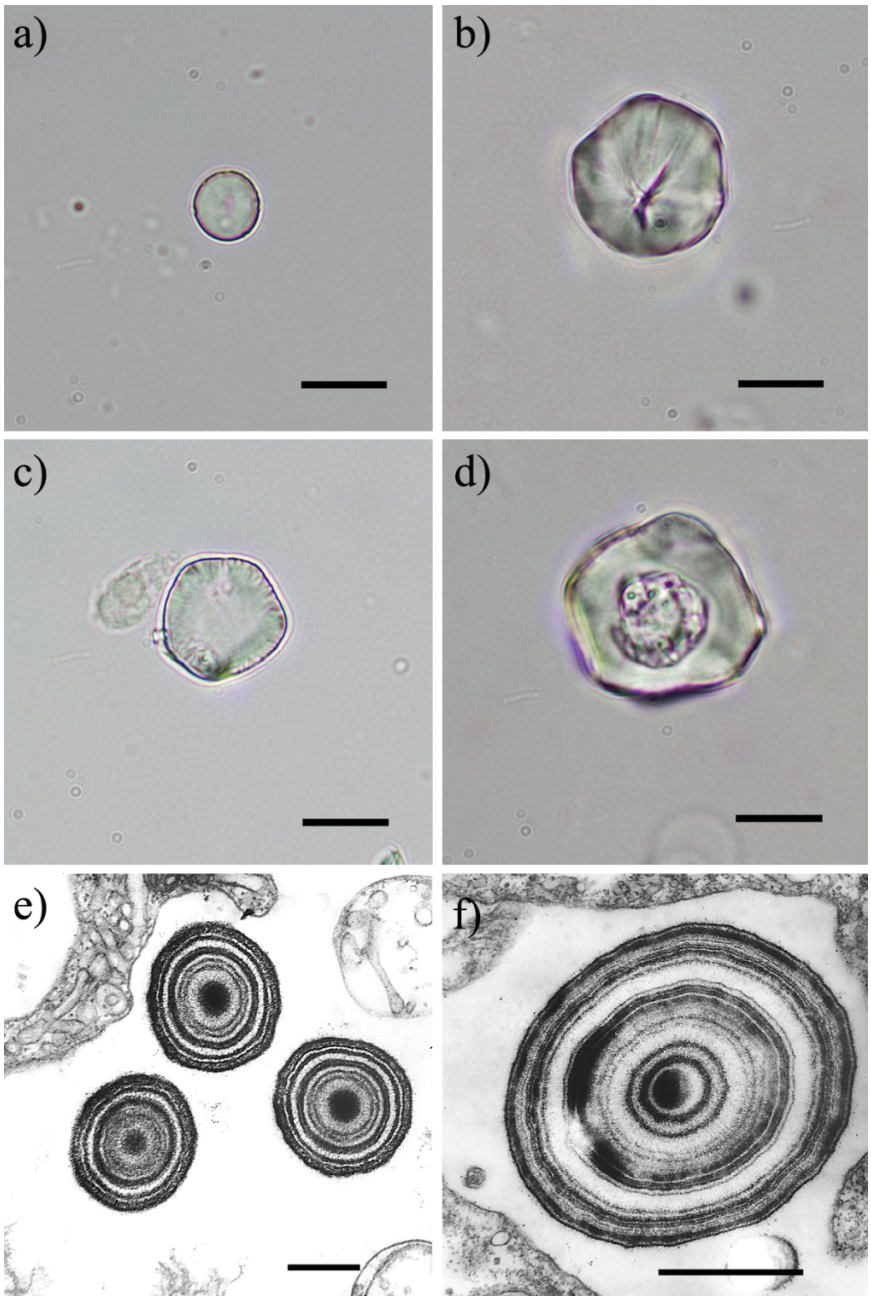


Figure 1 - Concretions from the haemolymph: a) to d) light microscopy images, showing different morphologies, scale bars 10 µm**,** e) and f) electron transmission microscopy images. Rings of deposition of organic (dark) and inorganic (white) material are evident, scale bars in e) 5 µm and in f) 2 µm.

The isolated haemolymph concretions were visualised TEM. The different rings of material deposition were visible, corresponding to layers of organic (dark rings) and inorganic (light rings) material deposition, (Figure 1e,f).The concretions from the gills and mantle were

observed in semithin sections from processed samples for electron transmission microscopy. Ultrathin sections were very difficult to obtain, the concretion components were not well impregnated in the resin, due to its inorganic nature and hard consistency. Semithin sections showed the arrangement of these concretions in the tissues; large amounts could be found in gill tissue, filling all the intrabranchial spaces and a more irregular and clustered shape (Figure 2a,b), while in the mantle they were more scarce but with a clear cut and round shape (Figure 2c,d).

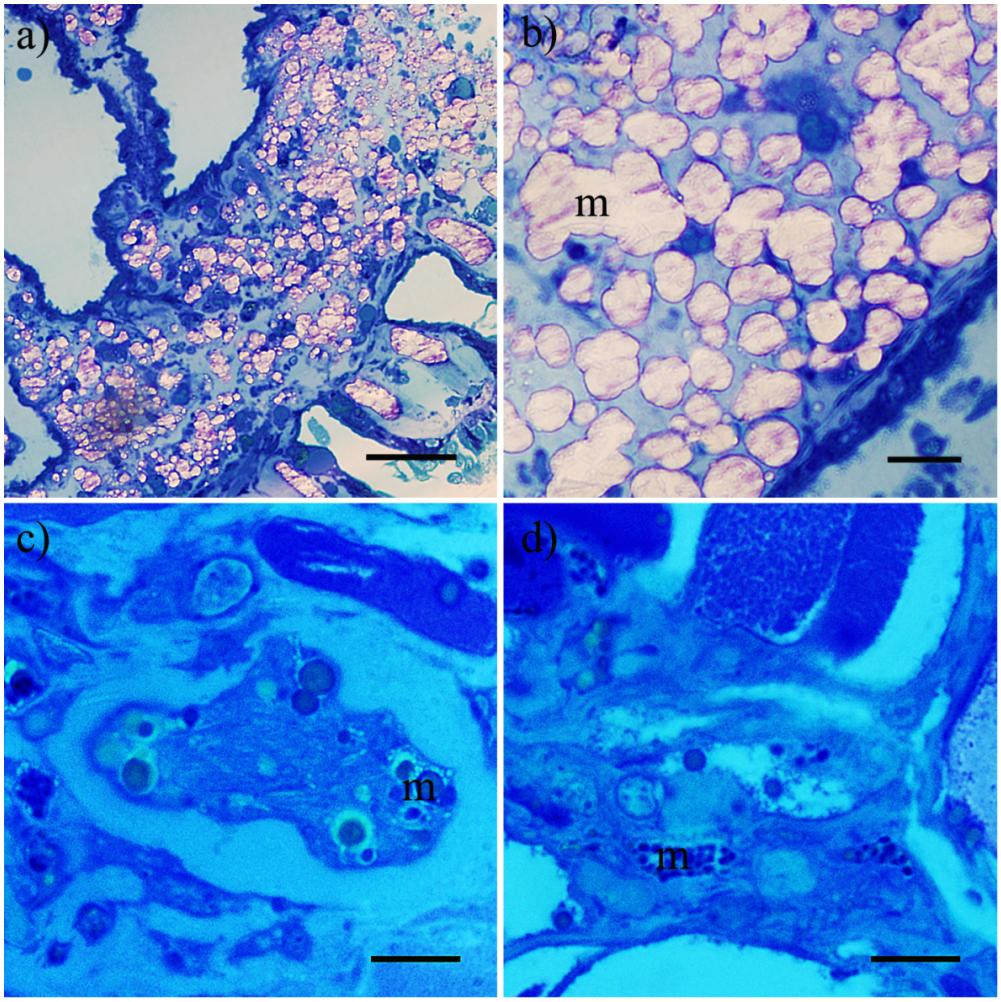


Figure 2 - Semithin sections from gill (a - b) and mantle (c - d) stained with blue methylene. In a) gill section full with concretions, scale bars 50 µm,; b) arrangement of the concretions (m) in detail, scale bars 20 µm; c) mantle tissue with some disperse micropearls (m); d) mantle tissue with small clusters concretions (m); scale bars 10 µm.

Using the scanning microscope, the arrangement of the concretions in the mantle and gill tissues was even more evident in 3 and 4. These mineral structures were well-fixed to the respective tissue surrounded by an interconnected organic matrix. Effectively, these concretions constitute a clear massive functional calcium reservoir.

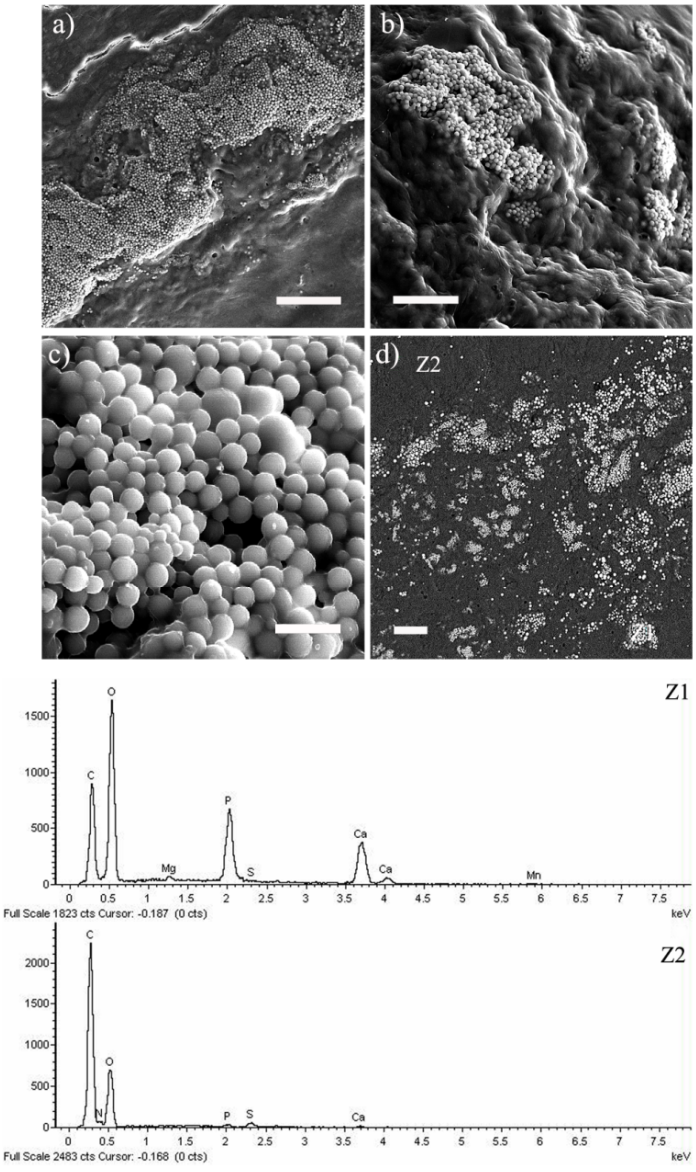


Figure 3 - Mantle concretions (a - d): a) and b) mantle covered with concretions, scale bars 20 µm and 10 µm, respectively; c) spherical concretions with magnified observation, scale bars 2 µm; d) backscattered image, scale bars 20 µm with respective EDS analyses; e) EDS spectrum corresponding to a zone with concretions (z_1); f) EDS spectrum corresponding to a zone without concretions (z_2).

The size and organization of the concretions vary on distinct tissues. In the mantle, these structures were predominantly very small (1-2 µm) with regular round shapes, and disposed in small aggregations (Figure 3). In contrast, in the gills, they were more numerous, bigger (more than 20 µm length) and highly irregular in shape, although some smaller round concretions with regular feature were also present (Figure 4). In the gills, the amount of concretions was much higher, so that almost all of the tissue covered by concretions.

Based on the EDS spectra (Figures 3 e,f and 4e-g), the concretions' composition is mainly calcium and phosphorous; magnesium is also present but in a much lower concentration. The concretion composition in both tissues was similar, although the gills had more granules. The main components analyzed by SEM-EMPA, see Table 1, revealed similar percentages of phosphorus for both tissues, higher contents of dinitrogen pentoxide and sodium oxide in the mantle concretions, while manganese oxide and calcium were found in higher amounts in the gills.

Table 1 - Micropearls present in mantle and gills of *A. cygnea*, analyzed by SEM-EMPA in terms of mass percentage (%).

Elements (%)	Na_2O	MnO	P_2O_5	N_2O_5	CaC
Mantle micropearls	0.500±0.036	2.537±0.339	25.498±2.912	9.510±2.880	26.238±2.392
Gill micropearls	0.156±0.025	3.815±0.195	28.768±0.971	1.104±0.628	37.600±1.560

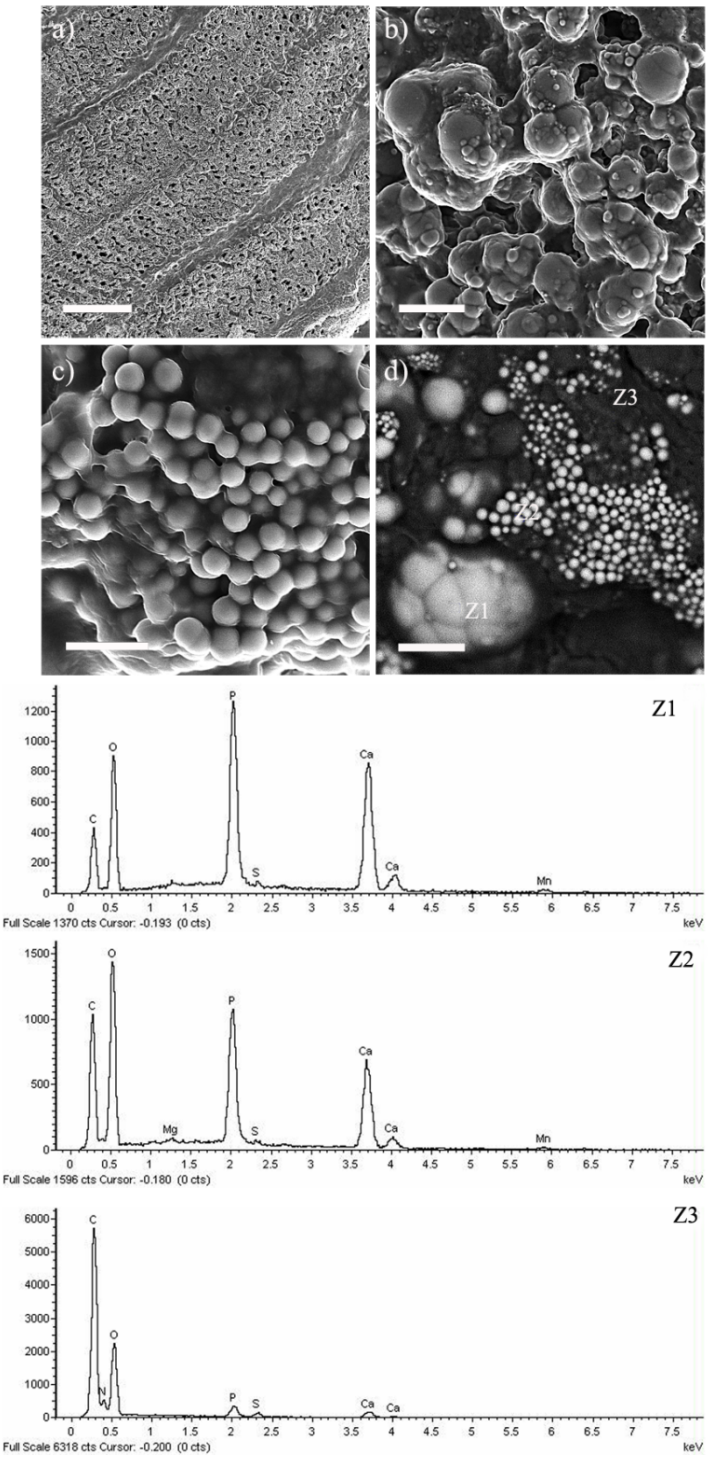


Figure 4 - From gill concretions (a - d) surrounded by dense organic matrix: a) lamellae from gill covered with concretions, scale bars 1 mm; b) irregular arrangement of concretions, scale bars 20 µm; c) regular spherical concretions, scale bars 5 µm; d) backscattered image of concretions, scale bars 10 µm with respective EDS analyses; e-g) corresponding to different zones with concretions (z_1 and z_2) and without concretions (z3).

X-Ray diffraction (XRD)

The XRD analysis of the concretions isolated from mantle and gill tissues showed a crystalline structure assigned to hydroxyapatite polymorphism. The hydroxyapatite $Ca_{10}(OH)_2(PO_4)_6$ has a hexagonal crystalline structure. In the samples, two forms of hydroxyapatite were found: hydroxyapatite as the dominant form, and a combined form of carbonated hydroxyapatite in a smaller quantity. The presence of this carbonated form could represent a transitional stage in microspherules formation, since some of the phosphate positions are occupied by a carbonate radical. In the gill samples, more crystalline structures of hydroxyapatite could be found mixed with the carbonated hydroxyapatite forms (Figure 5a), while in the mantle much less carbonated hydroxyapatite was detected (Figure 5b). The intense crystallinity in the gill microspherules also resulted in higher relative diffraction peaks observed at 31,704° and 32,864º regarding the reflecting positions of 2θ for the 211 and 300 planes, respectively. Some natural interference was stated in the X-ray analyses (Figure 5a,b) due to the presence of organic matrix.

Fourier transform infrared spectroscopy

Vibrational spectra of phosphates corresponding to υ_3 occur at 1,052 and 1,087 cm^{-1} (sample from mantle), for υ_1 at 961 cm^{-1} and at 600 and 570 cm^{-1} for υ_4. Both stretching bands corresponding to υ_3 are related to a monoclinic variety of phosphate (Figure 6).
The gill sample showed a broad stretching vibration of υ_3 suggesting a poorly crystallized specimen. The shoulder at 960 cm^{-1} is observed in both samples analyzed. Small amounts of carbonates were identified by the presence of the vibrational bands corresponding to 1,420 cm^{-1} and 880 cm^{-1}. The OH^- stretching band occurred at 3,400 cm^{-1}.

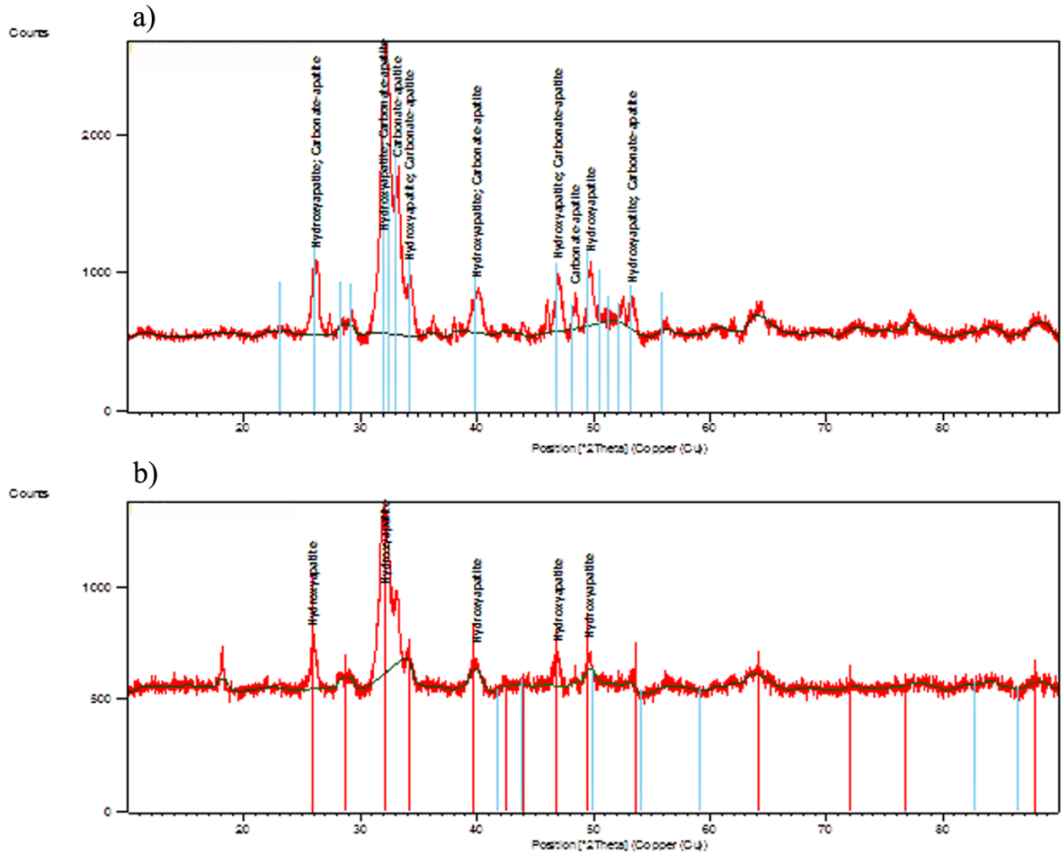


Figure 5 - Calcareous concretions isolated from gill (a) and mantle (b). The diffraction peaks are compatible with a solid phase composed by hydroxyapatite combined with carbonated forms (a) or pure form (b). The vertical axis represents X-ray intensity. The horizontal axis records angles in degrees (2θ) with time steps of 60 s between 10º and 90º.

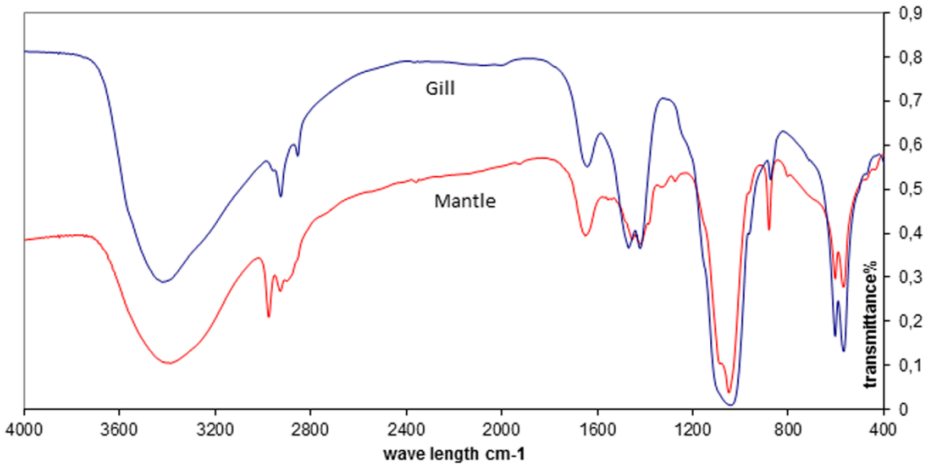


Figure 6 - FTIR spectra of the concretions isolated from gill and mantle.

Chemical analysis of the concretions and control tissues

Calcium is the dominant element in the concretions extracted from the tissue samples, see Table 2, other elements were detected but in lower amounts, when compared with the control tissues. The quantity of calcium found in the concretions is almost 70 times higher than the control tissues. The same pattern is observed for all of the other quantified metals (iron, magnesium, copper and zinc). Mantle concretions showed higher concentrations of Fe and Mg, while the gills concretions, showed higher values of Zn.

Table 2 - Metal levels in calcareous concretions extracted from gill and mantle and compared with the foot tissue (control without concretions) from *Anodonta cygnea* (in mg/g of dry sample) analyzed by SAA; DL – detection level.

Elements	Ca	Cd	Cu	Fe	Mg	Zn
Detection limit	0.0510	0.0035	0.0240	0.1250	0.0110	0.0250
Control tissue (foot)	1.18 ± 0.03	<DL	<DL	0.43 ± 0.08	0.48 ± 0.06	0.077 ± 0.002
Mantle Ca^{++} Concretions	72.0 ± 0.4	<DL	<DL	3.95 ± 0.02	4.14 ± 0.02	0.682 ± 0.005
Gill Ca^{++} Concretions	71.0 ± 9.0	<DL	<DL	1.51 ± 0.03	2.38 ± 0.18	1.174 ± 0.120

DL – detection level.

Discussion

Several studies showed that intracellular and extracellular mineralized structures, produced in many invertebrate species, play relevant roles such as defensive and supportive processes (skeletal structures), as natural mineral reservoirs on the homeostatic balance of essential ions and also as final targets for the elimination of toxic metallic ions (Becker et al., 1974; Noel-Lambot, 1976; Brown, 1977; Howard and Nickless, 1976; Brown, 1977; Jennings et al., 1979; Silverman et al., 1983, 1989; Greaves et al., 1984; Fowler, 1987; Pynnönen et al., 1987; Gardiner et al., 1989; Humbert and Pévet, 1995). Metals become toxic if the amount of metal entering an organism is higher than that required to saturate the enzymatic pool (Marigómez et al., 2002).

It is possible to conclude from these studies, that some invertebrates may have mechanisms for handling essential or trace elements, which are unique to their particular species (Fowler, 1987). To survive accumulation of toxic metals, animals use physiological and biochemical detoxification processes, which include excretion, sequestration in mineralized granules, binding to some specific cellular ligands, and so forth (Marigómez and BayBay-Villacorta, 2003).

According to Pynnönen et al. (1987) and Silverman et al. (1983), the unionid bivalves, *A. anatina*, *A. cygnea,* and *Unio pictorum*, exhibit a different distribution of concretions among the various tissues. The gills are the organs that generally have the highest content of concretions, up to 55% or 25% of the tissue dry weight for *A. cygnea* and *Ligumia subrostrata*, respectively. Conversely, a lower content of concretions is found in the mantle, in the midgut gland, and also in granulocytes. The origin of these concretions is still not well understood but may result from cellular mechanisms or extracellular phagocytic activity in response to toxic or particles invasion in the organism. In fact, one origin of intracellular concretions was described in the hepatopancreas of *Helix aspersa* (Greaves et al., 1984) and was related with the local cationic environment in intracellular phosphate granules that incorporated other metal elements. Silverman et al. (1985, 1989), however, described the origin of extracellular concretions in the gills of unionid mussels due to large accumulations of extracellular calcium phosphate concretions, which are initiated within the amorphous granules after phagocytosis in connective tissue cells.

This article shows the first-detailed analysis on concretions from the gills, mantle, and fluids of *A. cygnea*. The observations of gill and mantle concretions, based on SEM-EDS, SEM-EMPA, FTIR, and X-ray techniques, revealed heterogeneous structural shapes in a crystalline arrangement similar to a hydroxyapatite crystal, composed of mostly by calcium phosphate and incorporating a few metals like zinc, magnesium, manganese, and iron. Additionally, these observations supported that this arrangement in the gill concretions were composed of phosphate structures integrated with carbonated forms that resulted in carbonate hydroxyapatite crystals, while in the mantle the hydroxyapatite stayed much less carbonated. Shape, arrangement, and number of concretions can vary the different tissues and fluids, demands change during the life cycle of the animal according to the local tissue needs. The surrounding environment also has an important impact in the calcium deposition.

The formation of the calcareous structures in *A. cygnea* is mediated by an organic matrix composed of specific molecules depending on the respective biological fluids (proteins, chitin, GAGs, and lipids), which are involved in the calcareous nucleation and growth. So, these composites are responsible for the modulation of calcium phosphate polymorphic structures determining the crystalline shape and acting as triggers and glue that stabilizes the crystal structure (Moura et al., 1999, 2000; Lopes et al., 2010). Freshwater mussels have developed a unique highly efficient capacity to store calcium in tissues (Byrne, 2000), which seems to be functional and well-adapted to the calcification model proposed for *A.*

cygnea (Coimbra et al., 1988; Machado et al., 1988a, 1988b; Moura et al., 2000; Lopes-Lima et al., 2008, 2009). According to this model, the calcium concretions have a fundamental role in the larvae and adult shell formation, repair, reproduction, as well as in maintaining acid-base equilibrium with subsequent calcium balance and detoxification. This process is a seasonal response to the internal acidification by the respiratory and metabolic mechanisms (Lopes-Lima et al., 2008, 2009).

The origin of calcareous concretions is still uncertain, though it seems to be mediated by the haemocyte phagocytic process (Silvermann et al., 1983; Byrne, 2000). Indeed, according to Humbert and Pévet (1975), both the intracellular and the extracellular models constitute of two alternative ways for concretion formation in different bivalves species (Simkiss, 1976; Silverman et al., 1985). In this study, we found that concretions had inside organic structures within them. This is probably a consequence of phagocytosis activity followed by cell degeneration and the biomineralization process initiated in the phospholipidic droplets of the plasma membrane (Crenshaw, 1982; Humbert and Pévet, 1995; Wu et al., 2002).

Several studies indicated that initiation of the crystal nucleation mechanism for the calcium phosphate concretions is assisted by membrane induction. In detail, the enzyme is localized outside of the plasma phospholipidic membrane of cells where matrix vesicles anchor specifically to a glycophosphatidylinositol and/or phosphatidylserine forming microdomains (Coleman, 1992; Wu et al., 2002; Golub and Boesze-Battaglia, 2007; Kumon et al., 2013). According to Nancollas et al., 1996, this enzyme kinetically favours the formation of a very stable hydroxyapatite.

According to our results, alkaline phosphatase may be suitable enzyme, which catalyses the calcium phosphate precipitation, based on the blockage and destruction of the inhibitor(s) substances present in organic fluid (Fleish and Neuman, 1961) and on the calcium and phosphate ions concentrations, as well as, controlling an alkaline environment. Some authors suggest that calcium phosphate, due to low solubility, exhibits a high spontaneous precipitation at the centers of enzymatic activity, via the recycling of phosphates from organic and inorganic forms (Gomori and Benditt, 1953; Blake et al., 1998; Golub and Boesze-Battaglia, 2007). Hoang et al. (2003), and Kumon et al. (2013), have shown that the negatively charged protein surfaces and acidic/oxidized lipids can bind calcium ions in a spatial orientation providing structural scaffolds for mineral crystals. The growth of alternating organic and inorganic rings in the calcareous concretions should depend mostly on the continuous availability of organic matrix molecules, aside the

calcium and phosphate ions. It was clear that the organic fluids from *A. cygnea* offer rich organic molecules, which are capable to induce calcium phosphate crystal precipitation and further growth under specific conditions (Lopes et al., 2010).

According to Moura et al. (2004), the organic fluids exhibit several inorganic compounds, which are essential for the life of *A. cygnea,* some being present in large amounts in the tissues (e.g., Na, K, Ca, Cl, and Mg) and others only in trace amounts (Zn, Fe, and Mn) offering specific mechanisms for the formation of phosphates and carbonates deposits both present in *A. cygnea* (Coimbra et al., 1988; Lopes-Lima et al., 2008). Therefore, in this work, it was also shown that the mantle has regular mineral microspherules almost exclusively of phosphate composition, in contrast with the gill tissue concretions where they are mixed with carbonate forms. Significantly higher magnesium content was detected in the mantle that might serve to stabilize insoluble calcium phosphate (Carafoli et al., 1965; Weinbach and Von Brand, 1967), while the higher Fe deposits can reduce partially the calcium carbonate precipitation either by content gradient competitions (Herzog et al., 1989; van Langerak et al., 1999) or by chemical affinity of Fe to the phosphatidylserine (Kuross and Hebbel, 1988). In addition, calcium carbonate precipitation in the mantle is reduced by the high magnesium contents in the medium (Reddy and Wang, 1980), while the calcium phosphate is promoted (Ferguson and McCarthy, 1971). Conversely, the higher zinc level in gills concretions and lower magnesium can now indicate more specific conditions for the association of calcium phosphate and carbonate forms. The higher content in manganese promotes the phosphatase activity in the gills (Fitt and Peterkin, 1976), increasing calcium phosphate precipitation. Hence, the concretion formation seem to correctly support the synergistic role of alkaline phosphatase activity (with two Zn ions and one Mg) as well as carbonic anhydrase activity (with one Zn), facilitated by the Fe lower contents in the gill tissue.

Study also showed enormous amount of calcareous concretions with much larger size and irregular shape in the gill than in the mantle of *A. cygnea,* probably corresponding up to 50% of gill weight, as already indicated by Pynnönen et al. (1987), which serves as reservoir for shell formation of the glochidia. Regarding the variation in the shape and size, it is possible to assume that these distinct aspects may be determined by differences on the internal and external organic matrix and mineral content (Machado et al., 1991; Moura et al., 2004), which was also here supported by the interference on the mineral crystallinity in the XRD graphics. Thus, the enormous amounts of microspherules in the gills under more heterogeneous microstructure than in the mantle are possibly due to a greater

density of mineral and external organic matrix inducing a clustered habit. However, the more intense crystallinity may be favored by much lower internal matrix content (indicated by the lower dinitrogen pentoxide amount), and alkaline pH tendency induced by an intense oxygenation in the gills. This possibly promotes a slow crystal formation, and therefore, a more homogeneous crystalline structure. The almost exclusive presence of hydroxyapatite concretions in the mantle concretions, and mixed carbonated hydroxyapatite in the gills, seems to be supported by zinc, manganese, magnesium and iron balance in both tissues. In fact, the higher contents of magnesium and iron might greatly reduce the formation of carbonate forms in the mantle (Kuross and Hebbel, 1988), while the higher contents of zinc and manganese in the gill could accomplish a relevant complementary role promoting carbonate-hydroxyapatite forms in the gills concretions (Okrasa and Kazlauskas, 2006).

In conclusion, to emphasize is the possibility that calcareous microspherules, an elemental record, explain functional aspects on the bivalve physiology. It is possible to obtain valuable spatiotemporal biological and environmental information from biogenic calcified structures in caenogastropods (Galante-Oliveira et al., 2013). Concretions in *A. cygnea* eventually offer a tool to evaluate specific seasonal mechanisms, as well as for monitoring metal concentrations in the environment. Biomineral concretions in *A cygnea*, although understood here as an extracellular mechanism starting from the cell membrane reaction after a phagocytic activity against particulate invasion or toxic compound exposure, still studies crystalline nature.

Acknowledgments

This work was supported by a grant of the Portuguese Foundation for Science and Technology (FCT) (SFRH / BD / 76265 / 2011) under also projects PTDC/MAR/098066/2008, PTDC/AAC-AMB/117688/2010; COST Action TD0903. The authors declare no conflict of interest.

References

Adams, S.M., Shorey, C.D., 1998. Energy dispersive spectroscopy of granular concretions in the mantle of the freshwater mussel *Hyridella depressa* from Lake Burragorang as a technique to monitor metals in aquatic systems. Aquatic Toxicology 44, 93-102.

Bäeurlein, E., (ed.). 2004. Biomineralization. In: Progress in biology, molecular biology and application. Wiley-VHC Verlag GmbH & Co. KgaA, Weinheim.

Becker, G.L., Chen, C.H., Greenawalt, J.W., Lehninger, A.L., 1974. Calcium phosphate granules in the hepatopancreas of the blue crab *Callinectes sapidus*. Journal of Cell Biology 61, 316–326.

Blake, R.E., O'Neil, J.R., Garcia, G.A., 1998. Effects of microbial activity on the y18O of dissolved inorganic phosphate and textural features of synthetic apatites, American Mineralogist 83, 1516– 1531.

Bogan, A.E., 1993. Freshwater bivalve extinctions (Mollusca: Unionoida): a search for causes. American Zoologist 33, 599–609.

Brown, B.E., 1977. Uptake of copper and lead by a metal-tolerant isopod *Asellus meridianus*. Freshwater Biology 7, 235-244.

Byrne, M., 2000. Calcium concretions in the interstitial tissues of the Australian freshwater mussel *Hyridella depressa* (Hyriidae). In: The Evolutionary Biology of the Bivalvia. Vol. 177 (Harper E, Taylor J, Crame J, eds), p 329-337. Geological Society of London, Special Publications.

Carafoli, E.C., Rossi, C.S., Lehninger, A.L., 1965. Uptake of adenine nucleotides by respiring mitochondria during active accumulation of Ca^{++} and phosphate. Journal of Biological Chemistry 240, 2254-2261.

Coleman, J.E., 1992. Structure and mechanism of alkaline phosphatase. Annual review of biophysics and biomolecular structure 21, 441-83.

Coimbra, J., Machado, J., Ferreira, K.G., Ferreira, H.G., 1988. Electrophysiology of the mantle of *Anodonta cygnea*. Journal of Experimental Biology 140, 65-88.

Crenshaw, M.A., 1982. Mechanisms of normal biological mineralization of calcium carbonate. In: Biological Mineralization and Demineralization. (Nancolas GH, ed), p.243-251. Springer-Verlag, New York.

Epple, M., 2003. Biomaterialien und Biomineralisation. B. G. Teubner Verlag, Wiesbaden.

Fabritius, H., Walther, P., Ziegler, A., 2005. Architecture of the organic matrix in the sternal $CaCO_3$ deposits of *Porcellio scaber* (Crustacea, Isopoda). Journal of Structural Biology 150, 190-199.

Fitt, P.S., Peterkin, P.I., 1976. Isolation and properties of a small manganese-ion-stimulated bacterial alkaline phosphatase. Biochemical Journal 157, 161-167.

Ferguson, J.F., McCarthy, P.L., 1971. Effects of carbonate and magnesium on calcium phosphate precipitation. Environmental Science & Technology 5, 534–539.

Fleish, H., Neuman, W.F., 1961. Mechanisms of calcification: role of collagen, polyphosphates, and phosphatase. American Journal of Physiology 200, 1296-1300.

Fowler, B.A., 1987. Intracellular Compartmentation of Metals in Aquatic Organisms: Roles in Mechanisms of Cell Injury. Environmental Health Perspectives 71,121-128.

Gagnaire, B., Thomas-Guyon, H., Renault, T., 2004. *In vitro* effects of cadmium and mercury on Pacific oyster, *Crassostrea gigas* (Thunberg), haemocytes. Fish & Shellfish Immunology 16, 501-512.

Galante-Oliveira, S., Marçal, R., Guimarães, F., Machado, J., Barroso, C.M., 2013. Studies on the morphology and growth of statoliths in Caenogastropoda. Journal of Molluscan Studies 79 (4), 340-345.

Gardiner, D.B., Silverman, H., Dietz, T., Aoba, T., 1989. Intracellular concretion formation culture of a cell type which produces calcium phosphate concretions. Journal of Cell Biology 109, 303.

Golub, E.E., Boesze-Battaglia, K., 2007. The role of alkaline phosphatase in mineralization. Current Opinion in Orthopaedics 18, 444–448.

Gomori, G., Benditt, E.P., 1953. Precipitation of calcium phosphate in the histochemical method for phosphatase. Journal of Histochemistry & Cytochemistry 1, 114-122

Graf, D., Cummings, K., 2007. Review of the systematics and global diversity of freshwater mussel species (Bivalvia: Unionoida). Journal of Molluscan Studies 73, 291-314.

Greaves, G.N., Simkiss, K., Taylor, M., Binsted, N., 1984. The local environment of metal sites in intracellular granules investigated by using X-ray-absorption spectroscopy. Biochemical Journal 221, 855–868.

Greenaway, P., 1985. Calcium balance and molting in the Crustacea. Biological Reviews 60, 425-454.

Herzog, R.E., Shi, Q., Patil, J.N., Katz, J.L., 1989. Magnetic water treatment: the effect of iron on calcium carbonate nucleation and growth. Langmuir 5, 861-867.

Hinzmann M.F., Lopes-Lima, M., Gonçalves, J., Machado, J., 2013. Antiaggregant and toxic properties of different solutions on haemocytes of three freshwater bivalves. Toxicological and Environmental Chemistry 95, 790–805.

Hoang, Q.Q., Sicheri, F., Howard, A.J., Yang, D.S.C., 2003. Bone recognition mechanism of porcine osteocalcin from crystal structure. Nature 425, 977–980.

Howard, A.G., Nickless, G., 1976. The analysis of the betaine homarine by high pressure liquid chromatography. Analytical Biochemistry 76, 397.

Humbert, W., Pévet, P., 1995. Calcium concretions in the pineal gland of aged rats: an ultrastructural and microanalytical study of their biogenesis, Cell and Tissue Research 279, 565-573.

Istin, M., Girards, J.P., 1970. Carbonic anhydrase and mobilisation of calcium reserves in the mantle of lamellibranchs. Calcified Tissue Research 5, 247-260.

Jennings, J.R., Rainbow, P.S., Scott, A.G., 1979. Studies on the uptake of cadmium by the crab *Carcinus maenas* in the laboratory. II. Preliminary investigation of cadmium-binding proteins. Marine Biology 50, 141-149.

Kumon, H., Matsuura, E., Nagaoka, N., Yamamoto, T., Uehara, S., Araki, M., Matsunami, Y., Kobayashi, K., Matsumoto, A., 2013. Ectopic calcification: importance of common nanoparticle scaffolds containing oxidized acidic lipids. Nanomedicine - Nanotechnology 10, 441-450.

Kuross, S.A., Hebbel, R.P., 1988. Nonheme iron in sickle erythrocyte membranes: association with phospholipids and potential role in lipid peroxidation. Blood 72, 1278-1285.

Lima, P., Carvalho, F., Vasconcelos, V., Machado, J., 2004. Studies on growth in the early adult of the freshwater mussel, *Anodonta cygnea*. Invertebrate Reproduction & Development 45, 117-125.

Lopes-Lima, M., Bleher, R., Forg, T., Hafner, M., Machado, J., 2008. Studies on a PMCA-like protein in the Outer Mantle Epithelium of *Anodonta cygnea*: insights on calcium transcellular dynamics. Journal of Comparative Physiology 178, 17-25.

Lopes-Lima, M., Lopes, A., Casaca, P., Checa, A., Machado, J., 2009. Seasonal variations of pH, pCO_2, pO_2, HCO^{3-} and Ca^{2+} in the haemolymph: implications on the calcification physiology in *Anodonta cygnea*. Journal of Comparative Physiology B 179, 279-286.

Lopes, A., Lopes-Lima, M., Bobos, I., Ferreira, J., Gomes, S., Reis, R., Mano, J., Machado, J., 2010. The effects of *Anodonta cygnea* biological fluids on biomineralization of chitosan membranes. Journal of Membrane Science 364, 82-89.

Lowenstam, H.A., Weiner, S., 1989. On Biomineralization. Oxford University Press, New York.

Machado, J., Castilho, F., Coimbra, J., Sá, C., Monteiro, E., 1988a. Ultrastructural and cytochemical studies in the mantle of *Anodonta cygnea*. Tissue Cell 20, 797-807.

Machado, J., Coimbra, J., Sá, C., Cardoso, I., 1988b. Shell thickening in *Anodonta cygnea* by induced acidosis. Comparative Biochemistry and Physiology A 91:645-651.

Machado, J., Reis, M., Coimbra, J., Sá, C., 1991. Studies on chitin and calcification in the inner layers of the shell of *Anodonta cygnea*. Journal of Comparative Physiology 161, 413-418.

Machado, J., Lopes-Lima, M., 2011. Calcification mechanism in freshwater mussels: potential targets for cadmium. Toxicological & Environmental Chemistry 93, 1778–1787.

Marigómez, J.A., Soto, M., Cajaraville, M.P., Angulo, E., Giamberini, L., 2002. Cellular and subcellular distribution of metals in molluscs. Microscopy Research and Technique 56, 358-392.

Moura, G., Guedes, R., Machado, J., 1999. The extracellular mineral concretion in *Anodonta cygnea* (L.): Different types and manganese exposure-caused changes. Journal of Shellfish Research 18, 645-650.

Moura, G., Vilarinho, L., Santos, A., Machado, J., 2000. Organic compounds in the extrapallial fluid and haemolymph of *Anodonta cygnea* (L.) with emphasis on the seasonal biomineralization process. Comparative Biochemistry and Physiology B 125, 293-306.

Moura, G., Almeida, M.J., Machado, M.J.C., Vilarinho, L., Machado, J., 2004. The action of environmental acidosis on the calcification process of *Anodonta cygnea* (L.). In: Biomineralization (BIOM2001) Formation, diversity, evolution, and application (I. Kobayashi, H. Ozawa, eds) p 178-182. Tokai University Press, Japan.

Nancollas, G.H., Tsortos, A., Zieba, A., 1996. The nucleation and growth of calcium phosphate crystals at protein and phosphatidylserine liposome surfaces. Scanning Microscopy 10, 499–507.

Neves, R.J., Bogan, A.E., Williams, J.D., Ahlstedt, S.A., Hartfield, P.W., 1997. Status of aquatic mollusks in the southeastern United States: a downward spiral of diversity. In: Aquatic Fauna in Peril: The Southeastern Perspective. (G.W. Benz GW & D.E. Collins, eds.), p 45–86. Southeast Aquatic Research Institute, Decatur.

Noel-Lambot, F., 1976. Distribution of cadmium, zinc and copper in the mussel *Mytilus edulis*. Existence of cadmium-binding proteins similar to metallothioneins. Experentia, 32, 324-326.

Okrasa, K., Kazlauskas R.J., 2006. Manganese-substituted carbonic anhydrase as a new peroxidase. Chemistry – A European Journal 12, 1587-1596.

Pekkarinen M, Valovirta I. 1997. Histochemical and X-Ray studies on tissue concretions and shells of *Margaritifera margaritifera* (Linnaeus). Journal of Shellfish Research 16:169-177.

Pynnönen, D., Holwerda, D., Zandee, D., 1987. Occurrence of calcium concretions on various tissues of freshwater mussels, and their capacity for cadmium sequestration. Aquatic Toxicology 10,101-114.

Reddy, M.M., Wang, K.K., 1980. Crystallization of calcium carbonate in the presence of metal ions: I. Inhibition by magnesium ion at pH 8.8 and 25°C. Journal of Crystal Growth 50, 470–480.

Reis, P.A., Antunes, J.C., Almeida, C.M., 2009. Metal levels in sediments from the Minho estuary salt marsh: a metal clean area? Environmental Monitoring and Assessment 159, 191–205.

Silverman, H., Steffens, W.L., Dietz, T.H., 1983. Calcium concretions in the gills of a freshwater mussel serve as a calcium reservoir during periods of hypoxia. Journal of Experimental Zoology 227, 177–189.

Silverman, H., Steffens, W., Dietz, T., 1985. Calcium from extracellular concretions in the gills of freshwater Unionid mussels is mobilized during reproduction. Journal of Experimental Zoology 236, 137-147.

Silverman, H., Richard, P.E., Goddard, R.H., Dietz, T.H., 1989. Intracellular formation of calcium concretions by phagocytic cells in freshwater mussels. Canadian Journal of Zoology, 67, 198-207.

Simkiss, K., 1976. Intracellular and extracellular routes in biomineralization. Symposia of the Society for Experimental Biology 30, 423-444.

Sunda, W.G., Huntsman, S.A., 1998. Processes regulating cellular metal accumulation and physiological effects: Phytoplankton as model systems. The Science of the Total Environment 219, 165-181.

van Langerak, E.P., Beekmans, M.M., Beun, J.J., Hamelers, H.V., Lettinga, G., 1999. Influence of phosphate and iron on the extent of calcium carbonate precipitation during anaerobic digestion. Journal of Chemical Technology and Biotechnology 74, 1030-1036.

Vesk, P.A., Byrne, M., 1999. Metal levels in tissue granules of the freshwater bivalve *Hyridella depressa* (Unionoida) for biomonitoring: the importance of cryopreparation. The Science of the Total Environment 225, 219-229.

Weinbach, E.C., von Brand, T., 1967. Formation, isolation and composition of dense granules from mitochondria. Biochimica et Biophysica Acta 148, 256-266.

Wu, L.N., Genge, B.R., Kang, M.W., Arsenault, A.L., Wuthier, R.E., 2002. Changes in phospholipid extractability and composition accompany mineralization of chicken growth plate cartilage matrix vesicles. Journal of Biological Chemistry 277, 5126-5133.

Ziegler, A., Fabritius, H., Hagedorn, M., 2005. Microscopical and functional aspects of calcium transport and deposition in terrestrial isopods. Micron 36, 137-153.

Chapter 5

Synergistic impact of acute ammonia exposure and temperature stress on the freshwater mussel *Unio pictorum*

Synergistic impact of acute ammonia exposure and temperature stress on the freshwater mussel *Unio pictorum*

Mariana Hinzmann [1,2,3], Sebastian Beggel[2], Jorge Machado[1,3], Juergen Geist[2]

[1]Laboratory of Applied Physiology, Department of Aquatic Production, ICBAS-Abel Salazar Institute for Biomedical Sciences- University of Porto, Rua Jorge Viterbo Ferreira 228, 4050-313 Porto, Portugal.

[2]Aquatic Systems Biology Unit, Technical University of Munich, School of Life sciences Weihenstephan, Muehlenweg 22, D-85350 Freising, Germany

[3] CIIMAR - Interdisciplinary Centre of Marine and Environmental Research – University of Porto - Rua dos Bragas, n.289, 4050-123 Porto, Portugal.

Hinzmann, M., Beggel, S., Machado, J., Geist, J., 2016. Synergistic impact of acute ammonia exposure and temperature stress on the freshwater mussel *Unio pictorum.* Hydrobiologia (submitted).

Abstract

Ecotoxicological effects of nitrogen compounds (nitrate, nitrite, ammonia) are suspected to be a major stressor for aquatic organisms, but little is known about their impact on globally declining freshwater mussels (Unionoida). In this study, we tested the combined effects of ammonia and temperature stress on painter's mussel (Unio pictorum) survival, filtration behavior, haemocyte composition and energy status, at concentrations ranging from 0.3 to 9.0 $mg.L^{-1}$ total ammonia nitrogen (NH4-N) in 96-h acute exposures at two different temperatures, 17°C and 25°C. Sublethal physiological endpoints were more strongly affected by increased ammonia concentration than mortality, especially at the higher temperature. Despite of individual variation, higher ammonia concentrations resulted in decreased mussel activity and clearance rates, a situation that was most evident in beginning and at 25ºC. Glycogen was not affected by ammonia in the higher temperature 25°C. In the lower temperature (17 °C) glycogen decreased from control to 0.9 mg/L. Altered haemocytes ratios and morphologies, and an increase in hyalinocytes indicate activation of detoxification processes with potential immunological consequences. Since the two main stressors tested in this study, ammonia and temperature, are predicted to increase in most freshwater ecosystems, their observed synergistic impact on freshwater mussels should be better considered in defining water quality standards and effective conservation measures.

Introduction

Freshwater (Unionoid) mussels rank among the most endangered organisms in freshwater ecosystems and experience a global decline in species richness, distribution, dispersion and abundance (Bogan, 1993; Lydeard et al., 2004; Lima et al., 2016). Their specific life-history traits, feeding types, constrained mobility, complex life-cycles and comparatively long life spans make them extremely sensitive to disturbances in habitat and water quality. Mussels are known to play a key role in ecosystem processes by their influence on nutrient cycling, bioturbation and deposition of suspended materials (Strayer, 2006; Strayer et al., 2004; Vaughn, 2010; Richter et al., 2016). For instance, they are involved in reducing nitrogen forms to the benthic zone through the excretion of ammonium and biodeposition of feces and pseudofaeces. The understanding of possible causes for population declines as well as evidence-based conservation and restoration approaches

are urgently needed, due to the high conservation priority of this taxonomic group (Geist, 2010, 2011, 2015).

In terms of water contaminants, reactive nitrogen compounds, mainly ammonia and nitrate, are suspected to have negative effects on freshwater fish (Hasenbein et al., 2014) and mussels (Augspurger et al., 2003; Wang et al., 2007) at concentrations below national water quality criteria. Physiological responses associated to ammonia exposure are suspected to lead to reduction in feeding, fecundity and survivorship, resulting in decreased bivalve populations (Constable et al., 2003). Anthropogenic sources, including industrial wastes, sewage discharge, agricultural runoff, and animal farming, contribute approximately 75% to total nitrogen emission that are then transported into aquatic ecosystems at potentially hazardous concentrations.

Ammonia is mostly in equilibrium between the unionized form NH_3 (toxic) and the ionized form $NH4^+$, with the percentage of the toxic form increasing at higher pH values, and a greater toxicity at higher temperatures (Russell et al., 2008). Previous studies have demonstrated the toxicity of unionized ammonia to larval and juvenile stages of North American unionid mussels (Wang et al., 2007). Acute effect concentrations determined in standard bioassays ranged between 5 to > 16 mg total ammonia nitrogen for glochidia (24h EC50) at 20°C and pH 8.3 and 5.7 to 11.1 mg N/L for juveniles (96 h EC50) at 20°C and pH 8.3. However, there is a lack of information regarding the toxicity of ammonia to species of European freshwater mussels which is essential in terms of environmental regulation and for the conservation and restoration of mussel populations. In general terms US EPA consider that for protecting freshwater organisms from potential effects of ammonia is 17 mg/L total ammonia nitrogen (TAN) and the final chronic AWQC for ammonia is 1.9 mg/L TAN at pH 7.0 and temperature 20 °C (in the Aquatic Life Ambient Water Quality Criteria for Ammonia - Freshwater - USEPA 2013).

It is generally assumed that accurate measures of toxicity in adult mussels are difficult to obtain, due to their ability to detect toxicants in the water and their avoidance response by closing their valves (Naimo, 1995; Van Hassel and Farris, 2007; Hartmann et al., 2016a, b). However, Cope et al. (2008) reported that the toxicant avoidance response only lasted 24 h after which mussels reopened their valves and became exposed, probably forced by metabolic respiration requirements. Therefore we assumed that sublethal effects to stressor exposures > 24 h could be determined using adequate endpoints such as filtration behavior. The filtration capacity in freshwater bivalves may vary with the experimental design, species, size, age and sex of the animals, but they normally can filter up to several

liters of water per hour, making them excellent accumulators of contaminants from the environment. Their role in the habitat, particularly in nutrient cycling is essential (Kryger and Riisgard, 1988).

The identification of ecotoxicological biomarkers and assays in freshwater mussels is still not consensual, but the content of glycogen as storage carbohydrate has been established as a useful marker for the energetic status of the organism, also making it a physiological indicator for contaminant exposure (Naimo et al., 1998). In addition to this energy-status related marker, less standardized methods of assessing changes in the immunological response have been proposed as biomarkers for stressor exposure (Hinzmann et al., 2014). Bivalves possess an open vascular system (Cummings and Graf 2001; McMahon and Bogan, 2001), exposing their blood, the haemolymph, to the environment and thus to potential toxins and pathogens. This in turn increases their sensitivity to external contaminants. The cells in the haemolymph, the haemocytes, are the main intervenients in the immune response. The cells together with the humoral components from the haemolymph are very efficient in neutralizing pathogens or foreign particles. Haemocytes are involved in functions such as phagocytosis, encapsulation, nodule formation, pearl formation, tissue reparation and cell nutrition, but there is little knowledge on the mechanistic interactions among these processes (Hine, 1999; Cheng, 1981). Any effect that environmental stressors exert on the haemocyte composition or functioning may ultimately result in a reduction of immune response effectivity. Their filtration feeding exposes them to a variety of toxic compounds and pathogens (Antunes et al., 2010, 2014) that they seem to overcome well if the immune system if is not over-challenged.

We chose the painter´s mussel Unio pictorum (Linnaeus, 1758) as ideal test organism for various reasons: In contrast to other species of freshwater mussels, *U. pictorum* still have a wide distribution, inhabit a wide range of habitats including lakes and rivers, and are still more readily available than other species which is an important factor in using them as bioindicator in ecotoxicological studies. *U. pictorum* have been assessed as ´least concern´ by the IUCN in comparison with others native species with more vulnerable status (Van Damme, 2011) which should not primarily be used in ecotoxicological experiments.

In the present work, we tested the impacts of ammonia concentration and temperature stress on this species evaluating mortality, sublethal effects on filtration behaviour, haemocyte composition and morphology (as indicators for impacts on the immune system) as well as energetic status trough glycogen quantification. Specifically, we hypothesized a

synergistic negative effect of ammonia and temperature on the mussels. The work also pretends to evaluate the suitabilities of using different biomarkers to evaluate the animal welfare. The assay was conducted at two different temperatures, 17 and 25 °C, temperatures that are consider average high and extreme (reachable in the climate change scenario), respectively for that habitats where these mussels species occur, to evaluate the interaction of ammonia and temperature on the health condition, particularly since the increase of temperature was expected to modulate the response of the organisms and since it is known that ammonia toxicity and temperature are positively correlated.

Material and Methods

Test organisms

Adult painter's mussels (Unio pictorum) were obtained from a commercial supplier (OBI, Germany). Mussels were transported to the Aquatic Systems Biology Unit of TU Munich in Freising (Bavaria, Germany), maintained in aerated containers and allowed to acclimatize to laboratory conditions for three weeks prior to experimental setup. Upon arrival in the lab, mussels were held in a flow-through system (supplied with local well water) at a constant temperature of 13°C. Mussels were fed every other day with a commercially available shellfish diet which contains a mixture of four marine microalgae: *Isochrysis* sp., *Pavlova* sp., *Thalossiosira weissflogii*, and *Tetraselmis* sp. (Shellfish Diet 1800, Reed Mariculture). Each mussel was marked with an individual number (waterproof marker) and length, width, height (measured to the nearest mm) as well as wet weight (measured to the nearest g) was recorded. Measurements were repeated at the end of the experiments. Mussels had a mean (±SD) length, width and height of 83.7 (±9.3), 36.5 (±3.9) and 26.5 (±3.0) mm, respectively, and a mean initial wet weight of 52.1 (±16.7) g. Additional measurements of dry weight (after 48 h at 60°C, measured to the nearest g) were done for shell and soft-tissue of each individual 24 h after termination of the experiment, in order to be able to relate clearance rates and glycogen content to the body mass. Dry weight without shell was on average 2.1 (±0.9) g. Over these 24 h, delayed mortality effects were also recorded.

Mussel exposure

The experimental design for the two selected temperatures (17ºC and 25 ºC), comprised 6 water bath tanks of 50 L which were temperature controlled (Biotherm thermostat, Dohse Aquaristik, Germany). In each tank 8, 1.7 L glass beakers were randomly distributed in the tanks. Beakers were individually aerated by air stones. One randomly assigned mussel was placed in each beaker, each with a specific number. For each test concentration and the control 6 replicate mussels were used in the 17°C experiment and 5 replicates in the 25°C experiment. For the water control at each temperature, the very same setup was used, but without mussels. Acclimatization to experimental temperature conditions were conducted according to ASTM standard (Ref ASTM), by gradually adjusting the temperature at an increase of no more than about 3°C/h. For preparation of the test concentrations, an ammonia stock solution (1000 mg/L total ammonia nitrogen nominal) was prepared using ammonium chloride (Carl Roth GmbH, Germany). Respective amounts of the stock solution were added to the test beakers to reach nominal ammonium concentrations of 0.3, 0.9, 3.0 and 9.0 $mg.L^{-1}$. In each tank, all concentrations of ammonia were tested to avoid possible effects from individual water tanks. Temperature was maintained with thermostats and checked twice a day, as well as the oxygen concentration and pH (Oxi 330i, Multi 340i, WTW Germany). Water was exchanged every two days with a 50% renewal (850 ml). The mussels were exposed to the ammonia for a period of 96 hours. Within each temperature treatment, water chemistry and temperature remained constant during the experiment and no significant deviations in any of the measure variables between tanks were detected (with mean values at 17.0ºC of O_2 9.1±0.52 mg/L and pH of 8.8±0.1 and at 25.14±0.28ºC of O^2 7.2±0.49 mg/L and pH of 8.47±0.05).

Concentrations of total NH_4-N were measured for each individual test beaker using the photometric commercial kit (N 0.013 - 3.86 $mg.L^{-1}$; NH_4^+ SPECTROQUANT Spectroquant® Merk Millipore). Water samples with concentrations exceeding the range of the Spectroquant® test kit were adjusted by dilution with unionized water to meet the respective detection range. Fractions of ionized and un-ionized ammonia were calculated according to different values for temperature and pH of each test vessel. Nominal, measured and calculated values are given in Table 1.

Table 1 - Nominal and measured test concentrations of ionized and un-ionized ammonia nitrogen at 17°C and 25°C. Values are presented as mean ± standard deviation. Note that differences between nominal and measured values can result from mussel activity.

Nominal	Measured 17°C		Measured 25°C	
NH4+ [mg.L^{-1}]	NH4+ [mg.L^{-1}]	NH3 [mg.L^{-1}]	NH4+ [mg.L^{-1}]	NH3 [mg.L^{-1}]
0.0	0.68 (0.04)	0.12 (0.01)	1.08 (0.17)	0.28 (0.05)
0.3	0.94 (0.16)	0.17 (0.03)	1.23 (0.42)	0.32 (0.10)
0.9	1.26 (0.24)	0.22 (0.04)	1.48 (0.17)	0.39 (0.03)
3.0	3.63 (0.37)	0.64 (0.08)	3.68 (0.16)	0.97 (0.06)
9.0	8.63 (0.99)	1.51 (0.12)	10.56 (0.53)	2.78 (0.07)

Clearance rate and filtration activity

Clearance rates of *U. pictorum* were determined following the procedure described in McIvor (2004). For clearance measurement a 4 mL suspension of 153,882 cells/mL Shellfish Diet 1800® (Reed Mariculture Inc, USA) was mixed into the water in each beaker by agitation. Filtration in form of clearance rate was measured at three timepoints, 24 h before start of the exposure experiment, at initiation of the exposure experiment and after the 96h exposure period. Beakers were not aerated during these procedures (< 2 h), but oxygen levels did not drop below a critical concentration (never < 6 mg.L^{-1}). A 3mL water sample was removed from the middle of the water column in each beaker in duplicate, measured using a fluorescence photometer (Aquafluor Thermo Design, Canada), at the beginning and then again after 30, 60 and 90 minutes.

Clearance rates were calculated using Formula/Equation 1 (adapted from Coughlan, 1969; McIvor, 2004):

Formula/Equation 1: $CR = V \times \left[\frac{\ln C_0 - C_t}{t}\right]$

where V is the Volume of the test vessel, C0 is the initial concentration and Ct is the concentration at time t. Control measurements in beakers without mussel were subtracted to account for settlement of the algae over time. After determination of clearance rates for each individual, the values were normalized against dry weight of the mussels' soft tissue. Only clearance values from the 90 min time-interval were used for further analysis. Values determined within a 90 min time interval at the beginning and after 96h exposure were used for further analysis.

At the same time when clearance rates were experimentally determined, filtration activity for each mussel was recorded by observing the visibility of their siphons (two categories: shell open with visible siphons and active filtration, or shell closed with no visible siphon and no filtration). The fluorometer was previously calibrated against algal cell density determined by an improved Neubauer chamber (Merck Millipore, Germany). In addition, activity was confirmed by clearance rates greater than in the controls without mussels.

Haemolymph collection and haemocyte assessment

At termination of the experiment after 96h, a volume of 1 mL haemolymph was collected from each living specimen. Haemolymph from all mussels used was carefully extracted using a 21G needle (Braun) attached to a 2mL sterile syringe (Braun), by insertion between the valves across the inner layer of the mantle into the interepithelial space. Each haemolymph tube was maintained on ice, immediately after collection, to avoid aggregation (Gagnaire et al., 2004; Soares-da-Silva et al., 2002) and an anticoagulant solution of N-ethylmaleimide 0,05 M was added in a proportion of 1/10 of total volume (Hinzmann et al., 2013).

Per mussel, tree replicate subsamples of haemolymph were analysed under a light microscope (Olympus CKX41) coupled with digital camera (Olympus DP72). Haemocytes were classed into granulocytes and hyalinocytes. For cell counting, an improved Neubauer chamber (Merck Millipore, Germany) was used. Viability of the cells was estimated by the trypan blue exclusion assay (Ford and Haskins, 1988; Tirard et al., 1997). For morphological comparisons of haemocytes, three aliquots from the haemolymph of each mussel were set onto a glass slide for adhesion. The coloration technique was conducted following the procedure of Hemacolor® (Merck). Briefly, air dried smears were fixed with methanol, stained and then air-dried again. Prior to observation under the light microscope, the slides were mounted with DPX (Merck) for preservation.

Glycogen quantification from foot tissue

After collection of haemolymph samples, biopsies (0.091 ± 0.04 g, mean ± SD) from the foot tissue from each mussels were collected, weighed (nearest 0.001 mg), snap frozen in liquid nitrogen and then stored at -80°C until further analysis. Quantification followed the method by Keppler and Decker (1974). Briefly, the enzyme amyloglucosidase was used to digest glycogen into glucose which was then quantified photometrically.

Data analyses

Bivariate linear models (ordinary least squares regression) were used to comparatively test the relationships between concentrations of ammonia and physiological indicators (clearance rate, haemocyte composition and mortality, glycogen content) at the two temperatures. Normality and heteroscedasticity were tested using Shapiro Wilk's and Levene's test, respectively. Accordingly, nonparametric Kruskal-Wallis multiple comparison test were used to compare clearance rates, haemocytes abundances and ratios in relation to treatment. In case of significance ($p < 0.05$), Bonferroni-corrected Mann-Whitney U tests were used for individual comparisons between groups. Glycogen data were evaluated using analysis of variance (ANOVA) with Tukey's post hoc comparison. Statistical analyses were conducted using SPSS 21 (IBM, USA).

Results

Mortality

Only one mussel at the highest ammonia treatment died during the 96-h experiment at 17°C, and another one in the highest ammonia treatment at 25°C was in moribund state, only slowly closing the valves after disturbance. Two additional mussels from the two highest concentrations at 25°C died within 24 h after the end of the experiment, suggesting a delayed mortality effect. It needs to be noted, however, that this delayed mortality may also be a result of additional stress induced by haemolymph collection and biopsies.

Clearance rate and filtration activity

Over the 96h experimental period, filtration activity was temperature-dependent, with greater activity at higher temperatures (differences in normalized clearance rates: $p<0.01$ in all cases; Figure 1 and Table 2). At the beginning of the experiments, 31% and 70% of the mussels were actively filtrating (i.e., clearing the water from particles) at 17°C and 25°C, respectively, decreasing to 26% and 57% at test termination.

In general, clearance rates were relatively low in all treatments. Mean values ranged between 3 $mL.(min*g)^{-1}$ and 16 $mL.(min*g)^{-1}$ in the 17°C trial and between 3 $mL.(min*g)^{-1}$ and 24 $mL.(min*g)^{-1}$ in the 25°C trial at the beginning of the experiment (Fig. 1, A), and 2 $mL.(min*g)^{-1}$ and 21 $mL.(min*g)^{-1}$ in the 17°C trial and between 12 $mL.(min*g)^{-1}$ and 32 $mL.(min*g)^{-1}$ in the 25°C trial at the end of the experiment (Fig. 1, B). Clearance rates

showed some fluctuations within treatment concentrations at 17°C, but were not significantly different to controls. No significant differences were found at 25° either. Normalized clearance rates were only negatively correlated with concentrations of ammonia at the beginning of the 25°C treatment when most of the mussels were actively filtrating (Ordinary Least Square Regression, r2=0.189, p=0.016).

Table 2 - Activity (%) based on clearance data (90 min measurements at t=0 and t=96h) and visual observation, mussels were considered not actively filtering when clearance was same or below the control without mussels.

t=0h	**17ºC**		**25ºC**	
Nominal NH_4^+ [mg.L^{-1}]	**halflife**	**observation**	**halflife**	**observation**
0	28%	28%	44%	44%
0,3	33%	0%	50%	0%
0,9	33%	50%	83%	67%
3	33%	17%	50%	67%
9	33%	33%	33%	83%
t=96h	**17ºC**		**25ºC**	
Nominal NH_4^+ [mg.L^{-1}]	**halflife**	**observation**	**halflife**	**observation**
0	50%	40%	50%	70%
0,3	40%	20%	80%	80%
0,9	40%	20%	40%	40%
3	20%	0%	60%	100%
9	20%	20%	60%	0%

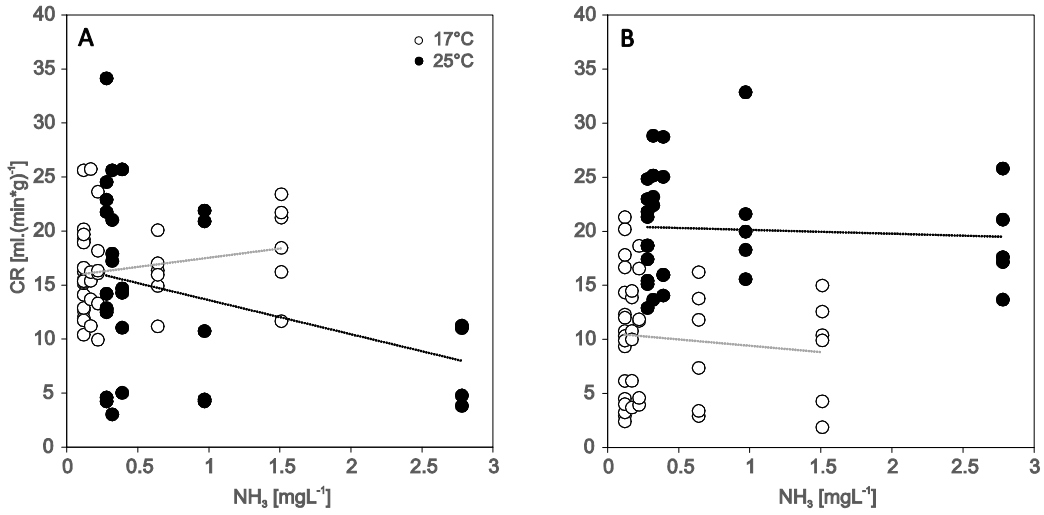


Figure 1 - Normalized clearance rates (CR [ml.(min*g)$^{-1}$]) for *U. pictorum* mussels exposed to increasing concentrations of ammonia-nitrogen (NH4-N) at 17°C (white) and 25°C (black). A: Initial clearance rates at the beginning of exposure. B: Clearance rates at 96h of exposure. Dotted lines indicate linear trends.

Haemocytes assessments

The cell counts of all types of haemocytes (granulocytes, hyalinocytes) was only slightly affected by temperature, particularly in granulocytes (Mann-Whitney: z= -0.86, p=0.28), but largely independent from ammonia concentration (Figure 2). Due to high variation among specimens, none of the cell counts were significantly different between treatments. A tendency towards higher concentrations of live granulocytes at 17°C between control and 3 mg/L of ammonia was found (p=0.09). Hyalinocytes generally were by a factor of 8 less abundant in haemolymph than granulocytes and did not reveal statistically significant differences according to temperature and ammonia concentrations.

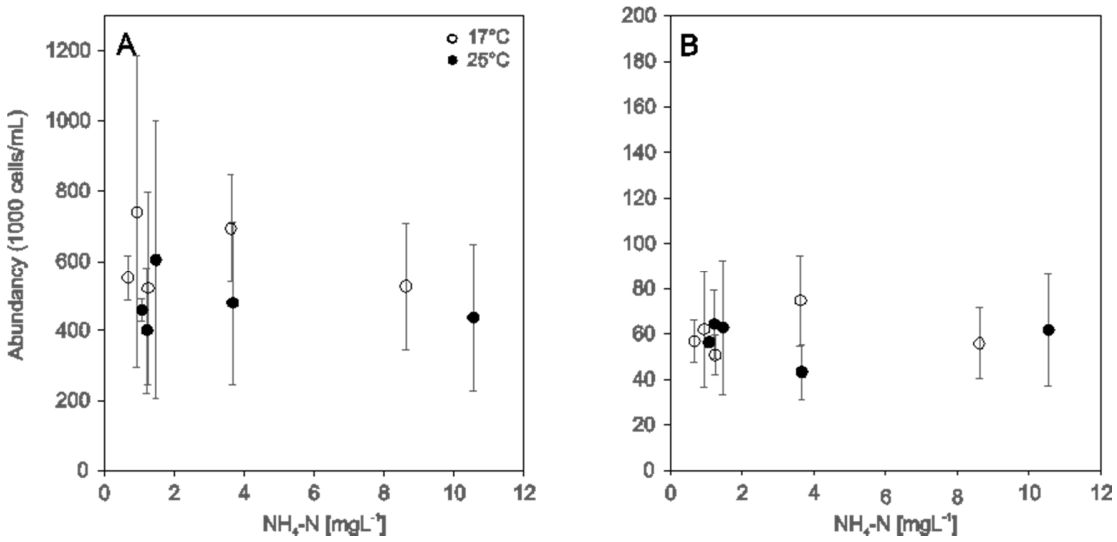


Figure 2 - Variation of the abundances on the haemocytes population (mean ± standard deviation; A: granulocytes and B: hyalinocytes) from *Unio pictorum* exposed to different ammonia concentrations at 17º C (white) and 25 ºC (black).

Temperature revealed a significant effect on hyalinocyte mortality (Kruskal-Wallis: p< 0.001), which was by a factor of 14 greater at 25°C compared to 17°C. Generally, the greatest differences between the different temperature treatments occurred at the lowest ammonia concentrations which may be explained by the greater filtration activities of mussels and a more intense exposure under these circumstances. No effect of temperature was evident in the granulocytes. In both cell types, no effect of ammonia concentration was observed (Figure 3). Great variation was generally observed between individuals from the same treatment, resulting in low statistical power.

Hyalinocyte and granulocyte mortality were weakly correlated with mussel activity as measured by their clearance rate. Interestingly, opposite trends were observed at the two different temperatures, with a positive correlation at 17°C, and a negative correlation at 25°C, irrespective of cell type (Figure 4).

A practical finding was that from specimens in the 25ºC treatment, collection of haemolymph was more difficult, and lower volumes were obtained, however fewer cells were counted, indicating some dehydration.

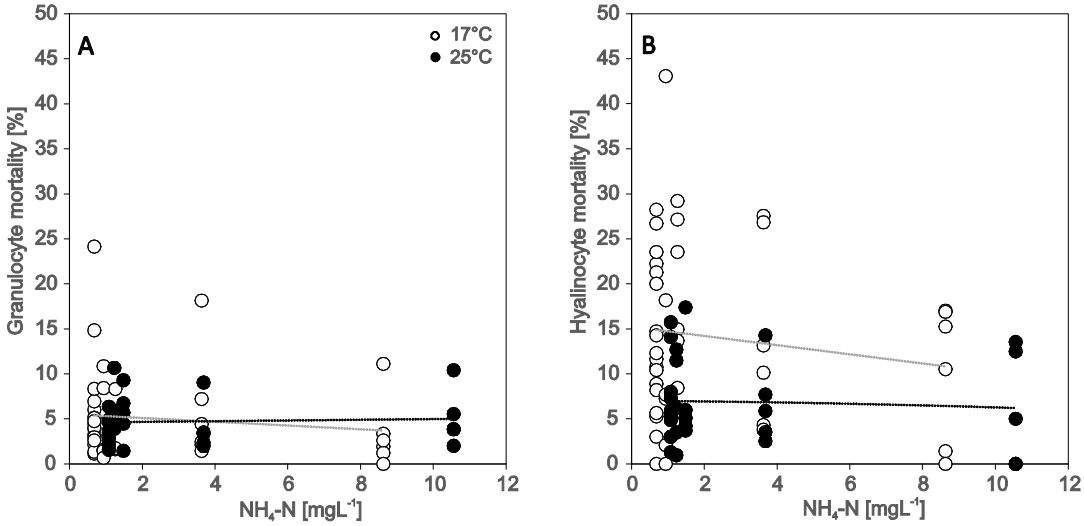


Figure 3 - Haemocytes mortality (A: Granulocytes, B: Hyalinocytes) from *Unio pictorum* exposed to different ammonia concentrations at 17º C (white) and 25 ºC (black). Dotted lines indicate linear trends.

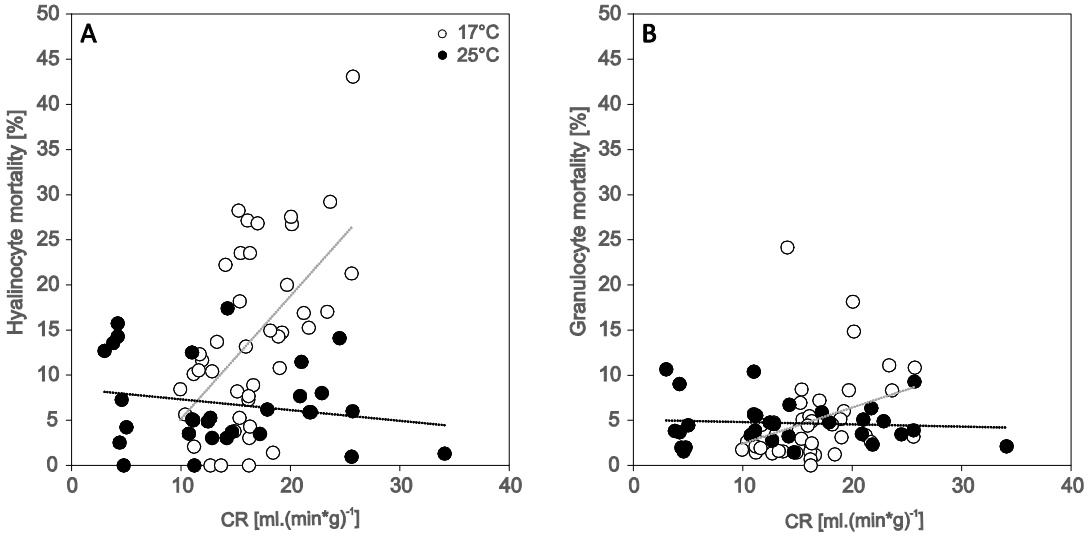


Figure 4 - Relationship between mussel filtration and observed haemocyte mortality (A: Hyalinocytes, B: Granulocytes). Correlations between these two endpoints show different trends at 17°C (white) and 25 °C (black).

When comparing stained cells from control organisms with the ones exposed to the different ammonia concentrations, several alterations were found: swelling or shrinkage of the cells, increase in the number of granules present in the cytoplasm of granulocytes and occurrence of pseudopods, summarized in Table 3 and Figure 5 (a to j). Cellular alterations increased with the concentration of ammonia, being more pronounced at the 25ºC treatment (Figure 5j). Alterations in relation to the control situation, that correspond to cells with a rounder shape, some tendency to aggregate, and no or few granules or pseudopods, can be interpreted as consequence of the exposure to ammonia that are associated with a decrease of the viability of the cell, which can lead to apoptosis or necrosis at ultimate stage. A qualitative analysis of the smears showed that even the smallest concentration of ammonia induced alterations in the cells, with most of the cells showing signs of shrinkage. With increasing concentration, these effects were more pronounced and additionally, other morphological alterations became evident. This included an increased number of pseudopods, and a greater number of granules in the cells (Table 3, Figure 5d, e, i, j). The highest concentrations of ammonia triggered even more drastic effects, including morphologic alterations associated with the necrosis pathway such as cell swelling or shrinkage. Granulocyte swelling was mostly observed at 25°C and at the higher ammonia concentrations, whereas cell shrinkage was most pronounced at 17°C at the higher ammonia concentrations.

Table 3 - Major alterations induced on the haemocytes of *U. pictorum.* Terminology refers to counts of 100 cells, with “many” refering to more than 50 in 100 cells; “some” refering to 10 to 20 per 100 cells, and “rare” to less than 5 in 100 cells.

Temperature (°C)	Nominal NH_3 [mg.L-^{1}]	Nominal NH_4^+ [mg.L-^{1}]	Cells Aggregation	Cells Alterations	Pseudopods	Granules
17	0.12	0.0	small	round shape	some	rare
	0.17	0.3	some	shrinkage	many	present
	0.22	0.9	none	severe shrinkage	many	present
	0.64	3.0	none	swollen	some	many
	1.51	9.0	some	severe shrinkage	many	rare
25	0.28	0.0	none	little shrinkage	some	some
	0.32	0.3	some	shrinkage	many	rare
	0.39	0.9	none	swollen/shrinkage	some	rare
	0.97	3.0	none	swollen	many	present
	2.78	9.0	some	swollen	many	many

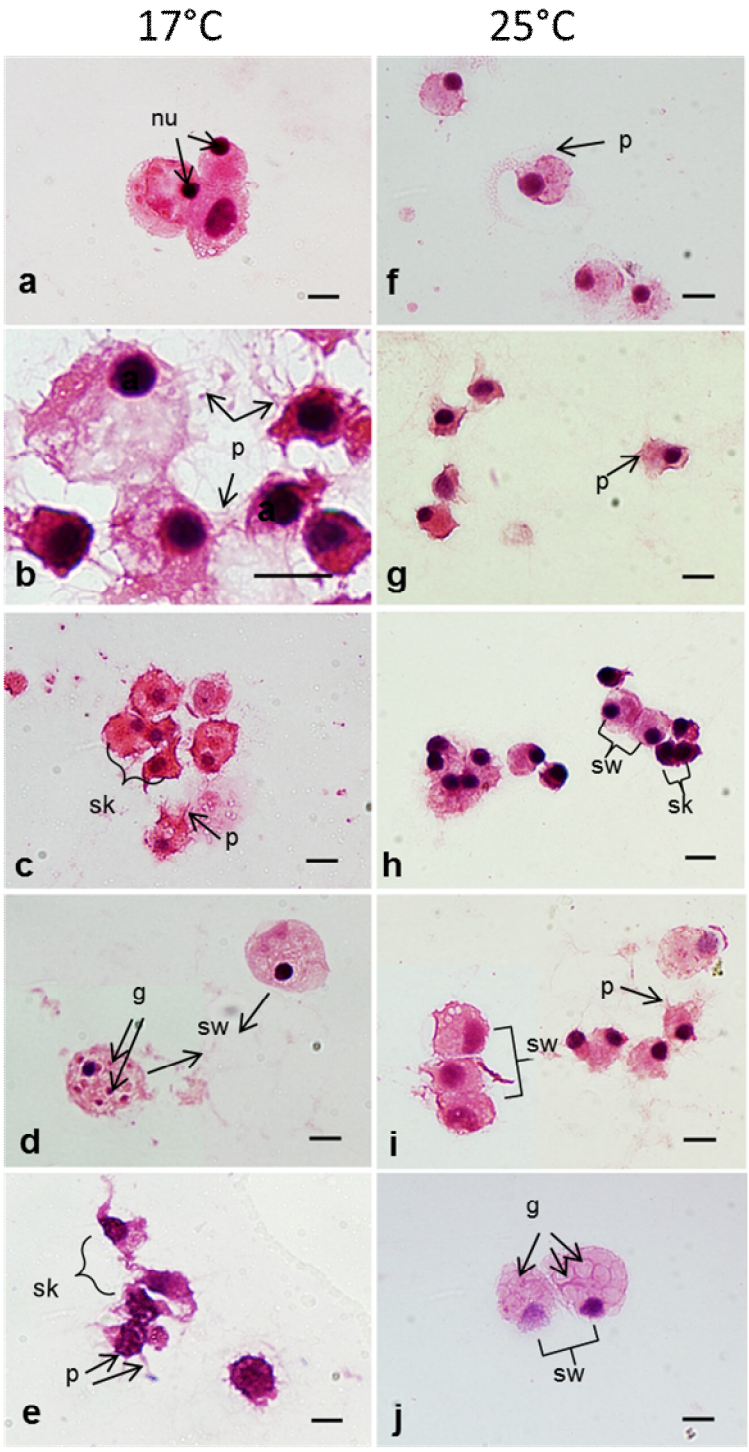


Figure 5 - Light microscopy images from stained haemocytes of *U. pictorum* exposed to different ammonia concentrations and temperatures. Images from a) to e) correspond to haemocytes from organisms kept at 17°C, in the follow nominal concentrations: a) control (0), b) 0.3, c) 0.9;d) 3.0 and e) 9.0 mg.L^{-1} of ammonia. Images from f) to j) correspond to haemocytes from organisms kept at 25°, in the nominal concentrations: f) control (0), g) 0.3, h) .0.9; i) 3.0 and j) 9.0 mg.L^{-1} of ammonia. Main cellular features observed where the nucleus (nu), the granules (g), occurrence of pseudopods (p), morphologic changes as shrinkage (sk) or swollen (sw) of the haemocytes; scale bars 10 µm.

Energy reserves

Glycogen concentration as a proxy for energy reserves in the foot tissue of the mussels revealed a positive effect of temperature (Figure 6). The greatest difference was found in the control, where the glycogen content reached the highest value for the mussels at 25ºC, but much lower values at 17°C ($p < 0.05$). Despite of a trend towards lower glycogen content with increasing ammonia concentration, particularly at the 17°C treatment, no statistically significant differences were found between controls and treated mussels, except for the ammonia concentration of 0.9 mg.L-1 ($p < 0.05$) at 17°C.

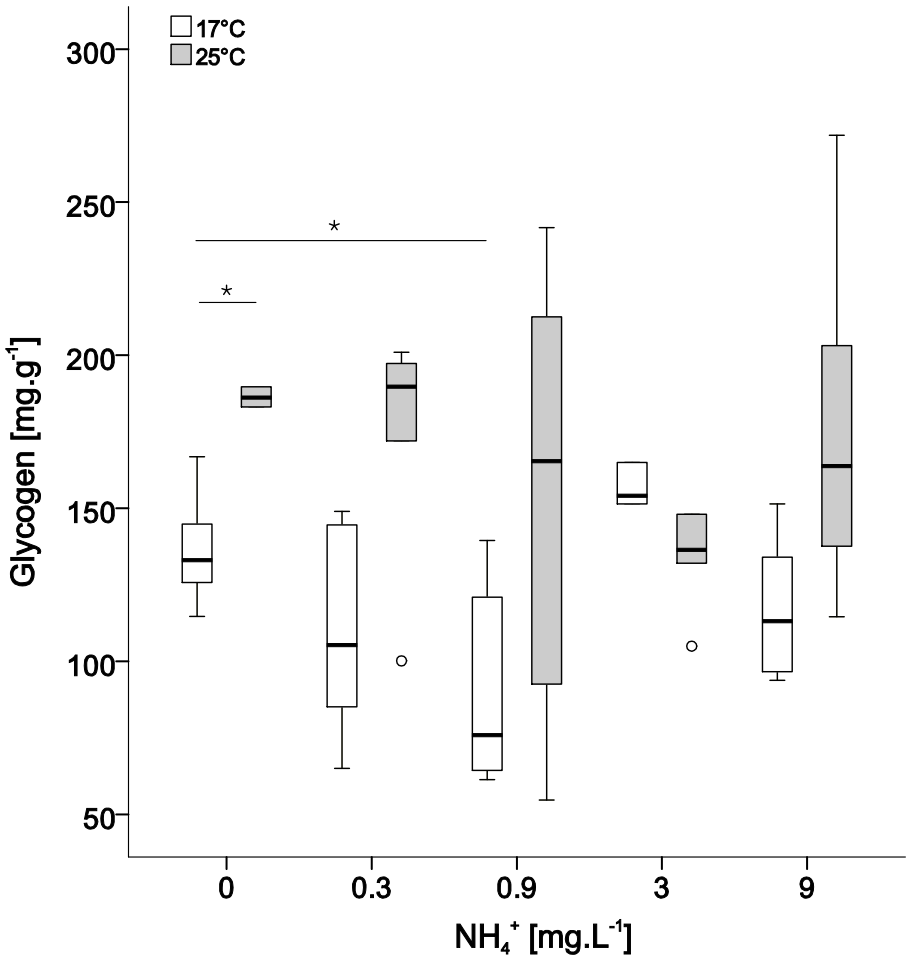


Figure 6 - Glycogen content in *U. pictorum* muscle tissue after 96 h exposure to ammonia at 17°C and 25°C. Boxplots show median, 25th and 75th percentile (box) and 5th to 95th percentile range (whiskers). Outlier values are indicated by circles, significant differences ($p<0.05$) are indicated by asterisks.

Discussion

The results of this study suggest that the integrative approach of combining behavioural, physiological and cellular endpoints is advantageous in assessing the ecotoxicological effects of stressors to freshwater mussels. They also indicate that the combined stressor effects of increased water temperatures and ammonia concentrations act synergistically, thus potentially contributing to the observed declines in freshwater mussel populations. Whilst the acute 96 h exposure scenario used in this study cannot be directly translated into chronic long-term effects, it shows that even short-term pollution that is a common scenario in stream ecosystems may be exacerbated by more subtle and global pressures such as climate change.

Ammonia increase, as a result of aquatic pollution, in streams is one of innumerous causes for mussel declines, although deeply knowledge of the sensitive of adult mussels is still not well known, even the actual methods to quantify ammonia can underestimate the real treat to mussel populations (Haag, 2012). These mussels demonstrated having a higher sensitivity to the toxic ammonia form (NH_3) that seems to decrease as they grow, the early stages are more vulnerable, and for those reasons have been used in more studies (Haag, 2012; Augspurger et al., 2007; Ward et al., 2007), this sensitivity seems to be higher with the pH increase (Wang et al., 2008). Even so, the ecological impact on adults is important since they represent one of the most threatened faunistic group of the world, and present several advantages related to their life style (filter-feeders, sedentary) over other organisms (Nobles et al., 2015).

Most toxic assays are based in mortality, even using lower ammonia concentrations than the regulated by US EPA, so lower concentrations also can cause negative impacts and these limits need to be clarified and used in the conservation and preservation measures for these species (Augspurger et al., 2003). Other limitation of the common toxic assays is that besides mortality they can be based on growth, but the grow rate of these species cannot be significant for the duration of most assays and other pathologies can be ignore, besides other factors that can also interfere, like life stage (Cope et al., 2008) that have already show different sensibilities between glochidia and juveniles (Raimondo et al., 2016). Different endpoints to access the impact of ammonia in mussels can provide functional information about the first sighs of intoxications in terms of behavior, energy resources and immune system presented in this study but other like hearth rate presented

in Ganser et al., 2013, can better reflect the mussel fitness before reaching a point of no return.

The accumulation with other anthropogenic stress factors, like the ones associated to climate changes, increase of water temperatures and pH variation, may affect freshwater mussels more drastically than fishes (Ganser et al., 2013). Nowadays the studies of several combined parameters are of interest, since they reflect better the real environmental conditions that mussels are exposed.

The finding that the tendency to accumulate adverse effects on the mussels was found at highest concentrations of ammonia and at highest temperature is in line with our initial hypothesis and can be explained by several reasons. As evident from the mussel activity observations in our study, a higher temperature increases metabolic processes including oxygen consumption which ultimately forces the mussels to increase their water uptake. This in turn also results in increased exposure to any kind of pollution. Moreover, at higher temperatures, the equilibrium of toxic unionized NH_3 and ionized NH_4^+ is shifted towards the more toxic form (Carpenter et al., 1998; Russel et al., 2008). The experimental setup in our study may even underestimate these effects compared to a natural scenario where primary production additionally increases pH, further shifting the equilibrium towards unionized ammonia. The concentration range used in our study represents different categories of water quality according to environmental quality standards for salts and nutrients (LAWA, 1998), where values above 2.4 $mg.L^{-1}$ represent a high anthropogenic disturbance, regulations according to US EPA (US EPA, 2013).

Due to their ability to accumulate toxins, *Unio* species have been used as bioindicator species since the 1960s, despite of little consideration of associated physiological alterations (Campanella et al., 2005). The different endpoints used in our study all revealed different sensitivities and thus vary in their usefulness as stress indicators. Mortality, despite of its direct relevance in assessing adverse outcomes and observability in the highest concentration, only seems to be a weak indicator, at least in the range of temperatures and ammonia concentrations used herein. However, as evident from the delayed mortality which was only recorded within an additional 24 h after exposure, this endpoint may still be useful. Increasing the post-exposure observation periods compared to our study may be important, especially if linked with a longer observation of glycogen as energy reserves. In this context, the clear trend of decreased glycogen content with increased ammonia concentrations and lower temperatures is likely to become statistically significant if longer exposure and holding periods are considered. Behavioural endpoints

including mussel activity and clearance rates were found to be both useful and meaningful since they indicated a response pattern to both stressors, and since they provide a direct link with a concrete ecosystem service provided by the mussels. However, the pronounced behavioural variability observed among individuals in this study, as well as in previous studies addressing mussel filtration (e.g., Hartmann et al., 2016 a, b) and glochidia attachment (Beggel et al., 2014) suggest that long enough time intervals and sufficiently high numbers of replicates are needed to use it as a powerful indicator. Based on our findings, clearance rate measurement as an ecotoxicological endpoint needs to be carefully interpreted due to several reasons. First, filtration behaviour can be controlled by an internal rhythm which is not necessarily synchronized within the test population (Hartmann et al., 2016b). As measurements include only distinct time points, it is not clear for how long individual mussels were active within this period. Second, clearance rate is measured by the addition of food particles which should trigger feeding behaviour (e.g. particle clearance), which might be hampered by the avoidance response. Video analysis or more sophisticated tools such as real-time assessment of shell gaping would allow the quantification of the duration in avoidance responses or feeding (Hartmann et al., 2016 a, b). Recent studies have demonstrated that the nature and concentration of seston may greatly influence bivalve filtration rates, with low and high algal concentrations causing reductions in filtration, making comparison between studies very complicate (Hawkins et al., 2001; Riisgard et al., 2003; Liu et al., 2010).

In contrast to the above discussed endpoints, haemocyte ratios, mortality and cell morphology all proved to be sensitive and informative indicators of physiological disturbance even at low exposure concentrations. Moreover, they can be directly linked with immunological consequences. In particular, morphological changes indicating apoptosis on the cellular level seem to be a currently underestimated biomarker in freshwater mussel ecotoxicology, despite of the great relevance of this endpoint in fish (Cheng et al., 2015; Hasenbein et al., 2014), molluscs (Kiss, 2010), and a previous study in the freshwater snail *Lymnea stagnalis* also suggesting its usefulness (Russo and Madec, 2007). The proportion of hyalinocytes in relation to granulocytes increased in the organisms exposed to ammonia. Although functional attributes of haemocytes are not entirely clear, some studies suggest that hyalinocytes are associated to detoxification mechanisms (Soares-da-Silva, 2002; Lanz et al., 1993) which is in line with our data. The granulocytes function is more associated to phagocytic activities (Bayne et al., 1979; Lanz et al., 1993), being less influenced under toxicity stress. A higher recruitment of these cells

for detoxification may compromise other physiological roles, and - in the long run - diminish these cell types and weaken the immune system with direct consequence for the health status.

As evident from this study and previous publications, mussels are suitable model species in ecotoxicology, as they are sessile filter-feeders, taking up water, dissolved and suspended particles during feeding and breathing. Therefore they accumulate substances from their surrounding environment, mirroring the pollution of their habitat. Additionally, mussels play a key role in nutrient cycling and biodeposition of suspended matter, representing essential processes in ecosystem functioning (Vaughn et al, 2010). This key roles of mussels for freshwater ecosystem functioning and their observed ongoing declines require delineation of effective conservation strategies that also include water quality standards. In light of global change, elevated water temperatures and increasing concerns about ammonia in aquatic ecosystems, this study underlines the importance of including freshwater mussel fauna in environmental regulation as well as conservation plans.

Acknowledgments:

This study was partly funded by the Portuguese Foundation for Sciences and Technology (FCT) for the PhD grant of MH (SFRH/BD/76265/2011). We also acknowledge the laboratory assistance of Barbara Ziegler and Manuela Rost during the experiments.

References

Antunes, F., Hinzmann, M., Lopes-Lima, M., Machado, J., Martins da Costa, P., 2010. Association between environmental microbiota and indigenous bacteria found in hemolymph, extrapallial fluid and mucus of *Anodonta cygnea* (Linnaeus, 1758). Microbial ecology 60, 304-309.

Antunes, F., Hinzmann, M., Lopes-Lima, M., Vaz-Pires, P., Ferreira, S., Domingues, B., Martins da Costa, P., Machado, J., 2014. Antibacterial effects of *Anodonta cygnea* fluids on *Escherichia coli* and enterococci multi-drug-resistant strains: environmental implications. Toxicological and Environmental Chemistry 96(6), 880-889.

Augspurger, T., Keller, A.E., Black, M.C., Cope, W.G., Dwyer, F.J., 2003. Water quality guidance for protection of freshwater mussels (Unionidae) from ammonia exposure. Environmental Toxicology and Chemistry 22, 2569-2575.

Augspurger, T., Dwyer, F.J., Ingersoll, C.G., Kane, C.M., 2007. Advances and opportunities in assessing contaminant sensitivity of freshwater mussel (Unionidae) early life stages. Environmental Toxicology and Chemistry 26(10), 2025-2028.

Bayne, B.L., Moore, M.N., Windows, J., Livingstone, D.R., Salkeld, P., 1979. Measurement of the responses of individuals to environmental stress and pollution: studies with bivalve molluscs. Philosophical Transactions of the Royal Society. London Serie B 286, 562-581.

Beggel, S., Geist, J., 2014. Acute effects of salinity exposure on glochidia viability and host infection of the freshwater mussel *Anodonta anatina* (Linnaeus, 1758). Science of the Total Environment 502, 659-665.

Bogan A.E., 1993. Freshwater Bivalve Extinctions (Mollusca: Unionoida): A Search for Causes. Integrative and Comparative Biology 33, 599-609.

Campanella, L., Gatta, T., Ravera, O., 2005. Relationship between anti-oxidant capacity and manganese accumulation in the soft tissues of two freshwater molluscs: *Unio pictorum mancus* (Lamellibranchia, Unionidae) and *Viviparus ater* (Gastropoda, Prosobranchia). Journal of Limnology Volume: 64 (2), 153-158.

Carpenter, S.R., Caraco, N.F., Correll, D.L., Howarth, R.W., Sharpley, A.N., Smith, V.H., 1998. Nonpoint Pollution of Surface Waters with Phosphorus and Nitrogen. Ecological Applications 8(3), 559-568.

Cheng, T.C., 1981. Bivalves. In: Invertebrate Blood Cells. Vol. I, Ratcliffe, N.A. and A.F. Rowley (Eds.). Academic Press, London, ISBN: 0125821506 9780125821506, pp: 231-300.

Cheng, C.H., Yang, F.F., Liao, S.A., Miao, Y.T., Ye, C.X., Wang, A.L., Tan, J.W., Chen, X.Y., 2015. High temperature induces apoptosis and oxidative stress in pufferfish (*Takifugu obscurus*) blood cells. Journal of Thermal Biology 53, 172-179.

Constable, M., Charlton, M., Jensen, F., McDonald, K., Craig, G., Taylor, K.W., 2003. An ecological risk assessment of ammonia in the aquatic environment. Human and Ecological Risk Assessment 9, 527-548.

Cope, W.G., Bringolf, R.B., Buchwalter, D.B., Newton, T.J., Ingersoll, C.G., Wang, N., Augspurger, T., Dwyer, F.J., Barnhart, M.C., Neves, R.J., Hammer, E., 2008. Differential exposure, duration, and sensitivity of unionoidean bivalve life stages to environmental contaminants. Journal of the North American Benthological Society, 27(2), 451-462.

Coughlan, J., 1969. The estimation of filtration rates from the clearance of suspensions. Marine Biology 2, 256-258.

Cummings, K.S., Graf, D.L., 2010. Chapter 11 - Mollusca: Bivalvia, In Ecology and Classification of North American Freshwater Invertebrates (Third Edition), edited by James H. ThorpAlan P. Covich, Academic Press, San Diego, Pages 309-384.

Ford, S.E., Haskin, H.H., 1988. Comparison of in Vitro Salinity tolerance of Oyster Parasite, *Haplosporidium nelsoni* (MSX) and Hemocytes from Host, *Crassostrea virginica*. Comparative Biochemistry and Physiology Part A 90, 183–187.

Gagnaire, B., Thomas-Guyon, H., Renault, T., 2004. In vitro effects of cadmium and mercury on Pacific oyster, *Crassostrea gigas* (Thunberg), haemocytes. Fish Shellfish Immunology 16(4), 501-512.

Ganser, A.M., Newton, T.J., Haro, R. J., 2013. The effects of elevated water temperature on native juvenile mussels: implications for climate change. Freshwater Science 32(4), 1168-1177.

Geist, J., 2010. Strategies for the conservation of endangered freshwater pearl mussels (*Margaritifera margaritifera* L.): a synthesis of Conservation Genetics and Ecology. Hydrobiologia 644, 69-88.

Geist, J., 2011. Integrative freshwater ecology and biodiversity conservation. Ecological Indicators 11, 1507-1516.

Geist, J., 2015. Seven steps towards improving freshwater conservation. Aquatic Conservation: Marine and Freshwater Ecosystems 25, 447-453.

Haag, W.R., 2012. North American Freshwater Mussels: Natural History, Ecology, and Conservation, Cambridge University Press.

Hartmann, J.T., Beggel, S., Auerswald, K., Stoeckle, B.C., Geist, J., 2016a. Establishing mussel behavior as a biomarker in ecotoxicology. Aquatic Toxicology 170, 279-288.

Hartmann, J.T., Beggel, S., Auerswald, K., Geist, J., 2016b. Determination of the most suitable adhesive for tagging freshwater mussels and its use in an experimental study of filtration behavior and biological rhythm. Journal of Molluscan Studies (in press). DOI:10.1093/mollus/eyw003.

Hasenbein M., Werner, I., Deanovic, L.A., Geist, J., Fritsch, E.B., Javidmehr, A., Foe, C., Fangue, N.A., Connon, R.E., 2014. Transcriptomic profiling permits the identification of pollutant sources and effects in ambient water samples. Science of the Total Environment 468-469, 688-698.

Hawkins A.J.S., Fang, J.G., Pascoe, P.L., Zhang, J.H., Zhang, X. L., Zhu, M.Y., 2001. Modelling short-term responsive adjustments in particle clearance rate among bivalve suspension-feeders: separate unimodal effects of seston volume and composition in the scallop *Chlamys farreri*. Journal of Experimental Marine Biology and Ecology 262 (1), 61–73.

Hine, P.M., 1999. The inter-relationships of bivalve haemocytes. Fish and Shellfish Immunology 9(5), 367-385.

Hinzmann, M.F., Lopes-Lima, M., Bobos, I., Ferreira, J., Domingues, B., Machado, J., 2014. Morphological and chemical characterization of mineral concretions in the freshwater bivalve *Anodonta cygnea* (Unionidae). Journal of Morphology 276, 65–76.

Hinzmann, M.F., Lopes-Lima, M., Gonçalves, J., Machado, J., 2013. Antiaggregant and toxic properties of different solutions on hemocytes of three freshwater bivalves. Toxicological and Environmental Chemistry 95(5), 790-805.

Keppler, D., Decker, K., 1974. Glycogen determination with amyloglucosidase. In Methods of Enzymatic Analysis, vol. 3 (ed. H. U. Bergmeyer), pp. 1127-1131. New York: Academic Press.

Kiss, T., 2010. Apoptosis and its functional significance in molluscs. Apoptosis 15(3): 313-321.

Kryger J., Riisgård, H.U., 1988. Filtration rate capacities in 6 species of European freshwater bivalves. Oecologia 77 (1), 34-38.

Lanz, H., Tsutsumi, V., Arechiga, A., 1993. Morphological and biochemical characterisation of *Procambarus clarki* blood cells. Developmental & Comparative Immunology 17, 389–397.

LAWA (Länderarbeitsgemeinschaft Wasser): Beurteilung der Wasserbeschaffenheit von Fließgewässern in der Bundesrepublik Deutschland – Chemische Gewässergüteklassifikation. Berlin, 1998.

Liu, H., Yang, J., Gan, J., 2010. Trace element accumulation in bivalve mussels *Anodonta woodiana* from Taihu Lake, China. Archives of Environmental Contamination and Toxicology 59(4), 593-601.

Lopes-Lima, M., Sousa, R., Geist, J., Aldridge, D.C., Araujo, R., Bergengren, J., Bespalaya, Y., Bódis, E., Burlakova, L., Van Damme, D., Douda, K., Froufe, E., Georgiev, D., Gumpinger, C., Karatayev, A., Kebapçi, Ü., Killeen, I., Lajtner, J., Larsen, B.M., Lauceri, R., Legakis, A., Lois, S., Lundberg, S., Moorkens, E., Motte, G., Nagel, K.-O., Ondina, P., Outeiro, A., Paunovic, M., Prié, V., von Proschwitz, T., Riccardi, N., Rudzīte, M., Rudzītis, M., Scheder, C., Seddon, M., Şereflişan, H., Simić, V., Sokolova, S., Stoeckl, K., Taskinen, J., Teixeira, A., Thielen, F., Trichkova, T., Varandas, S., Vicentini, H., Zajac, K., Zajac, T., Zogaris, S., 2016. Conservation status of freshwater mussels in Europe: state of the art and future challenges. Biological Reviews, n/a-n/a.

Lydeard, C., Cowie, R.H., Ponder, W.F., Bogan, A.E., Bouchet, P., Clark, S.A., Cummings, K S., Frest, T.J., Gargominy, O., Herbert, D.G., Hersheler, R., Perez, K.E., Roth, B., Seddon, M., Strong, E.E., Thomson, F.G., 2004. The global decline of nonmarine mollusks. Bioscience 54(4), 321-330.

McIvor, A.L., 2004. Freshwater mussels as biofilters. PhD thesis, Deparment of Zoology, University of Cambridge.

McMahon, R.F., Bogan, A.E., 2001. Mollusca: Bivalvia. In: Ecology and Classification of North American Freshwater Invertebrates, J.H. Thorp and A.P. Covich, eds, 2nd ed, Chapter 10, pp. 297–330. San Diego: Academic Press.

Naimo, T.J., 1995. A review of the effects of heavy metals on freshwater mussels. Ecotoxicology 4:341–362.

Naimo, T.J., Damschen, E.D., Rada, R.G., Monroe, E.M., 1998. Nonlethal evaluation of the physiological health of unionid mussels: methods for biopsy and glycogen analysis. Journal of the North American Benthological Society 17(1), 121-128.

Nobles, T., Zhang, Y., 2015. Survival, growth and condition of freshwater mussels: effects of municipal wastewater effluent. PloS one 10, e0128488.

Raimondo, S., Lilavois, C.R., Lee, L., Augspurger, T., Wang, N., Ingersoll, C.G, Bauer, C., Hammer, E., Barron, M.G., 2016. Assessing variability in chemical acute toxicity of unionid mussels: Influence of intra- and inter-laboratory testing, life stage, and species. Environmental Toxicology and Chemistry 3, 750-758.

Richter, A., Stoeckl, K., Denic, M., Geist, J., 2016. Association between the occurrence of the Thick-shelled River Mussel (*Unio crassus*) and Macroinvertebrate, Microbial and Diatom Communities; Freshwater Science 35 (3) 922-933.

Riisgard, H.U, Kittner, C., Seerup, D.F., 2003. Regulation of opening state and filtration rate in filter-feeding bivalves (*Cardium edule*, *Mytilus edulis*, *Mya arenaria*) in response to low algal concentration. Journal of Experimental Marine Biology and Ecology 284, 105–127.

Russell, J.E., John, W.N., Philip, M.C., Gerald, T.A., 2008. Bioavailability of Chemical Contaminants in Aquatic Systems. The Toxicology of Fishes, CRC Press, 9-54.

Russo, J., Madec, L., 2007. Haemocyte apoptosis as a general cellular immune response of the snail, *Lymnaea stagnalis*, to a toxicant. Cell Tissue Research 328(2), 431-441.

Soares-da-Silva, I.M., Ribeiro, J., Valongo, C., Pinto, R., Vilanova, M., Bleher, R., Machado, J., 2002. Cytometric, morphologic and enzymatic characterisation of haemocytes in *Anodonta cygnea*. Comparative Biochemistry and Physiology A-Molecular and Integrative Physiology 132(3), 541-553.

Strayer, D.L., Downing, J.A., Haag, W.R., King, T.L., Layzer, J.B., Newton, T.J., Nichols, S.J., 2004. Changing perspectives on pearly mussels, North America's most imperiled animals. BioScience 54, 429-439.

Strayer, D.L., 2006. Challenges for freshwater invertebrate conservation. Journal of the North American Benthological Society 25, 271-287.

Tirard, C.T., Grossfeld, R. M., Levine, J. F., Kennedy-Stoskopf, S., 1997. Effect of Osmotic Shock on Protein Synthesis of Oyster Hemocytes in Vitro. Comparative Biochemistry and Physiology Part A: Physiology 116, 43–49.

US EPA, 2013. Aquatic life ambient water quality criteria for ammonia – freshwater. EPA 822-R-13-001.

Van Damme, D., 2011. *Unio pictorum*. The IUCN Red List of Threatened Species 2011: e.T155543A4795613. http://dx.doi.org/10.2305/IUCN.UK.2011-2.RLTS.T155543A4795613.en.

Van Hassel, J.H., Farris, J.L., 2007. A review of the use of unionid mussels as biological indicators of ecosystem health. Pages 19–49 in J. L. Farris and J. H. Van Hassel (editors). Freshwater bivalve ecotoxicology. CRC Press, Boca Raton, Florida, and SETAC Press, Pensacola, Florida.

Vaughn, C.C., 2010. Biodiversity losses and ecosystem function in freshwaters: Emerging conclusions and research directions. Bioscience, 60(1), 25-35.

Wang, N., Ingersoll, C.G., Hardesty, D.K., Ivey, C.D., Kunz, J.L., May, T.W., Dywer, F.J., Roberts, A.D., Augspurger, T., Kane, C.M., Neves, R.J., Barnhart, M.C., 2007. Acute Toxicity of Copper, Ammonia, and Chlorine to Glochidia and Juveniles of Freshwater Mussels (Unionidae). Environmental Toxicology and Chemistry 26 (10), 2036-2047.

Ward, S., Augspurger, T., Dwyer, F.J., Kane, C., Ingersoll, C.G., 2007. Risk assessment of water quality in three North Carolina, USA, streams supporting federally endangered freshwater mussels (Unionidae). Environmental Toxicology and Chemistry 26(10), 2075-2085.

Chapter 6

Antibacterial effects of *Anodonta cygnea* fluids on *Escherichia coli* and enterococci multi-resistant strains: Environmental implications

Antibacterial effects of *Anodonta cygnea* fluids on *Escherichia coli* and enterococci multi-drug-resistant strains: environmental implications

F. Antunes [a,b], M. Hinzmann [a,b], M. Lopes-Lima [a,b], P. Vaz-Pires [a,b], S. Ferreira [a,b], B. Domingues [a,b], J. Machado [a,b] and P. Martins da Costa [a,b]

[a] Instituto de Ciências Biomédicas de Abel Salazar (ICBAS), Universidade do Porto, Porto,Portugal;

[b] Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR), Universidade do Porto, Porto, Portugal

Adapted from: Antunes, F., Hinzmann, M., Lopes-Lima, M., Vaz-Pires, P., Ferreira, S., Domingues, B., Machado, J., Martins da Costa, P., 2014. Antibacterial effects of *Anodonta cygnea* fluids on *Escherichia coli* and enterococci multi-drug-resistant strains: environmental implications. Toxicological & Environmental Chemistry 96 (6), 880-889.

Abstract

The persistence of bacteria in *Anodonta cygnea* largely depends on the haemolymph bactericidal activity against aquatic micro-organisms.

The aim of this study was to assess the *in vitro* bactericidal activity of whole haemolymph against multi-drug resistant *Escherichia coli* and enterococci strains. Four mussels were injected with multi-resistant strains of *E. coli* in order to determine their *in vivo* bactericidal reactivity. *In vitro* experiments showed that the haemocytes viability decreased almost 70% in 4 h, the same happened in the control haemolymph. Enterococci tested in *in vitro* experiments were more susceptible than *E. coli* to whole haemolymph and haemocytes fractions. None of the enterococci bacteria was detected in the haemolymph fractions from 24 to 72 h, while in *E. coli was* still detectable 72 h after inoculation both in the control and respective plasma fractions. The microbial inhibition on both haemolymph and cell pellets suggest that haemocytes may be the main responsible for that process.

In vivo experiments showed that haemolymph inhibited the *E. coli* load injected and it was correlated with a high tendency for the increase in haemocytes counts, after 72 h. High adherence of *E. coli* and an intense pseudopods manifestation, mainly to the granulocytes, suggest a clear phagocytosis process.

Introduction

The freshwater mussel *Anodonta cygnea* (Bivalvia; Unionoidae) has a very restricted distribution in Portugal and the existing populations can only be found in three lagoons in the north and center of the country (Reis, 2006). Additionally, the high human pressure on the aquatic environment where the mussels live (fishing activities, extraction of inert materials, and pollution) is threatening the viability of these species in those lagoons. Their conservation depends on maintenance of the ecological integrity of their habitat, and also on the reintroduction in sites where they might have existed previously (Reis, 2006).

As filter feeders, bivalves are exposed to a constant challenge by various pathogenic and/or opportunistic bacteria naturally present in the microflora of aquatic environments (Labreuche et al., 2006). Enterococci species and *E. coli* are part of the intestinal microflora of most mammals and birds and are released into the environment by human and animal dejections (Dancer, 2004). Available evidence supports the hypothesis that persistence of bacteria in bivalve tissues depends, at least in part, on their resistance to the bactericidal activity of the haemolymph (Canesi et al., 2001; Zampini et al., 2003).

Although lacking an adaptive immune system, these animals have evolved an effective mechanism for eliminating invading bacteria based upon a complex interplay between cellular and humoral defence responses. The cellular immune system relies specifically on immuno-competent cells, referred to collectively as haemocytes. In bivalves, three major haemocyte types are commonly recognized: granulocytes, hyalinocytes and agranulocytes. These cells are responsible for activities such as inflammation, wound repair, phagocytosis, and encapsulation of non-self-particles (Cheng, 1996).

Since the bivalve internal defence system is largely based on haemocytes, changes in haemocyte counts and activities have been widely used as an *in vivo* indicator of the microbial challenge of animals, following natural or experimental contact with pathogens (Suresh and Mohandas, 1990; Chu and La Peyre, 1993; Allam et al., 2000). In addition, humoral factors released by haemocytes may help the fight against invasive micro-organisms (Chu, 2000). These factors include hydrolytic enzymes, such as lysozyme, which can act directly against bacteria by lysing these micro-organisms or indirectly by increasing the killing activity of other antibacterial substances (Smith et al., 1995). *In vivo* changes in both cellular and humoral factors have already been reported in *Ruditapes philippinarum* naturally (Allam et al., 2000) or experimentally infected with *Vibrio tapetis*. Severely infected *R. philippinarum* exhibited low haemocyte counts and phagocytic activity, whereas infections of the more resistant clam species *R. decussatus* provoked increased haemocyte counts and phagocytic activity (Allam et al., 2006).

The aim of this study was to test the bactericidal reactivity of *A. cygnea* haemolymph against *E. coli* and enterococci strains, performing *in vitro* and assays based on the haemolymph, haemocytes, and plasma fractions behaviour when inoculated with the bacteria. This procedure was conducted under preliminary conditions in order to evaluate first the immuno-responses capacity to external bacterial agents in the habitat.

Material and methods

Bacterial strains

For this experimental study, 24 *E. coli* and 20 enterococci strains were randomly selected among a collection of strains isolated from inflow, effluent, and sludge from Portuguese municipal wastewater treatment plants, which had displayed simultaneous resistance against, at least, five different antimicrobial drugs (Martins da Costa et al., 2006, 2007). The original reference cultures were maintained in cryogenic storage at -80 ºC on glass

beads. *E. coli* and enterococci strains were grown on buffered peptone water (Oxoid, Basingstoke, UK) at 37 ºC for 8 h adjusted at 10^8 colony forming units (CFU) mL^{-1} with Triptone Salt (Biokar Dianostics, Beauvais, France).

The *in vivo* experiment was conducted using an *E. coli* strain resistant to ampicillin, tetracycline, trimethoprime/sulfamethoxazole, streptomycin, gentamicin, apramycin and ciprofloxacin. The original suspension of *E. coli* was adjusted to 4.0×10^7 CFU mL^{-1} in order to be injected in the tested bivalves.

The real of *E. coli* contents in the haemolymph was estimated to be 1.0×10^4 CFU mL^{-1}, taking into account the physiological dilution in the body haemolymph volume (200 mL in an adult bivalve).

Haemolymph collection

Freshwater mussels, *A. cygnea*, were collected from the Mira Lagoon in northern Portugal in spring period. The specimens were kept in refrigerated tanks during less than 1 h of transportation to the laboratory under appropriated conditions.

For the fluids collection, the animals were considered healthy when they closed their valves when disturbed, showed active ventilation and powerful valve closing or water ejection upon disturbance, and when their nacre presented a smooth and shiny look (Soares-da-Silva et al., 2002). During this procedure, the tip of the needle was always in contact with the inner epithelium surface, the needle was previously heated to avoid the risk of contamination by extraneous water bacteria. The haemolymph was carefully removed with a 0.8 × 40 mm needle attached to a sterile 10 mL Hamilton syringe. The needle was inserted between the valves across the inner layer of the mantle and into the inter-epithelial mantle space, carefully avoiding turbulence (Soares-da-Silva et al., 2002).

To obtain the haemolymph cellular fraction, samples were immediately centrifuged at 200 × *g* for 10 min at 4 ºC. Supernatants (plasma fraction) were transferred to Falcon tubes for the *in vitro* assays. The haemolymph, cell pellet, and the cell-free plasma were all kept on ice until being used.

Due to animal care proposes, after fluid collection, the bivalves were kept in tanks with lagoon mud, with aerated and dechlorinated water and acclimatized to these conditions with a microalgae diet, to simulate natural conditions.

In vitro assay

The effect of haemolymph, cellular fraction, and plasma, previously collected, on each bacterial strain was assayed *in vitro* using 96-well microplates. Ten microlitres of each bacterial suspension were distributed in three wells. Then, 90 µL of haemolymph, cellular fraction, or plasma were added to each well containing the same bacterial suspension. The plates were wrapped in aluminium foil, in order to avoid exposure to light, and incubated on a rotary shaker (300 rpm) at 25 ºC for 72 h. One microlitre of each bacterial suspension plus bivalve sample was collected at 1, 3, 24 and 72 h for further analysis. Bacterial suspensions and haemolymph fractions samples were used as control of bacterial growth and absence of growth, respectively. Bacterial enumeration was performed using the CFU technique, after serial dilutions when appropriate, and incubation at the conditions described in section "Bacteriological analysis".
E. coli ATCC 25922 and *E. faecalis* ATCC 29212 were used as control strains.

In vivo assay

Four mussels were challenged by one injection of *E. coli* with antimicrobial resistance mentioned in section "Bacterial strains", into the posterior adductor muscle, as previously described (Canesi et al., 2001; Zampini et al., 2003), with 50 µL containing 4.0×10^7 CFU mL^{-1}. Putative effect of experimental handling and/or bacteria growth media was checked by injecting bacteria-free phosphate saline buffer. After injection, mussels were returned to water and the haemolymph was collected 24 and 72 h post-injection. In one mussel (nr.3), haemolymph collection was only performed 72 h post injection, in order to check if there was any stressful effect from the manipulative operation on the immunological responses. About 2 mL of haemolymph were extracted from each mussel as above described and haemocytes on circulating haemolymph (granulocytes and hyalinocytes) were enumerated and their viability assessed.

Bacteriological analysis

Enumeration of *E. coli* and enterococci was determined by plating 100 µL of each sample on Slanetz and Bartley (SB) (Oxoid) and tryptone bile X-glucuronide agar (TBX) (Biokar Diagnostics), respectively. The plates were incubated at 37 ºC during 48 h in SB for *E. coli* and at 44 ºC during 24 h for TBX for enterococci. A Gram staining was performed on haemolymph samples to observe the cell activity relatively to the bacterial infection.

Haemocytes enumeration

Haemocyte counts in 0.1 mL haemolymph were determined using an improved Neubauer haemocytometer (American Optical, Buffalo, NY, USA) according to what was previously described by Mayrand et al. (2005). The cell viability of haemolymph samples was determined by trypan blue (Sigma-Aldrich, St. Louis, MO) exclusion, counting the number of viable cells (Tafalla et al., 2002). Haemocyte concentration is expressed as cells per millilitre of undiluted haemolymph.

Data analysis

Data analysis was carried out using the SPSS version 15.0 for Windows®. Each result represents the median ± standard deviation. Values were previously log-transformed (variable + 1) in order to homogenise their distribution.

Results

In vitro viability of *E. coli* and enterococci multi-drug-resistant strains in association with haemolymph

Enterococci strains were more susceptible than *E. coli* strains to the exposure to haemolymph, cell-free haemolymph, and cell pellet (Tables 1 and 2). Between all the fractions tested, the cell pellet was the more effective on the inhibition of bacterial growth. The viability of enterococci and *E. coli* strains inoculated in haemolymph and cell fractions became null at 1 and 24 h after inoculation, respectively, while both bacteria had highly proliferated after 72 h in the control suspensions. The microbial inhibition of both strains started mainly within the first hour. In the plasma fractions, it was observed that there was no inhibition for *E. coli* strains; in contrast, a fourfold increase was observed at 24-72 h, comparatively to the control. Among enterococci assays, the very low median values revealed a high inhibitory activity of haemolymph elements even in the plasma incubation. Furthermore, an increase on *E. coli* viability occurred after 72 h in both the plasma fraction and the control, whereas *in vitro* inoculation of haemolymph with bacteria revealed that haemocytes viability decreased almost 70% in 4 h (Figure 1) as well as in the control.

Table 1 - *In vitro* assays showing median values of viable counts of *E. coli* multi-resistant strains (n = 24) 1 and 3 h after exposure to haemolymph and cell pellet. The values obtained for plasma are expressed after 24 and 72 h. The control consisted of an *E. coli* suspension (2.5×10^7 CFU mL^{-1}). The median values are expressed in CFU mL^{-1}.

	Number of *E. coli* (median) (CFU mL^{-1})			
Time (h)	Haemolymph	Cells	Plasma	Control
1	1.4×10^4	9.6×10^3	1.1×10^6	5.2×10^6
3	1.4×10^3	2.2×10^3	5.1×10^6	5.2×10^6
24	0.0	0.0	1.1×10^7	1.8×10^6
72	0.0	0.0	4.1×10^7	1.8×10^8

Table 2 - *In vitro* assays showing median values of viable counts of enterococci multi-resistant strains (n = 20) 1 and 3 h after exposure to haemolymph and cell pellet. The values obtained for plasma are expressed after 24 and 72 h. The control consisted of an enterococci suspension (1.7×10^7 CFU mL^{-1}). The median values are expressed in CFU mL^{-1}.

	Number of enterococci (median) (CFU mL^{-1})			
Time (h)	Haemolymph	Cells	Plasma	Control
1	0.0	0.0	0.0	1.8×10^5
3	0.0	0.0	0.0	1.9×10^6
24	0.0	0.0	0.0	6.0×10^6
72	0.0	0.0	0.0	4.5×10^6

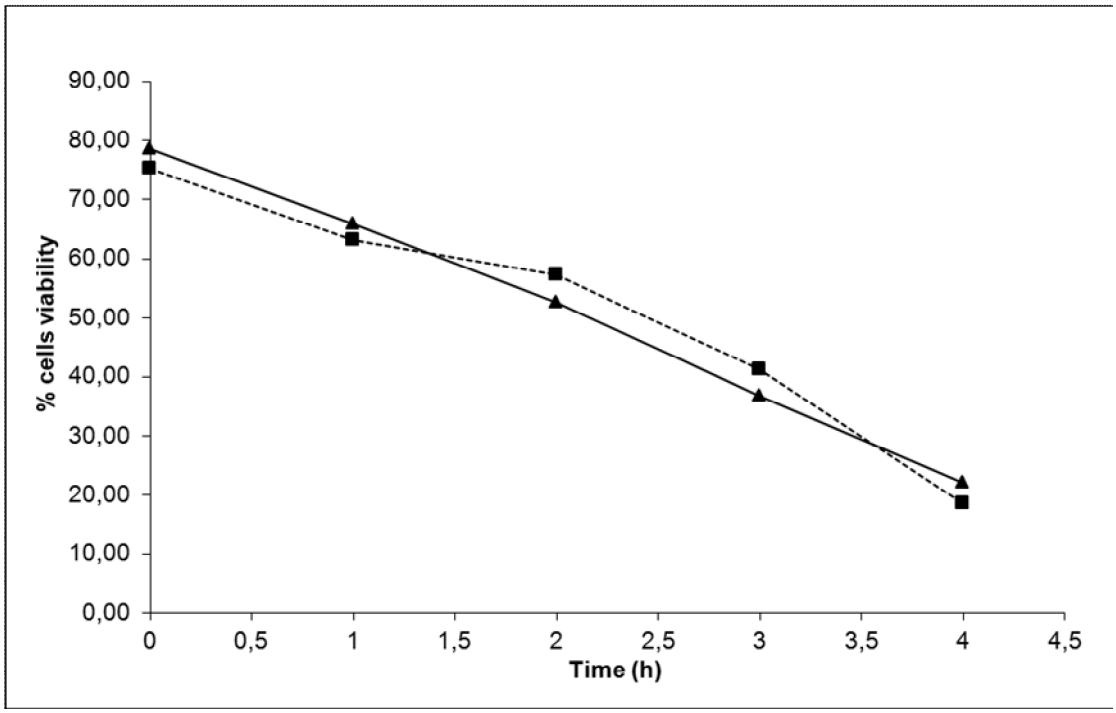


Figure 1 - Effect of *in vitro* experimental time on cell viability (%) after bacterial inoculation. Bacteria + Haemocytes (—▲—); Haemocytes (Control) (- - -■- - -).

Effect of *in vivo* challenge with *E. coli* on haemolymph

In general, a decrease in the *E. coli* contents occurred in the haemolymph of all bivalve *A. cygnea* after incubation, mainly from 0 h (1.0×10^4 CFU mL^{-1}) to 72 h ($0.0\text{-}7.7 \times 10^2$ CFU mL^{-1}). The plasma fraction showed a similar reduction in *E. coli* levels, whereas the cell fraction showed a higher bacterial content, although with evidence of *E. coli* reduction from 0 to 72 h.

From the haemocyte counts on haemolymph, it was observed a larger concentration of granulocytes than hyalinocytes in all mussels (1, 2, 3, and 4). In general, with the exception of mussel 1, all the mussels assayed exhibited a tendency for higher cellular counts particularly for the granulocytes after 72 h (Table 3), which was inversely correlated with bacterial counts (Table 4). In fact, mussel 1 showed a decrease of haemocyte numbers, from 9.3×10^5 to 5.3×10^5 granulocytes mL^{-1} and from 3.6×10^5 to 2.2×10^5 hyalinocytes mL^{-1}. In mussel 2, the number of hyalinocytes decreased after 72 h 1.3×10^5 from to 1.2×10^5 cells mL^{-1}. On the contrary, mussel 4 was the one in which the major increase of hyalinocytes counts was detected during the *in vivo* assay, from $7.8x10^4$ to

$3.0x10^5$ cells mL^{-1}. Mussel 3, stress controller assay, was sampled at 0 and 72 h post-injection and showed a slight increase on both granulocytes and hyalinocytes counts. Moreover, it is possible to highlight that after 72 hours all mussels exhibited at least the double of granulocytes comparatively to the hyalinocytes.

Table 3 - *In vivo* assay presenting median values of granulocytes (G) and hyalinocytes (H) counts in the haemolymph from *A. cygnea* (mussels 1, 2, 3, and 4) at time 0, 24, and 72 h, after inoculation with 1 mL of *E. coli* original suspension containing 4.0×10^7 CFU mL^{-1}.

Time (h)	Mussel							
	1		2		3		4	
	G	H	G	H	G	H	G	H
0	9.3×10^5	3.6×10^5	3.5×10^5	1.3×10^5	1.2×10^6	2.0×10^5	7.8×10^4	9.3×10^4
24	6.5×10^5	1.8×10^5	6.3×10^5	2.0×10^5	Control*	Control*	1.3×10^5	7.3×10^4
72	5.3×10^5	2.2×10^5	7.4×10^5	1.2×10^5	1.3×10^6	2.3×10^5	3.0×10^5	1.5×10^5

*Stress manipulation control

Concerning the efficiency of haemolymph against injected *E. coli* bacteria, under physiological concentrations around 1.0×10^4 CFU mL^{-1} (see the method section), the results showed that in all mussels *E. coli* was clearly inhibited (Table 4), *i.e.* the microbial load decreased in all the mussels tested, with a complete elimination of *E. coli* in mussel 2 and a reduction of at least 10 times in mussels 1 and 4. In the control, mussel 3, it was possible to admit that the *E. coli* was also reduced taking into account the same level of contents comparatively to the others.

Regarding the light microscope observation, the Gram staining reaction confirmed that gram-negative bacilli (*E. coli*) were phagocytised by granulocytes (Figure 2).

Table 4 - *In vivo* assay presenting median values of *E. coli* counts in haemolymph, plasma, and cellular fraction collected 24 and 72 h after inoculation with 1 mL of *E. coli* original suspension containing 4.0×10^{7} CFU mL^{-1}. The final physiological concentration of *E. coli* in the whole bivalve haemolymph after injection corresponds to 1.0×10^{4} CFU mL^{-1}.

		Number of *E. coli* (CFU mL^{-1})		
Time (h)	Mussel number	Haemolymph	Plasma	Cells
24	1	1.9×10^{3}	2.4×10^{2}	5.1×10^{3}
	2	3.9×10^{2}	5.5×10^{1}	2.0×10^{2}
	3	*Control	*Control	*Control
	4	1.7×10^{3}	5.1×10^{2}	5.2×10^{3}
72	1	7.7×10^{2}	4.2×10^{2}	3.5×10^{3}
	2	0	0	7.5×10^{1}
	3	1.5×10^{2}	8.0×10^{1}	7.9×10^{2}
	4	4.2×10^{2}	1.6×10^{2}	3.3×10^{3}

*Stress manipulation control

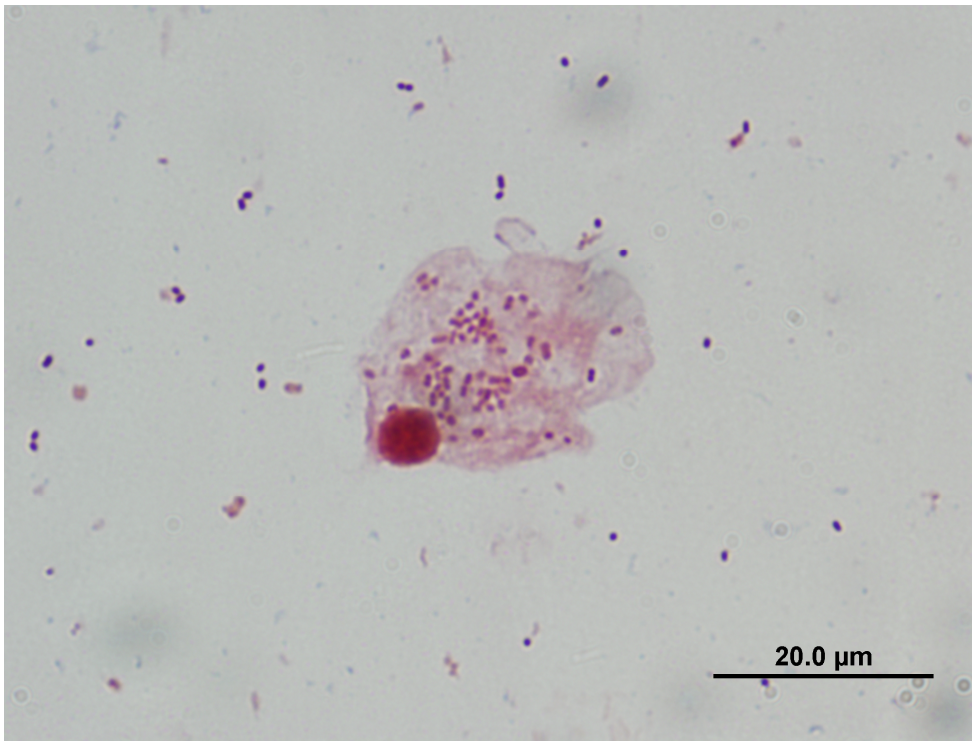


Figure 2 - Granulocyte with long projections (pseudopods) and Gram-negative bacilli phagocytosis by *Anodonta cygnea* granulocyte. Several Gram-positive cocci and Gram-negative bacilli with a vibrionic form are observed on the haemolymph. The scale bars represent 20 µm.

Discussion

In vitro assays enable the use of small amounts of fluids which leads to a lesser disturbance of the mussels and do not compromise their health status. This method also allows the testing of several bacterial strains with the same haemolymph samples. However, when the priority is the assessment of the immune system modulation on the pathogens elimination, *in vivo* assays are indispensable to elucidate all the complex processes that could be involved. It was decided to use only four mussels to perform the *in vivo* experiment as a preliminary research work, in order to adjust a correct methodology for deeper future studies on immune-responses in bivalves. Moreover, the present work was based on the findings from the research proposal of Antunes et al., 2010, in which they stated that *E. coli* and enterococci bacteria were completely absent in the haemolymph, although being clearly present in the surrounding environment of Mira Lagoon, Aveiro. Thus, in this context, the current work appears as an experimental case report which can offer an interesting new research line.

Our results revealed that *in vivo* challenge with *E. coli* has elicits a cellular response in the haemolymph of *A. cygnea* in terms of haemocyte counts, which probably results from stimulation of migration from the tissues, rather than proliferation of the cells (Coles et al., 1995). Although an *E. coli* content reduction occurred in all fractions throughout time, the higher charge of *E. coli* in the cell fraction when compared to the plasma and whole haemolymph, after bacterial injection, suggests its greater affinity and adherence as well as subsequent stronger reactivity against the bacteria. Furthermore, morphological evaluation of haemocytes through optical microscopy led to the verification that the mobilized haemocytes were mainly granulocytes. Granulocytes are considered to be the major effector cells for internal defence in bivalve molluscs because of their ability to produce a wide array of enzymes and antimicrobial substances (Mitta et al., 1999; Chu, 2000) and their high phagocytic activity (Lopez et al., 1997; Lopez-Cortes et al., 1999). *E. coli* counts over time and Gram staining observations provide evidence for an active phagocytosis of the resistant strain inoculated by granulocytes. Our observations are in agreement with Pipe et al. (1995), which reported the predominant mechanism of internal defence in bivalves involving phagocytosis by the circulating haemocytes.

The *in vitro* assays showed that whole haemolymph exhibited high microbial load, when compared with the cellular fraction, mainly in the first hour followed by a fast levelling between them followed by the complete disappearance of bacterial activity within 24 h.

This may suggest that the beginning process of immune reaction may result in a combined activity between the humoral and cell fraction in the haemolymph. In contrast, when the microbial load in cell fraction is compared with that in the plasma fraction, it is very evident that the cell fraction is the main contributor for the strong agglutination and elimination of *E. coli* bacteria. This is mainly accentuated at 24 hours after bacterial injection and suggests that the *E. coli* attachment to *A. cygnea* haemocytes is an effective mechanism, probably due to a cellular recognition process between granulocytes and bacteria which lead to further phagocytosis. This was not surprising considering that phagocytosis requires biochemical surface interactions (i.e. recognition, chemotaxis, and attachment) prior to incorporation and destruction (Canesi et al., 2001; Parisi et al., 2008). In the present discussion, any interference from the haemocytes viability during the *in vitro* experiments can be excluded, since the cell damage percentage was very similar both in bacteria inoculated and in the free-control solutions.

A second conclusion to be drawn from the present results is that the phagocytosis process was prompt. This is corroborated by modifications on cellular morphology (pseudopod exhibition) and the rapid decline in the numbers of viable bacteria. Based on the present work, we cannot exclude other hypothesis beyond the granulocytes phagocytosis process when submitted to prior *E. coli* inoculation. Eventually, some specific interactions between bacteria and haemolymph elements (cellular and/or humoral factors) may be responsible for differences regarding the capacity to kill, or even tolerate, a certain bacteria (Pruzzo et al., 2005; Li et al., 2008; Parisi et al., 2008).

In vitro experiments demonstrated that *A. cygnea* fluids, particularly the plasma fraction, are inefficient per se inefficient on microbial elimination, suggesting that the bactericidal activity of haemolymph is less dependent on cytolytic elements (e.g. antimicrobial peptides and lysosomal enzymes) described in previous investigations (Pruzzo et al., 2005; Labreuche et al., 2006). However, the role of opsonisation substances (agglutinins) in facilitating bacterial aggregation (and/or immobilization) to haemocytes could not be scrutinized by the design of this study.

Interestingly, when comparing bacterial viability following the exposure to whole haemolymph or cells and plasma fractions, enterococci were easily inhibited from the beginning. This might be explainable by the establishment of different surface interactions between haemocytes and Gram positive or Gram negative bacteria, which could elicit distinct responses both in terms of cell damage, activation, and release of humoral and/or kinase-mediated pathways components involved in the immune- responses (Canesi et al.,

2001; Zampini et al., 2003; Canesi et al., 2005). Additionally, considering that the viability of the plasma cells decreased to 70% in 4 hours, the fast decline in culturable bacteria is attributable to either a powerful phagocytic competence (enhanced by a great cell concentration) or a massive release of inhibitory factors existing in the haemocyte cytoplasm.

In conclusion, the results obtained with the *in vitro* and *in vivo* experiments point out interesting information, relatively to strong functional aspects of the immune response of mussels against very resistant bacteria contamination, in particular in *A. cygnea*. The application of this knowledge to the future understanding on the specific responses and mechanism in bivalves when exposed to strange micro-organisms from their natural environment can be of significant ecological, economical, and public health-related interest.

Funding

This work was supported by a grant of the Portuguese Foundation for Science and Technology and also under projects PTDC/MAR/098066/2008, PTDC/AAC-AMB/117688/2010 and Pluriannual ProgramCIIMAR and FCT grant SFRH/BD/76265/2011.

References

Allam, B., Paillard, C., Howard, A., Le Pennec, L., 2000. Isolation of the pathogen *Vibrio tapetis* and defense parameters in Manila clam, *Ruditapes philippinarum*, cultured in England. Diseases of Aquatic Organisms 41, 105-113.

Allam, B., Paillard, C., Auffret, M., Ford, S.E., 2006. Effects of the pathogenic *Vibrio tapetis* on defence factors of susceptible and non-susceptible bivalve species: II. Cellular and biochemical changes following *in vivo* challenge. Fish and Shellfish Immunology 20, 384-397.

Antunes, F., Hinzmann, M., Lopes-Lima, M., Machado, J., Martins da Costa, P., 2010. Association between environmental microbiota and indigenous bacteria found in haemolymph, extrapallial fluid and mucus of *Anodonta cygnea* (Linnaeus, 1758). Microbial Ecology 60(2), 304-309.

Canesi, L., Pruzzo, C., Tarsi, R., Gallo, G., 2001. Surface interactions between *Escherichia coli* and the haemocytes of the Mediterranean mussel *Mytilus galloprovincialis* Lam. leading to efficient bacterial clearance. Applied and Environmental Microbiology 67, 464–468.

Canesi, L., Betti, M., Ciacci, C., Lorusso, L.C., Gallo, G., Pruzzo, C., 2005. Interactions between *Mytilus edulis* haemocytes and different strains of *Escherichia coli* and *Vibrio cholerae* O1 El Tor: role of kinase-mediated signalling. Cellular Microbiology 7, 667–674.

Cheng, T.C., 1996. *Haemocytes: forms and functions*. in: The Eastern Oyster *Crassostrea virginica*, edited by V.S. Kennedy, R.I.E. Newell, A.F. Eble, 299-333., Maryland Sea Grant, College Park, MD, USA.

Chu, F., La Peyre, J.F., 1993. *Perkinsus marinus* susceptibility and defense-related activities in eastern oysters *Crassostrea virginica*: temperature effects. Diseases of Aquatic Organisms 16, 223-234.

Chu, F., 2000. *Defense mechanisms of marine bivalves*. in: Recent Advances in Marine Biotechnology, vol. 5, Immunology and Pathology, edited by M. Fingerman, R. Nagabhushanam, 142. Enfield, NH: Science Publishers Inc.

Coles, J.A., Farleyand, S.R., Pipe, R.K., 1995. Alteration of the immune response of the common marine mussel *Mytilus edulis* resulting from exposure to cadmium. Diseases of Aquatic Organisms 22, 59-65.

Dancer, S.J., 2004. How antibiotics can make us sick: the less obvious adverse effects of antimicrobial chemotherapy. Lancet Infectious Diseases 4, 611-619.

Labreuche, Y., Lambert, C., Soudant, P., Boulo, V., Huvet, A., Nicolas, J.L., 2006. Cellular and molecular haemocyte responses of the Pacific oyster, *Crassostrea gigas*, following bacterial infection with *Vibrio aestuarianus* strain 01/32. Microbes and Infection 8, 2715-2724.

Li, H., Parisi, M.G., Toubiana, M., Cammarata, M., Roch, P., 2008. Lysozyme gene expression and haemocyte behaviour in the Mediterranean mussel, *Mytilus galloprovincialis*, after injection of various bacteria or temperature stresses. Fish and Shellfish Immunology 25, 143-152.

Lopez, C., Carballal, M.J., Azevedo, C., Villalba, A., 1997. Differential phagocytic ability of the circulating haemocytes types of the carpet shell clam, *Ruditapes decussatus* (Mollusca, Bivalvia). Diseases of Aquatic Organisms 30, 209-215.

Lopez-Cortes, L., Castro, D., Navas, J.I., Borrego, J.J., 1999. Phagocytic and chemotactic responses of Manila and carpet shell clam haemocytes against *Vibrio tapetis*, the causative agent of brown ring disease. Fish and Shellfish Immunology 9, 543-555.

Martins da Costa, P., Vaz-Pires, P., Bernardo, F., 2006. Antimicrobial resistance in *Enterococcus* spp. isolated in inflow, effluent and sludge from municipal sewage water treatment plants. Water Research 40, 1735-1740.

Martins da Costa, P., Vaz-Pires, P., Bernardo, F., 2007. Antimicrobial resistance in *Escherichia coli* isolated in inflow, effluent and sludge from municipal wastewater treatment plants. Urban Water Journal 4, 275- 281.

Mayrand, E., St-Jean, S.D., Courtenay, S.C., 2005. Haemocyte responses of blue mussels (*Mytilus edulis* L.) transferred from a contaminated site to a reference site: can the immune system recuperate?" Aquaculture Research 36, 962-971.

Mitta, G., Vandenbulcke, F., Hubert, F., Roch, P., 1999. Mussel defensins are synthesised and processed in granulocytes then released into the plasma after bacterial challenge. Journal of Cell Science 112, 4233-4242.

Parisi, M.G., Li, H., Jouvet, L.B.P., Dyrynda, E.A., Parrinello, N., Cammarata, M., Roch, P., 2008. Differential involvement of mussel haemocyte sub-populations in the clearance of bacteria. Fish and Shellfish Immunology 25, 834–840.

Pipe, R.K., Coles, J.A., Thomas, M.E., Fossato, V.U., Pulsford, A.L., 1995. Evidence for environmentally derived immunomodulation in mussels from the venise lagoon. Aquatic Toxicology 32, 59-73.

Pruzzo, C., Gallo, G., Canesi, L., 2005. Persistence of *Vibrios* in marine bivalves: the role of interactions with haemolymph components. Environmental Microbiology 7, 761-772.

Reis, J., 2006. *Atlas dos Bivalves de água doce em Portugal continental.* in: Instituto da Conservação da Natureza, 130pp. Lisboa.

Smith, V.J., Findlay, C., Scnapp, D., Hutton, D.M.C., 1995. *Detection of antibacterial activity in the tissues or body fluids of marine invertebrates.* Pages 173-82 in: Techniques in Fish Immunology, vol. 4, edited by J.S., Stolen, T.C., Fletcher, S.A. Smith, J.T. Zelikoff, S.L, Kaattari, R.S., Anderson. Fair Haven, CT: SOS Publications.

Soares-da-Silva, I.M., Ribeiro, J., Valongo, C., Pinto, R., Vilanova, M., Bleher, R., Machado, J., 2002. Cytometric, morphologic and enzymatic characterisation of haemocytes in *Anodonta cygnea*. Comparative Biochemistry and Physiology part A: Molecular and Integrative Physiology 132, 541-553.

Suresh, K., Mohandas, A., 1990. Number and types of haemocytes in *Sunetta scripta* and *Villorita cyprinoides* var. *cochinensis* (Bivalvia), and leukocytosis subsequent to bacterial challenge. Journal of Invertebrate Pathology 55, 312-318.

Tafalla, C., Novoa, B., Figueras, A., 2002. Production of nitric oxide by mussel (*Mytilus galloprovincialis*) haemocytes and effect of exogenous nitric oxide on phagocytic functions. Comparative Biochemistry and Physiology part B: Biochemistry and Molecular Biology 132, 423-431.

Zampini, M., Canesi, L., Betti, M., Ciacci, C., Tarsi, R., Gallo, G. Pruzzo, C., 2003. Role for mannose-sensitive hemaglutitin in promoting interactions between *Vibrio cholerae* El Tor and mussel haemolymph. Applied and Environmental Microbiology 69, 5711–5715.

Chapter 7

Antimicrobial activity of unionid mussels from the North of Portugal

Antimicrobial activity of unionid mussels from the North of Portugal

Mariana Hinzmann [a, b], Lucinda J. Bessa [a, d], Amílcar Teixeira [c], Paulo Martins da Costa [a, b], Jorge Machado [a, b]

[a] ICBAS - Abel Salazar Institute for the Biomedical Sciences, Department of Aquatic Production, University of Porto, Rua de Jorge Viterbo Ferreira n.º 228, 4050-313 Porto, Portugal

[b] CIIMAR - Interdisciplinary Centre of Marine and Environmental Research, University of Porto, Rua dos Bragas, n.º 289, 4050-123 Porto, Portugal

[c] CIMO-ESA-IPB—Mountain Research Centre, School of Agriculture, Polytechnic Institute of Bragança, Campus de Santa Apolonia, Bragança, Portugal

[d] Requimte/UCIBIO, Department of Chemistry and Biochemestry, Faculty of Sciences, University of Porto, Rua do Campo Alegre, 4169-007, Porto, Portugal.

Hinzmann, M.F., Lopes-Lima, M., Bobos, I., Ferreira, J., Domingues, B., Machado, J., 2016. Antimicrobial activity of unionid mussels from the North of Portugal. Journal of Fish and Shellfish immunology (submitted).

Abstract

In the last decades, the freshwater mussels of the family Unionidae have suffered a drastic decrease in numbers and population diversity due to constant alterations on their natural habitat. However, to revert that situation and willing for survival, those bivalves are able to develop strategies of defence against potential aggressors.

The present work aimed to assess the potential antibacterial capacity of different species of this endangered group of mussels from the North of Portugal. For this purpose, circulating haemocytes, circulatory fluids and mucus of *Anodonta anatina*, *Anodonta cygnea*, *Potomida litorallis* and *Unio delphinus* were obtained by using a nonlethal approach and tested against bacterial reference strains and multidrug-resistant isolates.

The antibacterial activity of the cellular fraction from *A. anatina*, *A. cygnea* and *P. litorallis* was firstly detected by the agar disc diffusion method against *Bacillus subtilis* ATCC6683, *Pseudomonas aeruginosa* ATCC 27853 and *Acinetobacter baumannii* ATCC 19606. The cellular fraction from *P. littoralis* and *A. anatina* also inhibited *Listeria monocytogenes* ATCC 19111, and the *A. cygnea* cellular fraction inhibited a multidrug-resistant isolate of *Pseudomonas putida* as well.

The plasma, used directly or diluted, and the cellular fraction prepared with the haemocytes collected from the above mentioned freshwater mussels were subsequently tested for their ability to hamper or inhibit bacterial biofilms formation. Biofilm biomass quantification and biofilm microscopic observation through Live/Dead staining revealed that the plasma of all unionid species, inclusive *U. delphinus*, impaired biofilm formation by *Staphylococcus aureus* ATCC 25923, *P. aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922. The *Enterococcus faecalis* ATCC 29212 capacity to form a biofilm also decreased in the presence of plasma collected from *A. cygnea*. Contrarily, the cellular fraction of *A. cygnea* supported the proliferation of all the tested bacteria except *E. coli* ATCC 25922.

Overall, these results showed that not only haemocytes have a relevant impact in the immunity of these species, even in poor cellular conditions, but also their plasma, which is very likely to contain antimicrobial substances. *Anodonta cygnea* seems to be the mussel species with higher antibacterial activity, probably because these species faces a regular and continuous pressure in terms of bacteria pool.

Introduction

In the last decades, the concern regarding the conservation of freshwater mussels belonging to the superfamily Unionoidea has been increasing; still, many populations are endangered (Bogan, 1993; Geist, 2015; Lopes-Lima et al., 2016; Neves et al., 1997; Strayer, 2000; Strayer et al., 2007). Their particular life cycle, in which before being autonomous the larvae (glochidia) needs to fix to a specific fish host to develop to the next stage, limits greatly their dispersion and propagation. Many are the reasons for their decline in terms of numbers, being the pollution one of the major threats, together with the introduction of new species with invasion attributes (mussels and fish), followed by the loss of hosts and multiple other factors of anthropogenic origin (Bogan, 1993; Strayer, 2000).

Freshwater mussels are key elements in freshwater systems as their suspension-feeding behaviour is fundamental for the cleaning and recirculation of particles in rivers, lakes and streams, and additionally, their shells can be used as support structures for other organisms (animals, plants and algae) (Strayer, 2000; Strayer et al., 2004). Aggressions affecting the environment are potentiated in these animals due to the bioaccumulation processes and also because of their sessile lifestyle, thus making freshwater mussels ideal monitoring species, which are very sensitive to environmental perturbations (Farris and Van Hassel, 2006; Hartmann et al., 2016).

Unionids, being water cleaning organisms, end up filtrating large amounts of bacteria, which can be a food source or establish symbiotic relationships either mutualistic or antagonistic with those mussels (Grizzle and Brunner, 2009; Antunes et al., 2010). In the early stages of development (glochidia and juveniles), unionid mussels can be largely affected by bacteria (Grizzle and Brunner, 2009). However, diseases caused by bacteria, such as vibriosis, are much more studied in marine bivalves than in freshwater ones, since the economic implications are greater (Pruzzo et al., 2005; Mateo et al., 2009).

In a changing world where adaptation is a recurrent phenomenon, the increase of stressors in natural habitats raises the question whether the environmental alterations may have or not implications in the immunity system of the organisms. Several studies point to an immune modulation (Mydlarz et al., 2006), but in a long-term, it is likely that the effects may cause deep transformations. Not only freshwater mussels are loaded with toxins due to the water pollution, but are also subjected to a microbial pool that is very different from that of their ancestors.

The immune system of unionid mussels, like most invertebrates, is based on innate immunity (Mydlarz et al., 2006; Danilova, 2006), comprising exterior barriers like the shell and mucus on the surface of the tissues, and interiorly the cellular component and circulatory fluids. Particularly, the blood cells (haemocytes) play a fundamental role in the line of defence, especially in the mechanisms associated with phagocytosis (Hong et al., 2006; Canesi et al., 2000; Blaise et al., 2002) and detoxification processes. The plasma where humoral components are present is equally relevant. The discovery of antimicrobial peptides (AMPs) and other molecules with antimicrobial properties has been increasing (Cheng-Hua et al., 2009; Smith et al., 2010), and several studies have already identified those peptides in invertebrates, such as crayfish (*Pacifastacus leniusculus*) (Jiravanichpaisal et al, 2007), but more intensively in marine bivalves such as oysters (*Crassostrea gigas*) (Bachere et al, 2015) and mussels (*Metilus galloprovinciallis*) (Mitta et al. 1999; Mitta et al, 2000).

Therefore, in order to assess the potential of freshwater mussels as a source of bioactive compounds, the aim of the present study was to evaluate the antimicrobial capacity of several species of Portuguese freshwater mussels, by testing their fluids (plasma, and extrapallial fluid) as well as their cells and mucus against reference and multidrug-resistant bacterial strains, using diverse techniques. A few species within the family Unionidae were selected from two freshwater systems: *Anodonta cygnea* from a shallow lagoon (Barrinha - Mira), and *Anodonta anatina*, *Potomida littoralis* and *Unio delphinus* from a river (Tâmega).

Materials and methods

Location and collection of freshwater mussels

The species of freshwater bivalves used in this study were collected in the northern Portugal: *A. cygnea* was collected from the Mira lagoon, (40° 27′ 22″ N, 8° 48′ 7″ W), *A. anatina*, *U. delpinhus* and *P. littoralis* from the Tâmega River (41º 24′ 52″ N, 7° 57′ 51″ W). They were kept in aerated tanks with dechlorinated water and were acclimatised in these conditions for two weeks. These animals were fed daily with a microalgae diet. The organisms were considered healthy when the surface of the shell was smooth and shiny and when they closed the valves when disturbed.

Cells and fluids extraction

Haemolymph from three organisms of each species (*A. cygnea*, *A. anatina*, *P. littoralis* and *U. delphinus*) was carefully extracted using a 21G needle (Braun) attached to a 2 mL sterile syringe (Braun), by inserting it between the valves across the inner layer of the mantle into the intraepithelial space, avoiding contact with other surfaces. Each haemolymph sample was maintained on ice immediately after collection; no anti-aggregate was added to avoid introducing bias. The whole procedure was conducted using sterile material and in an aseptic environment.

The cells were isolated from the plasma by centrifugation at 200 ×g and 4ºC for 10 min (Antunes et al., 2014), and resuspended in PBS buffer 50mM for the susceptibility disc diffusion assay or Tryptic Soy broth (TSB – Biokar Diagnostics, Allonne, France) for further use in the biofilm assays. The plasma was filtered through a 0.22 µm membrane filter to eliminated eventual contaminants.

The extrapallial fluid could only be collected from the *Anodonta* species, since these species are big enough to ensure a safe extraction, by inserting the needle between the shell and the mantle; the fluid was filtered and kept on ice until use.

Mucus collection

Mucus was collected from the foot surface using a sterile blade and suspended in PBS 50mM, vortexed and kept on ice (it was not filtered since it was too viscous).

Bacterial water quality analysis at the collection points

Water and biofilm samples were analysed from 2009 to 2011 to determine the counts of total mesophilic aerobic bacteria, *Escherichia coli* and *Enterococcus* spp. from the natural habitat of the tested freshwater bivalves. Biofilm was collected from rocks and macrophytes immerse in the water, scraping with a brush into a 50-mL falcon tube.

Water samples of 100 mL were filtered through 0.45 µm-pore-size membrane filters (MilliporeCorporation, USA), which were then placed on Tryptone Bile X- glucuronide agar (TBX) (BioKar Diagnostics, Beauvais, France) and on Slanetz and Bartley agar (SB) (Oxoid, Basing- stoke, UK) for *E. coli* and *Enterococcus* spp. enumeration, respectively. The TBX plates were incubated at 37ºC for 24 h and the SB plates at 37ºC for 48 h. *Enterococcus* spp. colonies were confirmed in Kanamycin Aesculin Azide agar (KAA) (Liofilchem, Roseto degli Abruzzi, Italy), incubated at 44ºC for 4 h.

For the determination of total aerobic mesophilic bacteria, dilutions of the water samples were made in tryptone salt and then 1 mL of each dilution was incorporated in Plate Count Agar (PCA) medium (Biokar Diagnostics) and incubated at 20ºC for 72 h. The number of CFUs were counted and recorded.

The biofilm was diluted in tryptone salt and analysed by incorporation with the above mentioned mediums following the same procedure.

Biochemical quantifications on the plasma from the Unionidae species

Subsamples of the plasma from *A. cygnea*, *U. delphinus* and *P. littoralis* were used for the quantification of total proteins (Bradford method), calcium (QuantiChrom Calcium Assay kit Bioassays Systems), phosphates (PiBlue Phosphate assay kit Bioassays Systems) and total lipids (Total lips Liquid kit FAR diagnostics, after extraction using methanol and chloroform).

Antibacterial susceptibility testing

Agar disc diffusion method

For screening the potential antibacterial activity of the cells, fluids and mucus from the four bivalve species tested, four reference strains of bacteria (*Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6683, *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922) were initially used. Fresh bacterial cultures were used to prepare an inoculum equivalent to 0.5 MacFarland.

Mueller-Hinton agar (MH—BioKar Diagnostics, Allonne, France) plates were inoculated with the bacterial suspensions. Then, small sterile paper discs (6 mm of diameter) were equidistantly attached to the agar (5 per plate) and loaded with 15 µL of the sample (cells, fluids or mucus). The plates were kept for 30 min at room temperature before incubation at 37°C for 18–24 h (Pereira et al., 2015). A control was made using PBS.

The plates were observed under a stereoscope (SZ61 stereoscope, Olympus America Inc., Center Valley, PA, USA) and the antibacterial activity was given by the occurrence of inhibition zones surrounding the discs that, if present, were measured and photographed (PD70 Olympus America Inc., Center Valley, PA, USA). The species of bivalves that caused any bacterial inhibition were further studied against other potential pathogenic bacterial strains: *Salmonella enterica Typhimurium* CECT 443, *Klebsiella pneumoniae* ATCC 13883, *Acinetobacter baumannii* ATCC 19606, *Listeria monocytogenes* ATCC

19111, *Enterococcus faecalis* ATCC 29212, and multidrug-resistant clinical strains of *Pseudomonas putida*, and *Klebsiella* spp.; following the same procedure.

Antibiofilm Activity Assay

Biofilm biomass quantification

The fractions selected to be tested were the ones with a lower microbial burden and that had given positive results in the previous assay; they were derived from the haemolymph, the cellular fraction and the plasma (pure or diluted 1:2 and 1:4 in TSB). The bacterial strains used were: *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *E. faecalis* ATCC 29212 at a concentration of 1×10^6 CFU/mL in TSB.
The assays were conducted in 96-well plates, adding 200 µl of suspension in each well, with 6 to 8 replicates per condition tested. A positive control was made using only the bacterial inoculum. The microplates were incubated at 37°C for 24 h. The biofilm biomass was quantified through the crystal violet staining, as described by Gomes et al., 2014.

Microscopic and viability analysis of biofilms

The conditions that have affected the most the biofilm formation were then also used for qualitative assessment by microscopic visualization, using the Live/Dead BacLight viability kit (Life Technologies—Molecular Probes, Carlsbad, CA, USA). For this qualitative assay three *Anodonta cygnea* were used.
Biofilms of *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 were formed in 35-mm diameter polystyrene plates using TSB (control) and TSB supplemented with cellular fraction and plasma (pure or diluted 1:2 and 1:4 in TSB). The plates were incubated at 37ºC for 24 h. After that, the planktonic phase was removed from each plate, the biofilms were washed with PBS, stained with 500 µL of the mixture of SYTO 9 and propidium iodide and incubated for 20 min at room temperature in the dark; then, the biofilms were rinsed and examined under a fluorescence microscope (BX41 Microscope, Olympus America Inc., Center Valley, PA, USA) (Gomes et al., 2014).

Statistics

The statistical significance of difference between biofilms of controls and biofilms in the presence of haemolymph, cellular fraction or plasma was evaluated using Student's t test. Probability levels < 0.05 were considered statistically significant

Results

Microbial quality of the water at the collection points of the bivalves

The annual variations in the numbers of *E. coli*, *Enterococcus* spp. and total mesophilic aerobic bacteria in the water and in the biofilm obtained from the surface of rocks and tree branches from the Tâmega river and Mira lagoon, are shown in Figure 1. It is possible to observe similar profiles in both water and biofilm samples. The Tâmega river presented higher fluctuations in all counts and also the highest values of *E. coli* and *Enterococcus* spp. in the water and biofilm samples, and of total aerobic mesophylls in the biofilm. *Enterococcus* spp., *E. coli* and total aerobic mesophylls maximums occurred in the biofilm fraction.

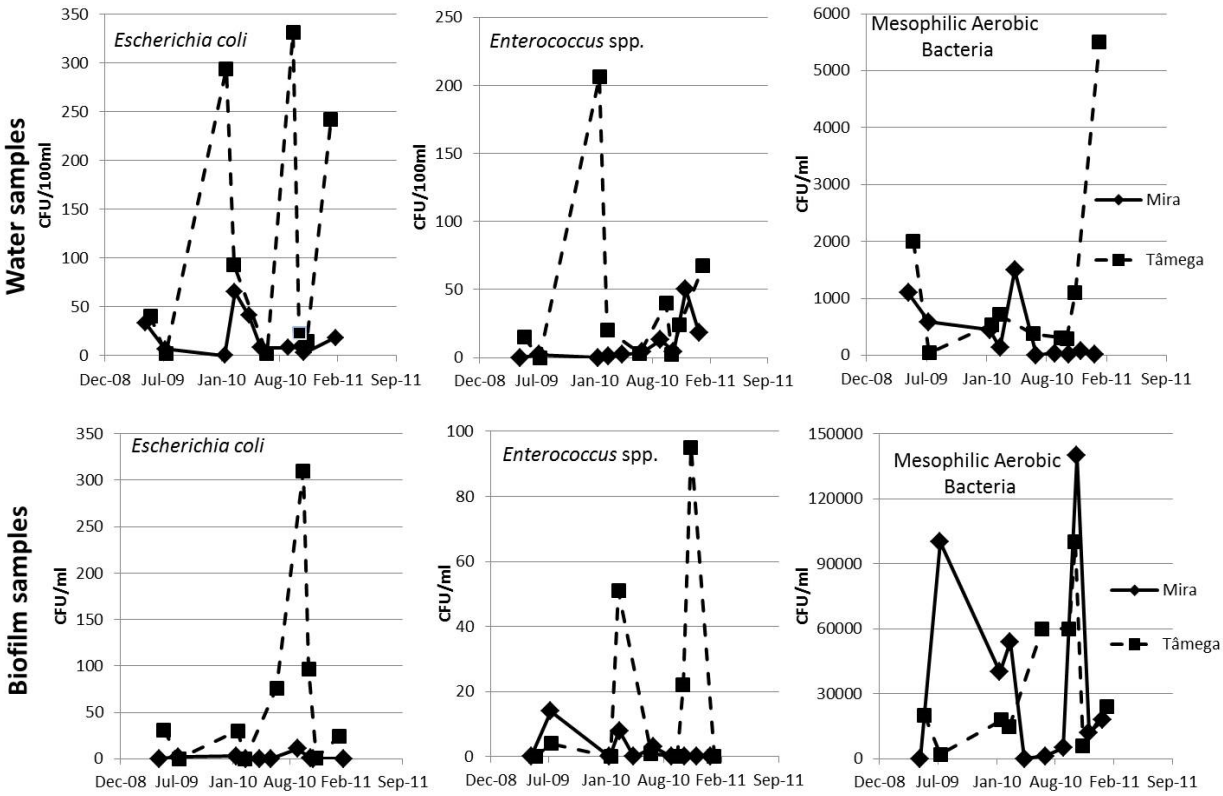


Figure 1 - Annual variations (CFU/100 mL or CFU/mL) of *Escherichia coli*, *Enterococcus* spp. and mesophilic aerobic bacteria in samples of water and biofilm collected from the Tâmega river and Mira lagoon.

Plasma composition

Regarding the quantifications of the components of the plasma (Figure 2), it was possible to observe that the plasma of the three species tested presented similar values in terms of calcium and total lipids. *Unio delphinus* presented higher differences in the quantity of the components tested comparing to the other species and the highest values concerning phosphates. *Potomida littorallis* presented a lower content in terms of total proteins.

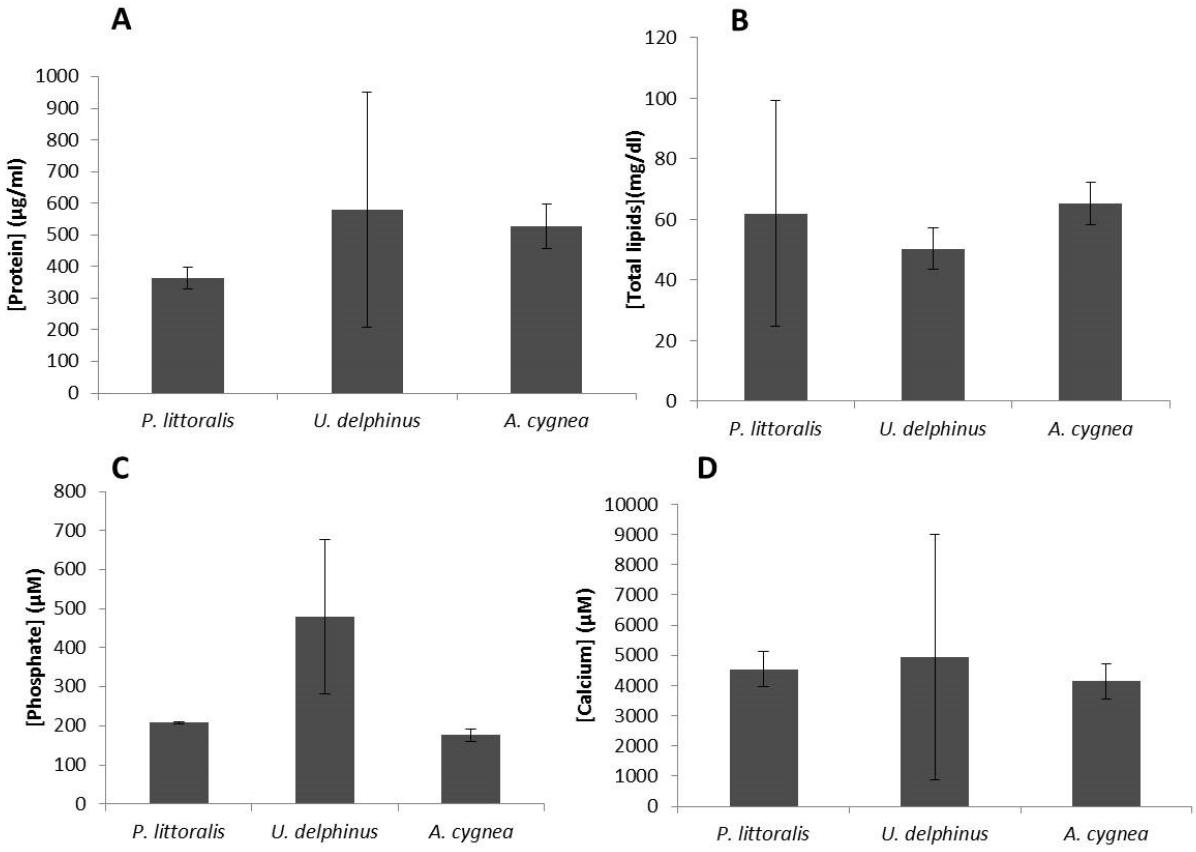


Figure 2 - Quantification of several plasma constituents from Unionidae species*: Anodonta cygnea*, *Potomida littoralis* and *Unio delphinus*: A - Total proteins, B – Total lipids, C – Phosphates and D – Calcium.

Antibacterial activity

Concerning the results from the agar disc diffusion assay, it was observed that only the cellular fraction have caused bacterial inhibition, but not from all bivalve species and not against all bacteria tested. The zones of inhibition were generally small. In the case of the mucus, as it is very rich in bacteria, no zones of inhibition were observed and, on the contrary, greater bacterial proliferation around the disc was recorded. A similar result was observed with the extrapallial fluid, which is in contact with the exterior fluids and the shell.

Anodonta cygnea from Mira lagoon showed to be the most promissory species, since its cellular fraction, resuspended in PBS buffer, inhibited *Bacillus subtilis* ATCC 6683 and *P. aeruginosa* ATCC 27853. When testing the effects on multidrug-resistant isolates, a clear and slight inhibition occurred for *Pseudomonas putida* and *A. baumannii*, in the presence of the haemocytes (Figure 3a).

Two freshwater species from the Tâmega river showed diverse results. *Potomida littoralis* cellular fraction also inhibited *B. subtilis* ATCC 66683, *P. aeruginosa* ATCC 27853, *L. monocytogenes* ATCC 19111, *A. baumannii* ATCC 19606 and *S. enterica Typhimurium* CECT 443 (Figure 3b). The cellular fraction of *A. anatina* showed similar results as the previous bivalve species, inhibiting the same strains; however, that inhibition was more marked in the case of *S. enterica Typhimurium* CECT 443 and slight in the case of *A. baumannii* ATCC 19606 (Figure 3c). *Unio delphinius*, though being collected from the same place did not cause any bacterial inhibitions in this assay (results not shown).

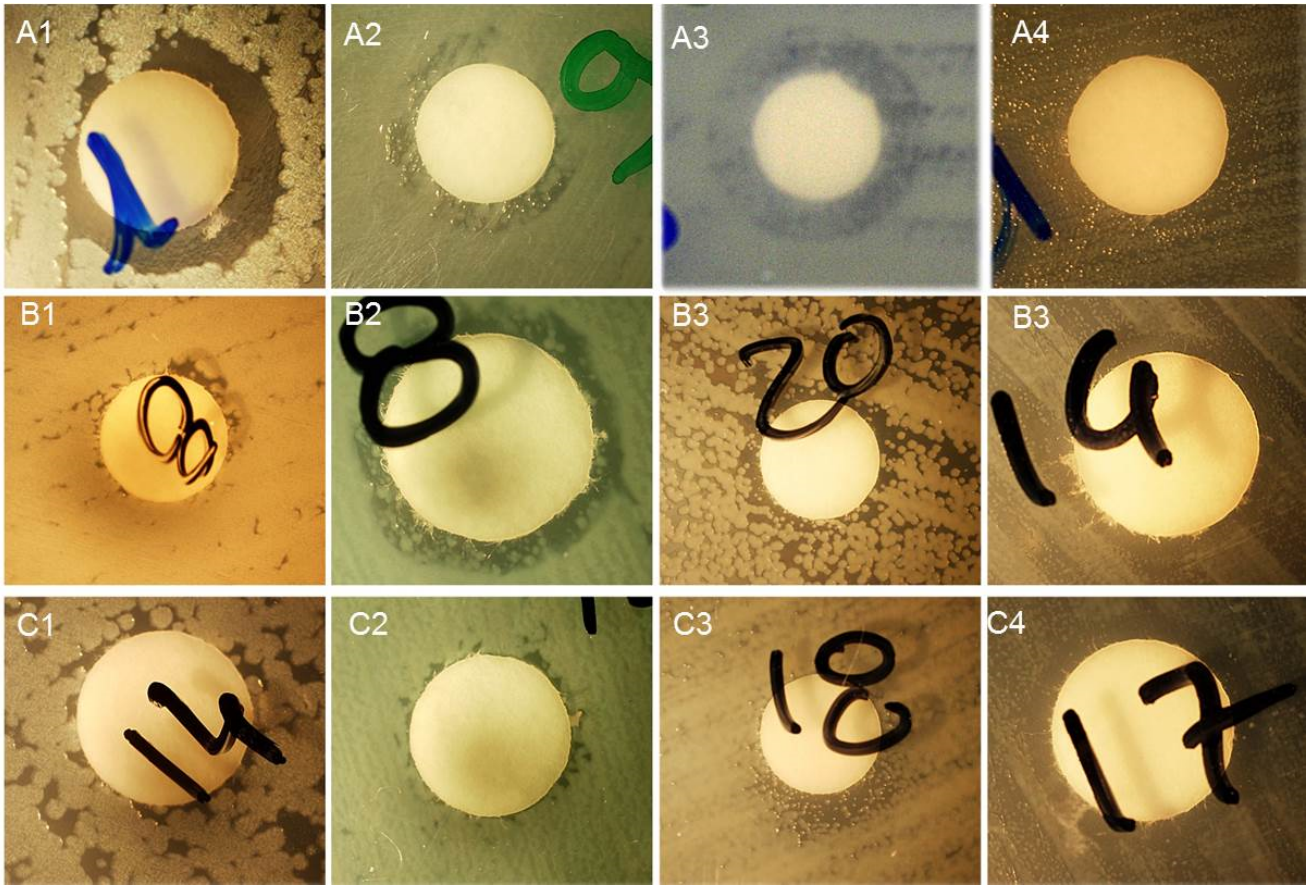


Figure 3 - Antimicrobial activity (inhibition zones around the discs) of cellular fraction from freshwater bivalves against different strains of bacteria. In A, the *A. cygnea* cellular fractions were tested against: A1 – *B. subtilis* ATCC 6683, A2 – *P. aeruginosa* ATCC 27853, A3 – *Pseudomonas putida* and in A4 – *L. monocytogenes* ATCC 19111. In B, *P. littoralis* cellular fraction against: B1– *B. subtilis* ATCC 6683, B2 – *P. aeruginosa* ATCC 27853, B3 – *A. baumannii* ATCC 19606 and in B4 – *L. monocytogenes* ATCC 19111. In C, *A. anatina* cellular fraction against: C1 – *B. subtilis* ATCC 6683, C2 – *P. aeruginosa* ATCC 27853, C3 – *S. enterica Typhimurium* CECT 443 and in C4 – *L. monocytogenes* ATCC 19111.

Antibiofilm activity

In the biofilm formation assay, the results were more evident, with almost all tested conditions being significantly different from the control ($P < 0.05$, Figure 4). The biggest inhibitions were recorded in the presence of the plasma of the bivalves, even when diluted. Interestingly, the cellular fraction hampered the biofilm formation solely by *E. coli* ATCC 25922. This bacteria species suffered the most when in contact with the cellular fraction and the higher dilution of plasma of *A. cygnea*. Again, *A. cygnea* showed great ability to

inhibit the biofilm formation of *S. aureus* ATCC 25923, being all its components tested able to cause a lower biofilm production than the control. *Pseudomonas aeruginosa* ATCC 27853 biofilm was mostly inhibited when in contact with plasma; the diluted plasma caused the highest inhibition. *A. cygnea* was also tested against *E. feacalis* ATCC 29212; inhibition was only recorded for pure plasma and plasma diluted 1:2; the higher dilution of the plasma and the cellular fraction caused higher biofilm production than the control (Figure 4A). *Anodonta anatina* although belonging to the same genus showed slightly different results, presenting a significant capacity to inhibit the biofilm formation by *S. aureus* ATCC 25923 and *E. coli* ATCC 25922, especially in the presence of undiluted and diluted 1:2 plasma. However little inhibition was observed for *P. aeruginosa* ATCC 27853; *A. anatina* cellular fraction was even able to significantly increase the biofilm biomass in respect to the control. The cellular fraction equally increased the biofilm biomass of *S. aureus* ATCC 25923 (Figure 4 B).

In terms of biofilm production, *U. delphinus* and *P. littoralis* (Figure 4C and D) caused a similar effect in the three different bacterial strains tested: *S. aureus* ATCC 25923 suffered the highest inhibition in the biofilm formation (the highest value was recorded for the more diluted sample of plasma); *P. aeruginosa* ATCC 27853 was inhibited in the presence of all extracts of these two species, with the plasma diluted 1:2 causing the highest inhibition; the cellular fraction showed little effect on the biofilm formation. Regarding *E. coli* ATCC 25922, the results were more inconsistent; though, the diluted plasma produced a marked reduction on the biofilm formed, however, the undiluted plasma of *U. delphinus* led to a higher production of biofilm in comparison to the control. The haemocytes from *P. littorallis* and *U. delphinus* inhibited significantly the biofilm production by *S. aureus* ATCC 25923 ($P < 0.05$).

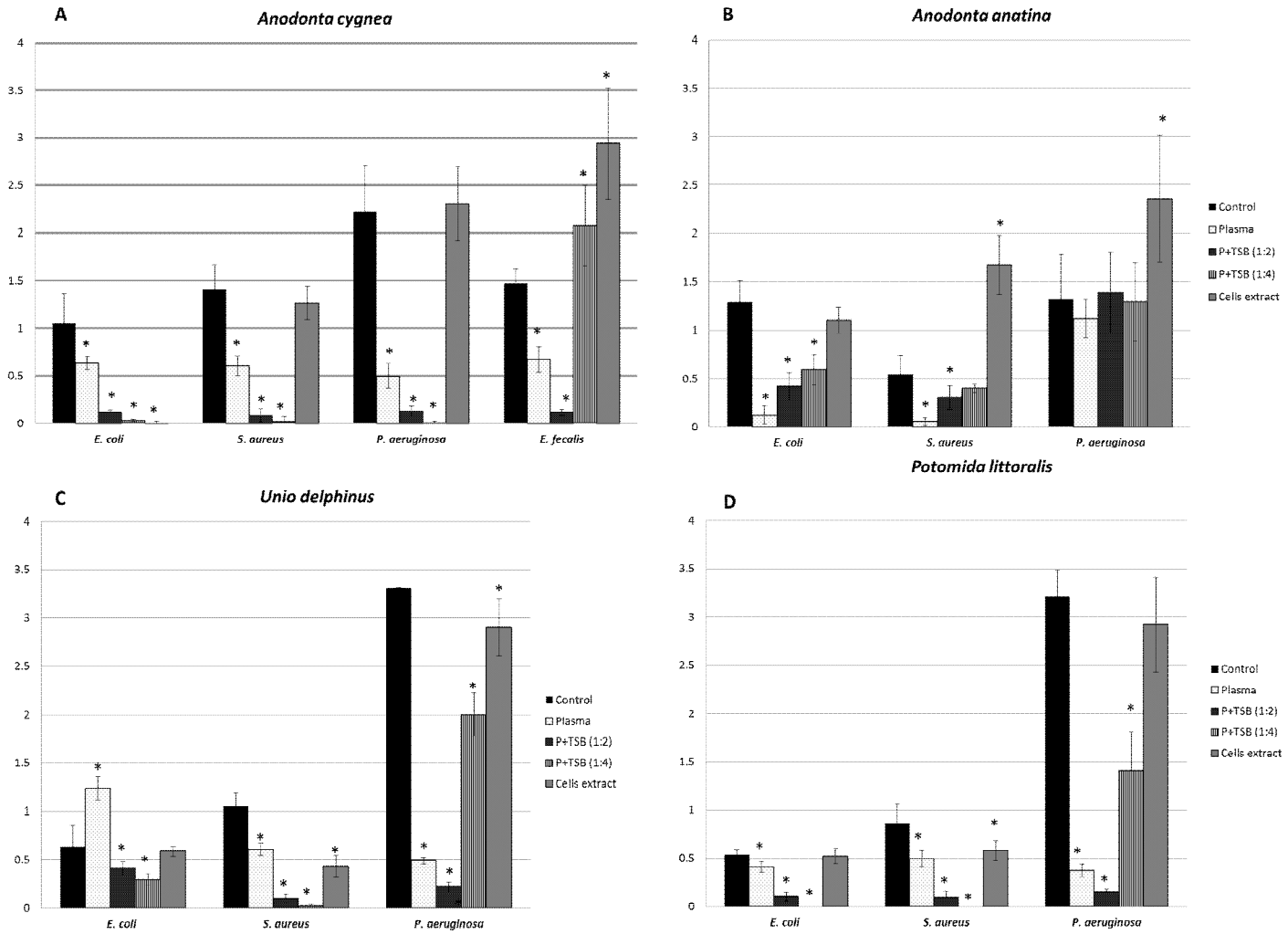


Figure 4 - Biomass quantification (by optic density measurement at 595 nm) of biofilms of *E. coli* ATCC 25922, *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853 and *E. faecalis* ATCC 29212 in the presence of different biological fractions (plasma, plasma diluted 1:2, plasma diluted 1:4 and cells (cells extract or cellular fraction)) from freshwater bivalves (A: *Anodonta cygnea*, B – *Anodonta anatina*, C – *Unio delphinus* and in D – *Potomida littoralis*) compared with a control (only the bacteria in suspension). Differences statistically significant in relation to the control are marked with *, using Student's t test with a $P < 0.05$.

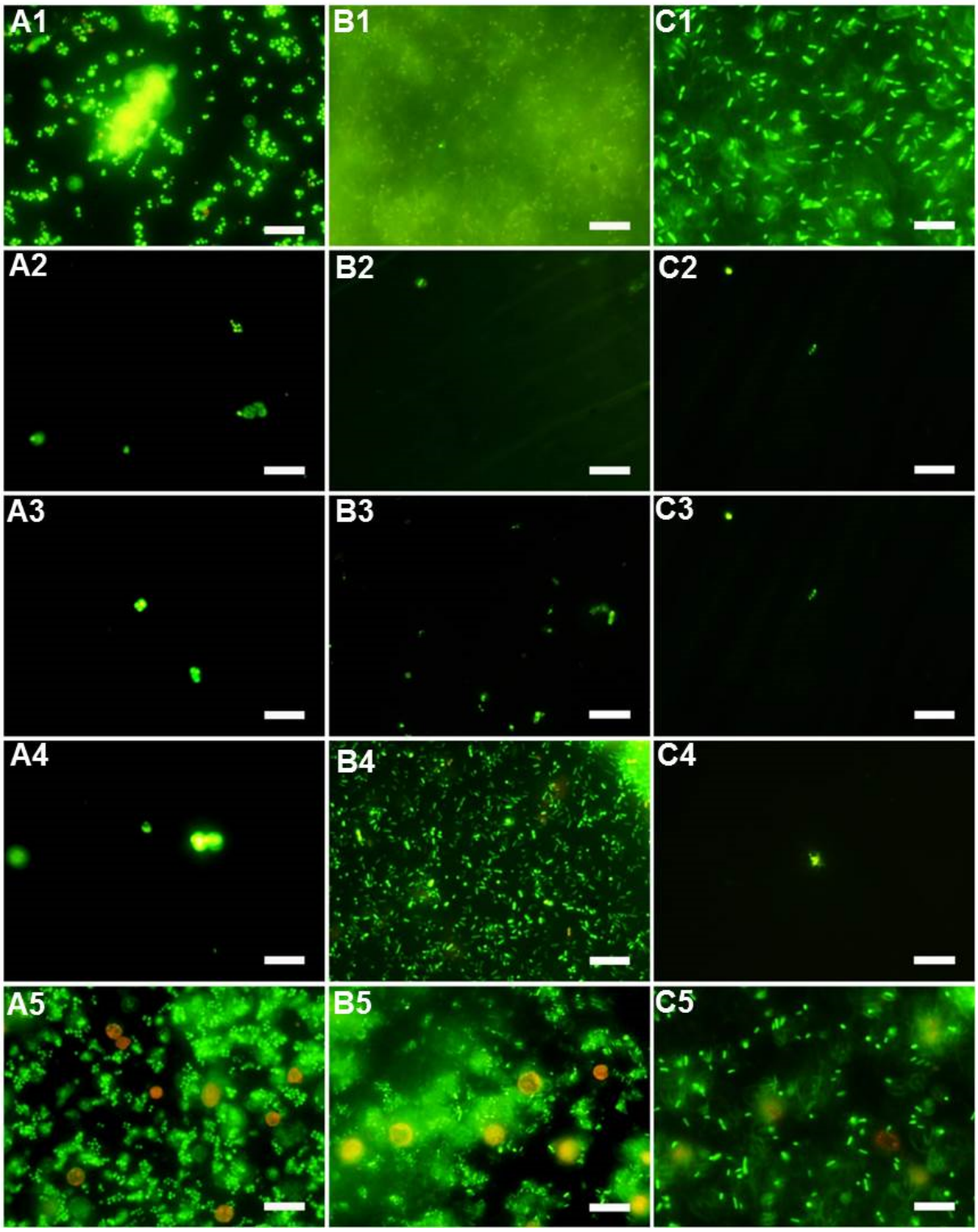


Figure 5 - Evaluation of biofilm formation by different bacterial strains in presence of cells and plasma extracted from the freshwater bivalve *A. cygnea*. Live/dead viability staining images after 24 h of incubation. In A, it was used *S. aureus* ATCC 25923, in B, *P. aeruginosa* ATCC 27853 and in C, *E. coli* ATCC 25922. A1, B1 and C1 – control; A2, B2 and C2 - presence of plasma; A3, B3 and C3 – presence of plasma diluted 1:2 in TBS; A4, B4 and C4 – presence of plasma diluted 1:4 in TBS and A5, B5 and C5 – presence of cellular fraction. Red dots observed in the number 5 situation (bottom line) refer to the cell nucleus that was stained with the propidium iodide of the staining mixture.

Viability assay

The Live/Dead fluorescence assay (Figure 5) allowed a qualitative analysis with the viability assessment of the biofilms in the presence of different biological extracts of the freshwater bivalves. It was visible that no biofilm was formed in presence of the plasma (non-diluted and at both dilutions) of *A. cygnea*. The undiluted plasma and the plasma diluted 1:2 in TBS inhibited almost totally the growth of the three bacterial strains tested, even the more diluted form of the plasma inhibited greatly *S. aureus* ATCC 25923 and *E. coli* ATCC 25922. *Pseudomonas aeruginosa* ATCC 27853, on the other hand, could grow in these conditions, although not as much as in the control condition. The fraction with the cellular fraction showed almost no inhibition, the big red cells (Figures 5-A5, -B5 and -C5) correspond to dead haemocytes, which may have been used as subtract material, favouring the proliferation of the three bacterial strains tested.

Discussion

Invertebrate animals, which lack an adaptive immune system, have developed a defence system that respond to common antigens on the surface of potential pathogens (Mitta et al., 1999). One of the main components of their immune system is composed by the circulating cells in their body fluids, like the haemocytes, which are responsible for mechanisms such as phagocytosis (Antunes et al., 2010). However, it is quite evident that humoral substances must also be present in the plasma, allowing it to play a significant role in the immune defence, as shown in our results that revealed an antibiofilm activity of the plasma of all unionid species tested. Nevertheless, little is known on the chemical identification of those substances as well as on their mechanisms of action, but rises a new interest area for further studying and protect this endangered mussel group.

In previous studies, we have demonstrated that *E. coli* and *E. faecalis* were not tolerated by *Anodonta* spp., being eliminated by the bivalve's granulocytes, which proved the action of the cellular immunity (Antunes et al., 2010, Antunes et al., 2014). Now, in the present work, we show evidence that the humoral immunity may also have a role in the defence process.

Cellular fraction caused bacterial inhibition, however in a lower degree, and in some cases even stimulated the bacteria growth. This last result was probably due to the fact that the conditions used did not allow the maintenance of the haemocytes viability; however, there is a gap in the literature in terms of the optimal conditions to maintain these cells viable. In the report by Hinzmann et al. (2013), some procedures were

recommended like the use of appropriate antiaggregative solutions and a low temperature (keeping the cells on ice), still, our several attempts to put these cells into culture failed (data not shown). Moreover, our work focused on the bacterial conditions, so nothing was added to avoid interference with the bacterial growth and the temperature selected was the optimal for the bacteria. Thus, the use of cellular fraction for biological activities needs to be optimised. Nonetheless, using the agar disc diffusion method, the cellular fraction from *Anodonta* spp. and *P. littoralis* inhibited *B. subtilis* ATCC 6683 and *P. aeruginosa* ATCC 27853, showing that those extracts possessed inhibitory components, which could have resulted from cells, like reactive oxygen species that are released when the cell collapse. Moreover, the agar disc diffusion assay showed that those species of freshwater mussels have also the potential to inhibit multidrug-resistant strains, such as multidrug-resistant isolates of *P. putida* and *A. baumannii*. Obviously, the agar disc diffusion assay is a basic screening assay that as several limitations, e.g. only a small volume of sample can be tested, and some substances do not diffuse in the agar medium. Probably, that can be one of the reasons explaining why in some cases no inhibition was recorded using the plasma extract in the disc, while very high inhibition was recorded in the biofilm quantification assay.

Taking into account our results regarding the response of bivalves towards the biofilm formation by bacteria, it is likely that those bivalves developed molecular mechanisms capable of defending them from bacteria with the capacity to form biofilm inside them that inevitably would put at stake their survival. As no antibacterial effect on the bacterial growth was observed, the antibiofilm activity of plasma components was most likely due to their interference in the communication/quorum sensing system of the bacterial cells. Moreover, once the diluted plasma still had the same effects, it may indicate that the components interfering with the biofilm formation are able to do it even at a low concentration. The fact that the inhibitory biofilm formation effects of these plasma components were found in diverse bivalve species and towards bacterial strains that are typically not present in their living water (e.g. *S. aureus*), is suggestive of an innate non-specific mechanism; otherwise, the bivalve species inhabiting waters with higher microbial contamination, would have expressed a more pronounced inhibitory capacity against bacteria.

Regarding the cellular fraction obtained from *A. cygnea*, they were also able to inhibit the biofilm formation by *E. coli*, while have not affected the other bacterial strains. An explanation could be that the *A. cygnea* cells maintained the viability, even if for a short period, or from their lysis, some compounds inhibiting bacterial cells were released. These hypotheses need to be further explored.

The knowledge on the immune response of freshwater bivalves is rather limited when compared with what is currently known about marine bivalves. Being one of the main reasons the fact that the cells of marine bivalves are easily cultured, promoting several studies of these kind. Also, in terms of the identification of antimicrobial substances (antimicrobial peptides-AMPs), most cultivated species have their AMPs already identified like: metilins in mussels (*M. edulis* and *M. galloprovicialis*) (Mitta et al., 2000; Charlet et al., 1996) or defensins from oysters (*C.verginica* and *C. gigas*) (Bachere et al., 2015; Anderson and Beaven, 2001) and big defensin from the scallop *Argopecten irradians* (Zhao et al., 2007). Thus, the studies on AMPs have been increasing, since those peptides have the potential to replace actual antibiotics as new antimicrobial drugs with application on aquaculture (Cheng-Hua et al., 2009).

Although little knowledge is given here in terms of the plasma components that may be inducing the bacterial inhibition, we know that the plasma of freshwater bivalves has potential to be further explored and its components must be identified in order to understand the immune system of this highly endangered group of species. Since the bacterial burden is increasing in their natural habitat and threatening even more their survival, it is expectable that freshwater bivalves may find strategies to overcome that hostile situation by producing antimicrobial substances, therefore further studies in this field are fundamental to fully understand how humoral factors act in the immune response.

Herein, *A. cygnea* gave more evident results in comparison to the other bivalve species, which however slightly follow the same pattern. *Anodonta cygnea* inhabits a more constant environment, which provides the appropriate conditions for these species to persist. *Unio delphinus* and *P. littoralis* came from a river with higher fluctuation in terms of bacteria load and that may explain the less pronounced results.

Similar studies on freshwater mussels were reported by Estari et al., 2011, regarding *Lamellidens marginalis* and by Santhiya and Sanjeevi (Santhiya and Sanjeevi, 2014) using *Parreysia corrugatea*. Estari et al., 2011, tested fluids and tissue extracts from *L. marginalis*, which were diluted in solvents (water, chloroform, acetone or methanol) and followed protein extraction protocols; among the different extracts tested, they obtained antimicrobial inhibition against *S. aureus*, *S. pyroenes*, *S. marginii*, *B. subtilis*, *E. coli*, *P. vulgaris* and *C. albicans*. The other study reported bacterial inhibition of tissue extracts through the diffusion agar method, against pathogenic bacteria, such as *S. aureus*, *B. subtilis*, *E. coli*, *P. aeruginosa*, and *K. pneumonia* (Santhiya and Sanjeevi, 2014). Regarding freshwater bivalves, the molecules that may be involved in this antibacterial response are still unidentified. Though, breakthroughs in this field may

start to appear, since a big defensin gene was recently identified in the freshwater mussel *Hyriopsis cumingii* (Wang et al., 2014).

Acknowledgments

This work was supported by the Portuguese Foundation for Science and Technology (FCT) with a research grant to MH (SFRH / BD / 76265 / 2011. This article was also supported by the project INNOVMAR-Innovation and Sustainability in the Management and Exploitation of Marine Resources (NORTE-01-0145-FEDER-000035, within Research Line NOVELMAR), supported by North Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF).

References

Anderson, R.S., Beaven, A.E., 2001. Antibacterial activities of oyster (*Crassostrea virginica*) and mussel (*Mytilus edulis* and *Geukensia demissa*) plasma. Aquatic Living Resources 14, 343–349.

Antunes, F., Hinzmann, M., Lopes-Lima, M., Machado, J., Martins da Costa, P., 2010. Association between environmental microbiota and indigenous bacteria found in hemolymph, extrapallial fluid and mucus of *Anodonta cygnea* (Linnaeus, 1758). Microbial. Ecology 60, 304–309.

Antunes, F., Hinzmann, M., Lopes-Lima, M., Vaz-Pires, P., Ferreira, S., Domingues, B., Machado, J., Martins da Costa, P., 2014. Antibacterial effects of *Anodonta cygnea* fluids on *Escherichia coli* and enterococci multi-drug-resistant strains: environmental implications. Toxicological Environmental Chemistry 96, 880–889.

Bachere, E., Rosa, R.D., Schmitt, P., Poirier, A.C., Merou, N., Charriere, G.M., Destoumieux-Garzon, D., 2015. The new insights into the oyster antimicrobial defense: Cellular, molecular and genetic view. Fish & Shellfish Immunology 46, 50–64.

Blaise, C., Trottier, S., Gagné, F., Lallement, C., Hansen, P.D., 2002. Immunocompetence of bivalve hemocytes as evaluated by a miniaturized phagocytosis assay. Environmental Toxicology 17, 160–169.

Bogan, A.E., 1993. Fresh-Water Bivalve Extinctions (Mollusca, Unionoida) - a Search for Causes. American Zoologist 33, 599–609.

Canesi, L., Gavioli, M., Pruzzo, C., Gallo, G., 2000. Bacteria–hemocyte interactions and phagocytosis in marine bivalves. Microscopy Research and Technique 57, 469–476.

Cheng-Hua, L., Jian-Min, Z., Lin-Sheng, S., 2009. A review of advances in research on marine molluscan antimicrobial peptides and their potential application in aquaculture. Molluscan Research 29, 17–26.

Charlet, M., Chernysh, S., Philippe, H., Hetru, C., Hoffmann, J.A., Bulet, P., 1996. Innate immunity - Isolation of several cysteine-rich antimicrobial peptides from the blood of a mollusc, *Mytilus edulis*. Journal of Biological Chemistry 271, 21808–21813.

Danilova, N., 2006. The evolution of immune mechanisms. Journal of Experimental Zoology. Part B 306, 496–520.

Estari, M., Satyanarayana, J., Kumar, B.S., Bikshapathi, T., Reddy, A.S., Venkanna, L., 2011. *In vitro* study of antimicrobial activity in freshwater mussel (*Lamellidens marginalis*) extracts. Biology and Medicine 3, 191–195.

Farris, J.L., Van Hassel, J.H., 2006. Freshwater Bivalve Ecotoxicology. First ed., Taylor & Francis, Bosa Roca, USA,

Geist, J., 2015. Seven steps towards improving freshwater conservation. Aquatic Conservation: Marine and Freshwater Ecosystems 25, 447–453.

Gomes, N., Bessa, L., Buttachon, S., Costa, P., Buaruang, J., Dethoup, T., Silva, A., Kijjoa, A., 2014. Antibacterial and antibiofilm activities of tryptoquivalines and meroditerpenes isolated from the marine-derived fungi *Neosartorya paulistensis*, *N. laciniosa*, *N. tsunodae*, and the Soil Fungi *N. fischeri* and *N. siamensis*. Marine Drugs 12, 822.

Grizzle, J.M., Brunner, C.J., 2009. Infectious diseases of freshwater mussels and other freshwater bivalve mollusks. Reviews in Fisheries Science 17, 425–467.

Hartmann, J.T., Beggel, S., Auerswald, K., Stoeckle, B.C., Geist, J., 2016. Establishing mussel behavior as a biomarker in ecotoxicology. Aquatic Toxicology 170, 279–88.

Hinzmann, M., Lopes-Lima, M., Goncalves, J., Machado, J., 2013. Antiaggregant and toxic properties of different solutions on hemocytes of three freshwater bivalves. Environmental Toxicology and Chemistry 95, 790–805.

Hong, X.T., Xiang, L.X., Shao, J., 2006. The immunostimulating effect of bacterial genomic DNA on the innate immune responses of bivalve mussel. *Hyriopsis cumingii* Lea, Fish & Shellfish Immunology 21, 357–364.

Jiravanichpaisal, P., Lee, S.Y., Kim, Y.A., Andren, T., Soderhall, I., 2007. Antibacterial peptides in hemocytes and hematopoietic tissue from freshwater crayfish *Pacifastacus leniusculus*: characterization and expression pattern. Developmental & Comparative Immunology 31, 441–455.

Lopes-Lima, M., Sousa, R., Geist, J., Aldridge, D.C., Araujo, R., Bergengren, J., Bespalaya, Y., Bódis, E., Burlakova, L., Van Damme, D., Douda, K., Froufe, E., Georgiev, D., Gumpinger, C., Karatayev, A., Kebapçi, Ü., Killeen, I., Lajtner, J., Larsen, B.M., Lauceri, R., Legakis, A., Lois, S., Lundberg, S., Moorkens, E., Motte, G., Nagel, K.-O., Ondina, P., Outeiro, A., Paunovic, M., Prié, V., von Proschwitz, T., Riccardi, N., Rudzīte, M., Rudzītis, M., Scheder, C., Seddon, M., Şereflişan, H., Simić, V., Sokolova, S., Stoeckl, K., Taskinen, J., Teixeira, A., Thielen, F., Trichkova, T., Varandas, S., Vicentini, H., Zajac, K., Zajac, T., Zogaris, S., 2016. Conservation status of freshwater mussels in Europe: state of the art and future challenges. Biological Reviews, n/a-n/a.

Mateo, D.R., Siah, A., Araya, M.T., Berthe, F.C., Johnson, G.R., Greenwood, S.J., 2009. Differential *in vivo* response of soft-shell clam hemocytes against two strains of *Vibrio splendidus*: changes in cell structure, numbers and adherence. Journal of Invertebrate Pathology 102, 50–56.

Mitta, G., Vandenbulcke, F., Hubert, F., Salzet, M., Roch, P., 2000. Involvement of mytilins in mussel antimicrobial defense. Journal of Biological Chemistry 275, 12954–12962.

Mitta, G., Vandenbulcke, F., Hubert, F., Roch, P., 1999. Mussel defensins are synthesised and processed in granulocytes then released into the plasma after bacterial challenge. Journal of Cell Science 112, 4233–4242.

Mydlarz, L.D., Jones, L.E., Harvell, C.D., 2006. Innate immunity, environmental drivers, and disease ecology of marine and freshwater invertebrates. Annual Review of Ecology, Evolution, and Systematics 37, 251–288.

Neves, R.J., Bogan, A.E., Williams, J.D., Ahlstedt, S.A., Hartfield, P.W., 1997. Status of aquatic mollusks in the southeastern United States: a downward spiral of diversity. in: G.W. Benz, D.E. Collins (Eds), Aquatic fauna in peril: the Southeastern perspective, Special publication 1. Decatur, GA Southeast Aquatic Research Institute, Lenz Design and Communications, pp. 43–85.

Pereira, A.L., Bessa, L.J., Leão, P.N., Vasconcelos, V., Martins da Costa, P., 2015. Bioactivity of *Azolla aqueous* and organic extracts against bacteria and fungi. Symbiosis 65, 17–21.

Pruzzo, C., Gallo, G., Canesi, L., 2005. Persistence of vibrios in marine bivalves: the role of interactions with haemolymph components. Environmental. Microbiology. 7, 761–772.

Santhiya, N., Sanjeevi, S.B., 2014. Antibacterial activity of freshwater Mussel *Parreysia corrugata* (Muller 1774) from Lower Anaicut Reservoir, India. International Journal of Research and Development in Pharmacy & Life Sciences 5, 3899–3902.

Smith, V.J., Desbois, A.P., Dyrynda, E.A., 2010. Conventional and unconventional antimicrobials from fish, marine invertebrates and micro-algae. Marine Drugs 8, 1213–1262.

Strayer, D.L., 2000. The ecology of freshwater molluscs. Nature 406, 126–126.

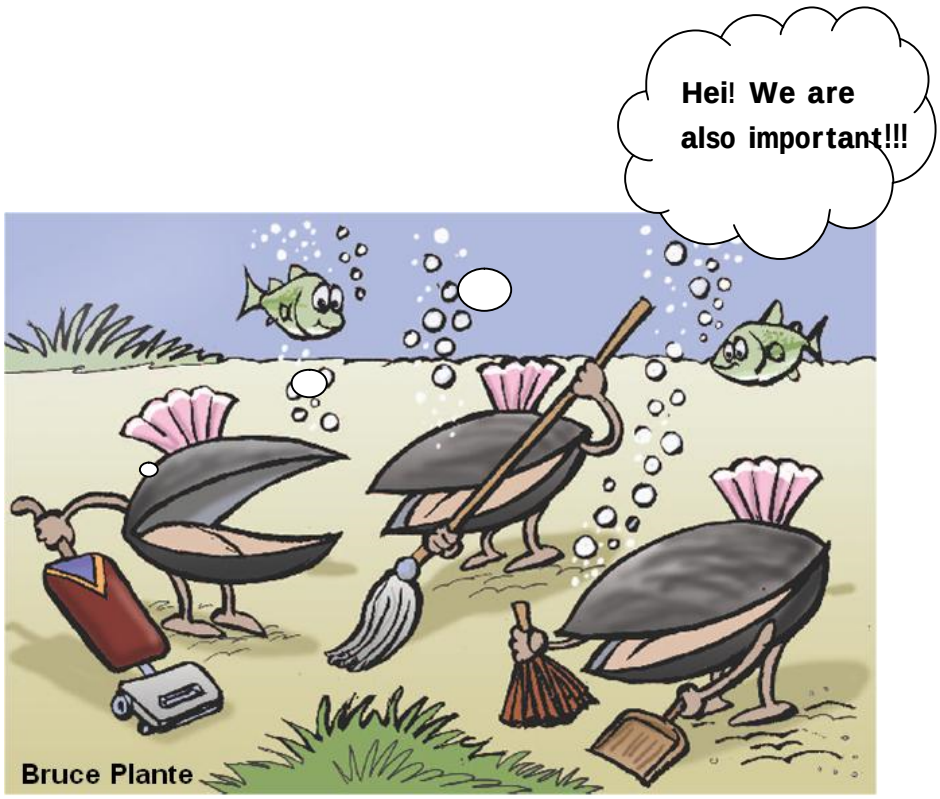
Strayer, D.L., Malcom, H.M., 2007. Shell decay rates of native and alien freshwater bivalves and implications for habitat engineering. Freshwater. Biology 52, 1611–1617.

Strayer, D.L., Hattala, K.A., Kahnle, A.W., 2004. Effects of an invasive bivalve (*Dreissena polymorpha*) on fish in the Hudson River estuary. Canadian Journal of Fisheries and Aquatic Sciences 61, 924–941.

Wang, G.L., Xia, X.L., Li, X.L., Dong, S.J., Li., J., 2014. Molecular characterization and expression patterns of the big defensin gene in freshwater mussel (*Hyriopsis cumingii*). Genetics and molecular research 13, 704–715.

Zhao, J., Song, L., Li, C., Ni, D., Wu, L., Zhu, L., Wang, H., Xu, W., 2007. Molecular cloning, expression of a big defensin gene from bay scallop *Argopecten irradians* and the antimicrobial activity of its recombinant protein. Molecular Immunology 44, 360–368.

Chapter 8



Final Remarks and Future perspectives

Final Remarks

Freshwater mussels are among most threatened animal group worldwide. More than 300 native species are considered extinct, endangered, threatened or under special concern (by the IUCN). Freshwater mussels are extremely important, with ecological and economic potential (Bogan, 1993; Graf and Cummings, 2007b; a; Bogan, 2008; Hanlon et al., 2009; Bauer and Wächtler, 2012). Mussels in general and freshwater in particular serve as a food resource for animals; they improve water quality through filtration of contaminants, sediments, and nutrients; their shells are even used in pearl culture. Their decline may be a reflection of the degradation of freshwater ecosystems, since they function as sentinel organisms, ultimately the consequences will affect other organisms (including fish species and in the long run the man) (Cao et al., 2013).

When working with animals and especially with species belonging to such endangered family, the effort to minimize the animal manipulation should be maximised. On the other hand, the lack of information surrounding them is so high, that justifies a line of research, but having in attention their conservation status (IUCN). Although the global status may still be not critic, regionally the abundances must be assessed before using them. A recent problem is a decrease in the number of animals and also in the recruitment (due to hosts lost), many populations are only made of adults, with already several years (Lopes-Lima et al., 2016).

Taking into account that knowledge on freshwater mussels (Unionids) immunology was in a “standing by” situation, since these species doesn’t generate as much interest as the commercial marine species or even the invasion freshwater species, which have been more studied, this work aimed to revive interest of former times (Cheng, 1981; Hine, 1999).

The work presented here contributed to characterize several immune aspects and practical considerations concerning the haemocytes and respective sub cellular types which were identified for the genus *Anodonta:* granulocytes and hyalinocytes. The antimicrobial potential of the haemolymph components (haemocytes and plasma), detected against several human pathogenic bacterial strains and tested in different unionids species was proved. A practical application is shown as how immune elements can be helpful in an ecotoxicity assay, demonstrating that environment contaminants can rapidly cause alterations at the cellular level. A full characterization of micropearls, as calcium reservoir, necessary for the life of these mussels, was made which may also be used to detect toxic elements from the environment, since their origin may also be haemocyte linked.

Several limitations affected this work, such as lack of available techniques to maintain or study these cells since there isn't yet available cellular lines from freshwater mussels, not even specific mediums to maintain the haemocytes viable for longer periods, achieving that would allow further functional studies.

The problem of the lack of information in relation to the origin of the haemocytes also limits this line of research. Attempts to mark *in vivo* the cells with BrdU, to identify proliferating cells where in vain, for other invertebrates species this process is already known, like for instance for arthropods (Jiravanichpaisal et al., 2006) or even freshwater crayfish species (Söderhäll et al., 2003) and in *M. edulis* haemocytes (Renwrantz et al., 2013).

The progress of immunological research is closely linked to the development of adequate, reliable and reproducible laboratory techniques, similarly to the standardized techniques already use with marine commercial species (fish, shrimp, crayfish, oysters and clams) (Villena, 2003). Many are the obstacles associated to the development of an ideal medium to keep the bivalves haemocytes, at their best physiologic capacities (homeostasis), demonstrating that in the absence of a commercial cell line available from marine invertebrates (Rinkevich, 2011), the freshwater snail *Biomphalaria glabrata* originate the only cell line (1818 embryonic (Bge)) available from molluscan species (Yoshino et al., 2013), there is general lack of information regarding nutritional requirements (Cai and Zhang, 2014).

Marine mussels haemocytes from the genus *Mytilus* can be kept into culture in medium Leibovitz's L.15 supplemented with antibiotics (Cao et al., 2003; Renwrantz et al., 2013) or even haemocytes from the invasion species *D. polymorpha* (Parolini et al., 2011) for more than 15 days, but not for longer periods. The main obstacle for freshwater mussels haemocytes relies on finding an ideal medium without causing osmotic stress, the low osmotic concentration of the haemolymph (osmolarity lower than 50 mOsm), as verified in the work from the chapter 2, limits even the selection of an antiaggregant (Hinzmann et al., 2013b). The best antiaggregant solution for the haemocytes of the three freshwater bivalves species was based on NEM, although toxic in high concentrations, when diluted proved to be to most efficient in keeping the cells separated and viable for longer time, but only few hours, *C. fluminea* haemocytes showed a higher flexibility and lower mortality. Most commercial media are prepared for plasma with values surrounding 300 mOsm (mammal cell lines), thus the medium needs to be adapted, other more frequent problems are: contamination, evaluation of impact of using antiagregant solutions or even from the release of ROS to the plasma (Yoshino et al., 2013; Cai and Zhang, 2014), all that have to be consider.

The development of better techniques to maintain the elements of the immune system of freshwater mussel are in the base for the development of standard methods to evaluate the mussel fitness through its immune system. Several mediums (L15 and M199 in different concentrations) were tested to prolong the life of the unionids haemocytes, but without success.

Secondly when studying haemocyte populations, standard methods refer the importance of separate different cell populations, also here the standard methods failed, attempts done with Percoll and Iodixanol only served to diminish their viability with very low separation capacity, in opposition the marine haemocytes are easily separated with Percoll (Friebel and Renwrantz, 1995; Lopez et al., 1997; Pipe et al., 1997; Gong et al., 2008).

The only method proved to be efficient in separating cells populations was flow cytometry-based on cell sorting, an expensive and not always available resource, fundamental in the haemocytes characterization work (Chapter 3) since without it we could not confirm that the populations studied by cytometry and microscopy corresponded.

Lectins have been historically used for functional recognition of molecules inside cells, on cell surfaces, and in physiological fluids (Yakovleva et al., 2001; Sharon and Lis, 2004). The use of lectins (chapter 3), mainly WGA lectin, revealed to be a good marker to separate the haemocytes population, between hyalinocytes, with low lectin affinity and granulocytes, even inside the granulocytes was possible to distinguish two sub-populations that marked differently, but only for *A. cygena*, *A. anatina* haemocytes are a more heterogeneous group, without clear differentiation with the lectins tested. In future studies other lectins can be tested like Con A and RCA, already used with marine mussels species. Carbohydrates connected to membrane were already identified, granules or capsules produced by granulocytes of marine species binding to lectins like HPA and RCA in *Tapes semidecussatus* (Montes et al., 1995), and WGA, Con A and others in *Mytilus edulis* (Wootton et al., 2003b; Renwrantz et al., 2013) and also in the deep sea species from the genus *Bathymodiolus* (Tame et al., 2015), as feature that can be used to differentiate haemocytes (Pipe, 1990a; Pipe et al., 1997).

There is a recent interest of using non-lethal techniques when studying these organisms (Gustafson et al., 2005), and this is possible since in these group are included big mussels, like *Anodonta cygnea*, that as already an history of being used as animal model for physiologic studies, especially in biomeralization mechanisms and membranes (Bleher and Machado, 2004; Oliveira et al., 2004a; Oliveira et al., 2004b; Lopes-Lima et al., 2008; Lopes et al., 2010). In this way we select these species that which have the lower conservation status to conduct a detailed study on the

micropearls or concretions of *Anodonta cygnea* (chapter 4) – a physiological necessity for freshwater mussels, to store calcium, but also by means of eliminate potential toxic pathogens, compounds or particles (Hinzmann et al., 2014). These structures can be used to tell their environmental story, in terms of exposure to contaminants, like cadmium sequestration (Pynnönen et al., 1987). These structures can be found in diverse tissues, even inside the haemocytes (difficulting the process of studding the haemocytes by TEM), but the largest reservoir are the gills (Pynnönen et al., 1987). The gills are the main reservoir, used for shell growth and offspring shell formation, the concretions formed due to metal detoxification process can be found more in the mantle and have a more irregular shape (Moura et al., 1999).

Bivalves as filter feeders have a fundamental role in the cleaning of water, in our study (Chapter 6), a host-parasite assay, conducted with *E. coli* and enterococcus species, in both *in vitro* and *in vivo* conditions proved the capacity of *Anodonta cygnea* immune system to neutralise drastically the proliferation of these bacteria (Antunes et al., 2010; Antunes et al., 2014). What reveals that these species immune system is not necessary less efficient than invasion species of freshwater mussels. Studies conducted with the invasor spcies *D. polymorpha* revealed that are excellent species in cleaning effluents waters, deacresing significantly the amount of viruses (poliovirus and retovirus) and *E. coli* (Mezzanotte et al., 2016). Also *C. fluminea* can work as bioindicators of fecal protozoan, accumulating patogens like *Cryptosporidium* and *Giardia* spp. from the sourrounded environment (Miller et al., 2005).

Similarly to our antibaterial results from chapter 7, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus,* species known to be pathogenic to man are were also inibhited with AMPs from the freshwater crayfish *Pacifastacus leniusculus* (Jiravanichpaisal et al., 2007), althought the composition of the substance involved in our study is still unknown, our results are important, proving that their immune system is working against bacteria that are know not to cause directly diseases on them. The antimicrobial activity detected seems very important in this new era of searching for new alternatives to conventional antibiotics, however deeper studies are still needed. First the isolation of the compounds responsible for the inhibition, like what was already done with marine species and secondly a functional clarification of how this inhibitory process occurs, to avoid easy development of bacteria resistance. For instance in *Mytilus galloprovincialis* the EP (extrapalleal protein) present in serum (MgEP) has recently been shown to work as an opsonin promoting D-mannose sensitive (MS) interactions of the bivalve with pathogen *Vibrio aestuarianus* and also with *Escherichia coli* via haemocytes (Canesi et al., 2016c), so these compounds isolatated may not have antimicrobial potential, only in association with the immune cells their role is

achieved. So, even if in some cases antimicobrial activity is not detected it doesn't mean that it doesn't exist, since they may result from the combined action of several immune elements.

Cellular alterations may be the first response to an exogenous alteration, and can be triggered by chemical, physical or biological sources. In this group of species there is the problem of the high intra-animal variability, but this variability can reflect the normal variability in the habitat, so it should be consider, since in the habitat we will not have all animals genetically equals, with the same predispositions, however there can only be used a reduce number of animals in experiments.

Among ecotoxicologists there is an increased interest in finding a freshwater bivalve suitable as bioindicator, similarly to what is already done with *M. edulis*, selected as a marine bioindicator species, many look to the invasion species *D. polymorpha* as candidate species (Binelli et al., 2015), they even have the same attaching mechanism, though the byssus. However, in my opinion, that will serve only for isolated laboratory studies, in regions where this species is endemic, the continuous usage of these species in the wild may promotes even more its dispersion, with repercussions to native species, in Portugal there aren't still occurrence of these species, but it proliferates already in Spain (Rajagopal et al., 2009; Gerard van der Velde et al., 2010).

In chapter 5, it is established a synergistic connection between increase of ammonia concentration and temperature, reflecting the increase of ammonia in the freshwater ecosystems and also the temperature rise, expected due to climate changes, several parameters were analysed: organism behaviour (measured by the filtration rate), health status (measured by the glycogen content) and immunological response (measured by the haemocytes abundance, morphotype and viability). Although the results are not as clear as desire, a direct link toward this combination of factors alter the homeostasis of the bivalves, if the assay was extended probably higher records of mortality would be recorded.

The problem of having unclear results in ecotoxicologic in recurrent, for bivalves and even other higher organisms, many times the biological response to environmental conditions can be overshadowed, due to the complexity of the toxic compound tested and also because of previous exposures to environmental toxics, which trigger a mechanism of addiction with physiological adaptation that my reduce the sensitivity to these and other contaminants that are being tested (Rocher et al., 2006).

The combination of toxic assays with bacterial tests would reflect better the environmental challengers that mussels have to deal every day on their habitat and would show as how affected is their capacity to adapt when having to deal with so

many stressors. These works reflect the isolated effect, in future studies the combined effect should be evaluated. Probably their capacity to adapt is diminished as well as their immunity response, when fighting with so many stressors acting simultaneously and this may be the main reason for their decline.

In conclusion, further studies are needed, similarly to what was already done with marine molluscs (Figure 1) (Li et al., 2011) in order to evaluate the cellular and humoral elements found in all unionids species from Portugal and clarification surrounding the mechanisms involved in the antimicrobial activity, however the data obtained in this thesis present new prespectives of study using this endangered group, aiming their protection. The more we know about their ecology, physiology and of course immunology, better tools can be develop in direction to their conservation.

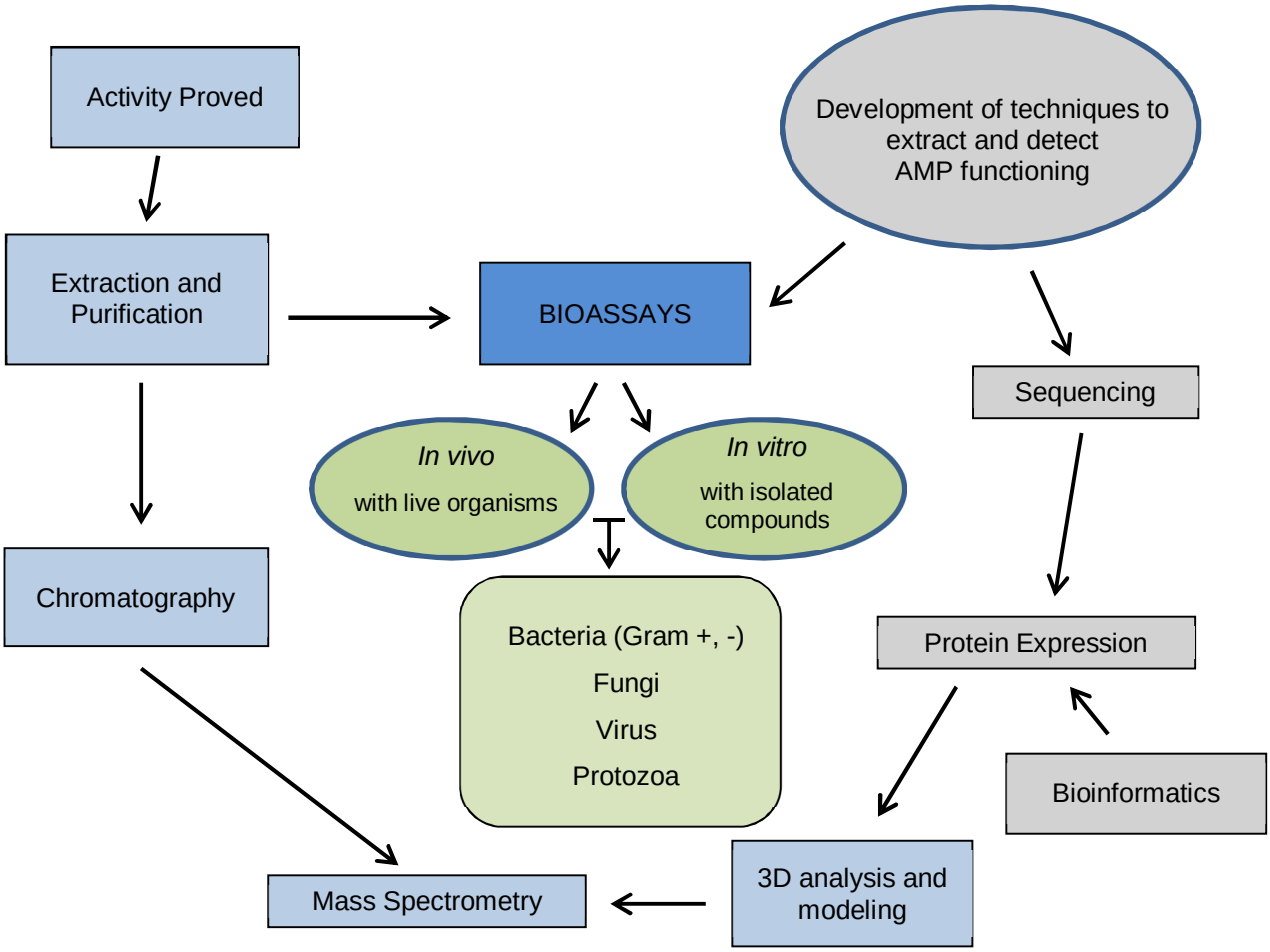


Figure 1 - Summary of the pathways already used to study marine mollusc AMPs, link between chemical, molecular techniques and bioinformatics technologies (adapted from Li et al., 2011).

Future perspectives

Future research will be necessary in order to determine the functions of the different haemocytes identified and also a detailed work on the nature of the plasma compounds involved in the inhibition of biofilm from human opportunistic bacteria, with resource chemical techniques.

When resources are available the genetic approach can give very helpful outputs. With full sequencing of the genome of these unionid species, conserved regions could be identified, when compared with genes already know to be involved in immune response from other invertebrate or even vertebrate species, since many are conserved among eukaryotic organism. Another strategy can be identification of proteins involved in immune response trough 2D gell electrophoresis and then sequentiation.

Searching for DNA damage analysis in cells preserved by cryopreservation through comet assay, similarly to what was done by Kwok et al. (2013) with *M. edulis* haemocytes, could be an alternative if haemocytes maintain their integrity, to evaluated toxic demage after exposure to stress conditions.

The ideal situation is trying to reproduce these studies for as many freshwater mussels species as possible, to have a general look of how the immunologic aswer function among this family.

A field of interest in the research of the impact of pollutants in bivalves is associated with the new sources of pollution, like nanomaterials or nanoparticles (NMs) (not toxic compounds or pathogens). Studies already conducted with *Mytilus spp.*, proved that invertebrate's haemocytes may represent a suitable model for investigating the impact of NMs on innate immunity (Canesi et al., 2016a; Canesi et al., 2016b). Freshwater mussels may be also used for these purposes, since nanoparticles are an emergent way of contamination and it is widely demonstrated that bivalves immune system are a target for nano- and micro-scale particles, like all the other sources of contamination, and they can all work together causing what is called immunomodulation (Canesi and Procházková, 2014; Canesi et al., 2015; Canesi et al., 2016a).

References

Antunes, F., Hinzmann, M., Lopes-Lima, M., Machado, J., Martins da Costa, P., 2010. Association between environmental microbiota and indigenous bacteria found in hemolymph, extrapallial fluid and mucus of *Anodonta cygnea* (Linnaeus, 1758). Microbial Ecology 60, 304-309.

Antunes, F., Hinzmann, M., Lopes-Lima, M., Vaz-Pires, P., Ferreira, S., Domingues, B., Machado, J., Martins da Costa, P., 2014. Antibacterial effects of Anodonta cygnea fluids on *Escherichia coli* and enterococci multi-drug-resistant strains: environmental implications. Toxicological & Environmental Chemistry 96, 880-889.

Bauer, G., Wächtler, K., 2012. Ecology and Evolution of the Freshwater Mussels Unionoida. Springer Berlin Heidelberg.

Binelli, A., Della Torre, C., Magni, S., Parolini, M., 2015. Does zebra mussel (Dreissena polymorpha) represent the freshwater counterpart of *Mytilus* in ecotoxicological studies? A critical review. Environmental Pollution 196, 386-403.

Bleher, R., Machado, J., 2004. Paracellular pathway in the shell epithelium of *Anodonta cygnea*. Journal of Experimental Zoology. Part A, Comparative experimental biology 301, 419-427.

Bogan, A.E., 1993. Fresh-Water Bivalve Extinctions (Mollusca, Unionoida) - a Search for Causes. American Zoologist 33, 599-609.

Bogan, A.E., 2008. Global diversity of freshwater mussels (Mollusca, Bivalvia) in freshwater. Hydrobiologia 595, 139-147.

Cai, X., Zhang, Y., 2014. Marine invertebrate cell culture: a decade of development. Journal of Oceanography 70, 405-414.

Canesi, L., Ciacci, C., Balbi, T., 2016a. Invertebrate Models for Investigating the Impact of Nanomaterials on Innate Immunity: The Example of the Marine Mussel *Mytilus* spp. Current Bionanotechnology 02, 1-7.

Canesi, L., Ciacci, C., Bergami, E., Monopoli, M.P., Dawson, K.A., Papa, S., Canonico, B., Corsi, I., 2015. Evidence for immunomodulation and apoptotic processes induced by cationic polystyrene nanoparticles in the hemocytes of the marine bivalve *Mytilus*. Marine Environmental Research 111, 34-40.

Canesi, L., Ciacci, C., Fabbri, R., Balbi, T., Salis, A., Damonte, G., Cortese, K., Caratto, V., Monopoli, M.P., Dawson, K., Bergami, E., Corsi, I., 2016b. Interactions of cationic polystyrene nanoparticles with marine bivalve hemocytes in a physiological environment: Role of soluble hemolymph proteins. Environmental Research 150, 73-81.

Canesi, L., Grande, C., Pezzati, E., Balbi, T., Vezzulli, L., Pruzzo, C., 2016c. Killing of *Vibrio cholerae* and *Escherichia coli* Strains Carrying D-mannose-sensitive Ligands by Mytilus Hemocytes is Promoted by a Multifunctional Hemolymph Serum Protein. Microbial Ecology. DOI 10.1007/s00248-016-0757-1.

Canesi, L., Procházková, P., 2014. The Invertebrate Immune System as a Model for Investigating the Environmental Impact of Nanoparticles. 91-112.

Cao, A., Mercado, L., Ramos-Martinez, J.I., Barcia, R., 2003. Primary cultures of hemocytes from *Mytilus galloprovincialis*: expression of IL-2Rα subunit. Aquaculture 216, 1-8.

Cao, Y., Huang, J., Cummings, K.S., Holtrop, A., 2013. Modeling changes in freshwater mussel diversity in an agriculturally dominated landscape. Freshwater Science 32, 1205-1218.

Cheng, T.C., 1981. Bivalves, in: Ratcliffe, N.A., A. F. Rowley (Eds.), Invertebrate Blood Cells Academic Press, New York., pp. 233-300.

Friebel, B., Renwrantz, L., 1995. Application of density gradient centrifugation for separation of eosinophilic and basophilic hemocytes from *Mytilus edulis* and characterization of both cell groups. Comparative Biochemistry and Physiology Part A: Physiology 112, 81-90.

Gerard van der Velde, Sanjeevi Rajagopal, Vaate, A.b.d., 2010. The zebra mussel in Europe. Backhuys Publishers ;, Leiden, The Netherlands :.

Gong, N., Li, Q., Huang, J., Fang, Z., Zhang, G., Xie, L., Zhang, R., 2008. Culture of outer epithelial cells from mantle tissue to study shell matrix protein secretion for biomineralization. Cell and Tissue Research 333, 493-501.

Graf, D.L., Cummings, K.S., 2007a. Preliminary review of the freshwater mussels (Mollusca: Bivalvia: Unionoida) of northern Africa, with an emphasis on the Nile. Journal of the Egyptian German Society of Zoology 53, 89-118.

Graf, D.L., Cummings, K.S., 2007b. Review of the systematics and global diversity of freshwater mussel species (Bivalvia: Unionoida). Journal of Molluscan Studies 73, 291-314.

Gustafson, L.L., Stoskopf, M.K., Bogan, A.E., Showers, W., Kwak, T.J., Hanlon, S., Levine, J.F., 2005. Evaluation of a nonlethal technique for hemolymph collection in *Elliptio complanata*, a freshwater bivalve (Mollusca : Unionidae). Diseases of Aquatic Organisms 65, 159-165.

Hanlon, S.D., Petty, M.A., Neves, R.J., 2009. Status of Native Freshwater Mussels in Copper Creek, Virginia. Southeast Nat 8, 1-18.

Hine, P.M., 1999. The inter-relationships of bivalve haemocytes. Fish & Shellfish Immunology 9, 367-385.

Hinzmann, M.F., Lopes-Lima, M., Bobos, I., Ferreira, J., Domingues, B., Machado, J., 2014. Morphological and chemical characterization of mineral concretions in the freshwater bivalve *Anodonta cygnea* (Unionidae). Journal of Morphology.

Hinzmann, M.F., Lopes-Lima, M., Goncalves, J., Machado, J., 2013b. Antiaggregant and toxic properties of different solutions on hemocytes of three freshwater bivalves. Toxicological & Environmental Chemistry 95, 790-805.

Jiravanichpaisal, P., Lee, B.L., Soderhall, K., 2006. Cell-mediated immunity in arthropods: hematopoiesis, coagulation, melanization and opsonization. Immunobiology 211, 213-236.

Jiravanichpaisal, P., Lee, S.Y., Kim, Y.A., Andren, T., Soderhall, I., 2007. Antibacterial peptides in hemocytes and hematopoietic tissue from freshwater crayfish *Pacifastacus leniusculus*: characterization and expression pattern. Developmental and Comparative Immunology 31, 441-455.

Kwok, A., Lyons, B.P., Hodges, N.J., Bean, T.P., 2013. Cryopreservation and storage of mussel (Mytilus spp.) haemocytes for latent analysis by the Comet assay. Mutation Research 750, 86-91.

Li, H., Parisi, M.G., Parrinello, N., Cammarata, M., Roch, P., 2011. Molluscan antimicrobial peptides, a review from activity-based evidences to computer-assisted sequences. Invertebrate Survival Journal 8, 85-97.

Lopes, A., Lopes-Lima, M., Bobos, I., Ferreira, J., Gomes, S., Reis, R., Mano, J., Machado, J., 2010. The effects of *Anodonta cygnea* biological fluids on biomineralization of chitosan membranes. Journal of Membrane Science 364, 82-89.

Lopes-Lima, M., Bleher, R., Forg, T., Hafner, M., Machado, J., 2008. Studies on a PMCA-like protein in the outer mantle epithelium of *Anodonta cygnea*: insights on calcium transcellular dynamics. Journal of Comparative Physiology. B 178, 17-25.

Lopes-Lima, M., Sousa, R., Geist, J., Aldridge, D.C., Araujo, R., Bergengren, J., Bespalaya, Y., Bódis, E., Burlakova, L., Van Damme, D., Douda, K., Froufe, E., Georgiev, D., Gumpinger, C., Karatayev, A., Kebapçi, Ü., Killeen, I., Lajtner, J., Larsen, B.M., Lauceri, R., Legakis, A., Lois, S., Lundberg, S., Moorkens, E., Motte, G., Nagel, K.-O., Ondina, P., Outeiro, A., Paunovic, M., Prié, V., von Proschwitz, T., Riccardi, N., Rudzīte, M., Rudzītis, M., Scheder, C., Seddon, M., Şereflişan, H., Simić, V., Sokolova, S., Stoeckl, K., Taskinen, J., Teixeira, A., Thielen, F., Trichkova, T., Varandas, S., Vicentini, H., Zajac, K., Zajac, T., Zogaris, S., 2016. Conservation status of freshwater mussels in Europe: state of the art and future challenges. Biological Reviews, n/a-n/a.

Lopez, C., Carballal, M.J., Azevedo, C., Villalba, A., 1997. Morphological characterization of the hemocytes of the clam, *Ruditapes decussatus* (Mollusca: Bivalvia). Journal of Invertebrate Pathology 69, 51-57.

Mezzanotte, V., Marazzi, F., Bissa, M., Pacchioni, S., Binelli, A., Parolini, M., Magni, S., Ruggeri, F.M., De Giuli Morghen, C., Zanotto, C., Radaelli, A., 2016. Removal of enteric viruses and *Escherichia coli* from municipal treated effluent by zebra mussels. The Science of the Total Environment 539, 395-400.

Miller, W.A., Atwill, E.R., Gardner, I.A., Miller, M.A., Fritz, H.M., Hedrick, R.P., Melli, A.C., Barnes, N.M., Conrad, P.A., 2005. Clams (*Corbicula fluminea*) as bioindicators of fecal contamination with Cryptosporidium and Giardia spp. in freshwater ecosystems in California. International Journal for Parasitology 35, 673-684.

Montes, J., Durfort, M., García-Valero, J., 1995. Cellular defence mechanism of the clam *Tapes semidecussatus* against infection by the protozoan *Perkinsus* sp. Cell and Tissue Research 279, 529-538.

Moura, G., Guedes, R., Machado, J., 1999. The extracellular mineral concretions in *Anodonta cygnea* different types and manganese exposure-caused changes. Journal of Shellfish Research. 18, 645-650.

Oliveira, M., Santos, M.A., Pacheco, M., 2004a. Glutathione protects heavy metal-induced inhibition of hepatic microsomal ethoxyresorufin O-deethylase activity in *Dicentrarchus labrax* L. Ecotoxicology and Environmental Safety 58, 379-385.

Oliveira, P.F., Lopes, I.A., Barrias, C., Rebelo da Costa, A.M., 2004b. H+-ATPase of crude homogenate of the outer mantle epithelium of *Anodonta cygnea*. Comparative Biochemistry and Physiology. Part A 139, 425-432.

Parolini, M., Quinn, B., Binelli, A., Provini, A., 2011. Cytotoxicity assessment of four pharmaceutical compounds on the zebra mussel (*Dreissena polymorpha*) haemocytes, gill and digestive gland primary cell cultures. Chemosphere 84, 91-100.

Pipe, R.K., 1990a. Differential binding of lectins to haemocytes of the mussel *Mytilus edulis*. Cell and Tissue Research 261, 261-268.

Pipe, R.K., Farley, S.R., Coles, J.A., 1997. The separation and characterisation of haemocytes from the mussel Mytilus edulis. Cell and Tissue Research 289, 537-545.

Pynnönen, K., Holwerda, D.A., Zandee, D.I., 1987. Occurrence of calcium concretions in various tissues of freshwater mussels, and their capacity for cadmium sequestration. Aquatic Toxicology 10, 101-114.

Rajagopal, S., Pollux, B.J.A., Peters, J.L., Cremers, G., Moon-van der Staay, S.Y., van Alen, T., Eygensteyn, J., van Hoek, A., Palau, A., bij de Vaate, A., van der Velde, G., 2009. Origin of Spanish invasion by the zebra mussel, *Dreissena polymorpha* (Pallas, 1771) revealed by amplified fragment length polymorphism (AFLP) fingerprinting. Biological Invasions 11, 2147-2159.

Renwrantz, L., Siegmund, E., Woldmann, M., 2013. Variations in hemocyte counts in the mussel, *Mytilus edulis*: similar reaction patterns occur in disappearance and return of molluscan hemocytes and vertebrate leukocytes. Comparative biochemistry and physiology. Part A, Molecular & Integrative Physiology 164, 629-637.

Rinkevich, B., 2011. Cell cultures from marine invertebrates: new insights for capturing endless stemness. Marine Biotechnology 13, 345-354.

Rocher, B., Le Goff, J., Peluhet, L., Briand, M., Manduzio, H., Gallois, J., Devier, M.H., Geffard, O., Gricourt, L., Augagneur, S., Budzinski, H., Pottier, D., Andre, V., Lebailly, P., Cachot, J., 2006. Genotoxicant accumulation and cellular defence activation in bivalves chronically exposed to waterborne contaminants from the Seine River. Aquatic Toxicology (Amsterdam, Netherlands) 79, 65-77.

Sharon, N., Lis, H., 2004. History of lectins: from hemagglutinins to biological recognition molecules. Glycobiology 14, 53R-62R.

Söderhäll, I., Bangyeekhun, E., Mayo, S., Söderhäll, K., 2003. Hemocyte production and maturation in an invertebrate animal; proliferation and gene expression in hematopoietic stem cells of *Pacifastacus leniusculus*. Developmental & Comparative Immunology 27, 661-672.

Tame, A., Yoshida, T., Ohishi, K., Maruyama, T., 2015. Phagocytic activities of hemocytes from the deep-sea symbiotic mussels *Bathymodiolus japonicus*, *B. platifrons*, and *B. septemdierum*. Fish & Shellfish Immunology 45, 146-156.

Villena, A.J., 2003. Applications and needs of fish and shellfish cell culture for disease control in aquaculture. Reviews in Fish Biology and Fisheries 13, 111-140.

Wootton, E.C., Dyrynda, E.A., Ratcliffe, N.A., 2003b. Bivalve immunity: comparisons between the marine mussel (*Mytilus edulis*), the edible cockle (*Cerastoderma edule*) and the razor-shell (*Ensis siliqua*). Fish & Shellfish Immunology 15, 195-210.

Yakovleva, N.V., Samoilovich, M.P., Gorbushin, A.M., 2001. The diversity of strategies of defense from pathogens in molluscs. Journal of Evolutionary Biochemistry and Physiology 37, 358-367.

Yoshino, T.P., Bickham, U., Bayne, C.J., 2013. Molluscan cells in culture: primary cell cultures and cell lines. Canadian Journal of Zoology 91.