Cristiana Andreia Valente Oliveira

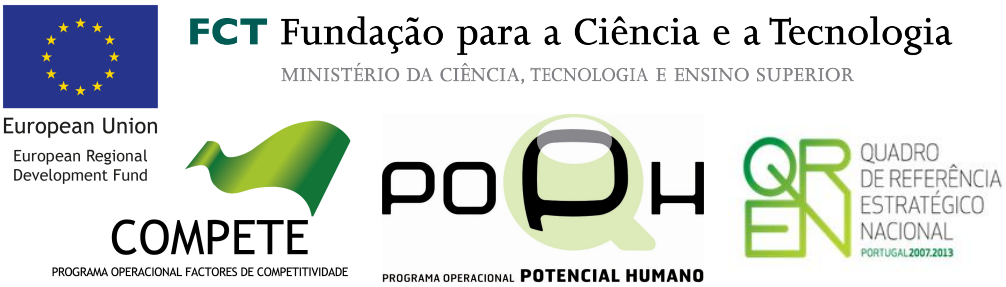
May pollution restrict the invasive behaviour of the non-indigenous species *Corbicula fluminea*?

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

Orientador - Prof. Doutora Lúcia Guilhermino
Categoria - Professora Catedrática
Afiliação - Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Coorientador - Prof. Doutor Ionan Marigómez
Categoria - Professor Catedrático
Afiliação - Universidade do País Basco.

The present Thesis was developed in the scope of the Project NISTRACKS funded by the European Regional Development Fund (ERDF) through the COMPETE programme (Operational Competitiveness Programme) and national funds through FCT – Foundation for Science and Technology (FCT) (PTDC/AAC-AMB/102121; FCOMP-01-0124-FEDER-008556) that provided the most part of the funding for the experimental work done. The author was also supported by a PhD grant (SFRH/BD/68423/2010) from “Programa Operacional Potencial Humano do QREN Portugal 2007-2013” and national founds from the “Ministério da Ciência e Tecnologia e Ensino Superior” (MCTES – POPH-QREN-Tipologia 4.1)



“The task is not so much to see what no one yet has seen, but to think what nobody yet has thought about that which everybody sees.”

(Arthur Schopenhauer)

Acknowledgements

I would like to sincerely thank all of the many people that contributed to this Thesis during the last (almost) five years. First of all, I would like to express my thankfulness to my supervisor Prof. Dr. Lúcia Guilhermino for all the support and advices during this journey and my co-supervisor Prof. Dr. Ionan Marigómez for having received me in the Research Centre for Experimental Marine Biology and Biotechnology from the University of the Basque Country with such goodwill and for all the help interpreting and discussing results. A special thank to Larraitz and Urtzi for all the knowledge, advices, friendship, encouragement, enthusiasm and patience when introducing me to a whole new world that was histology. I would like also to thank Luisma and Ester for teaching me the histology techniques with such sympathy and patience. A tender thanks to Eduardo, who by his wisdom, showed me things in my work that I could never see. A sincerely thank to Nerea for having received me at her home and making me company when I was not working, it was not many but quality times. Thank you all for the hospitality and for making me feel at home!

I would also like to thank to Dr. Pim Leonards, Dr. Timo Hamers and Prof. Dr. Dick Vethaak for having received me at the Institute for Environmental studies in the University of Amsterdam with such friendliness and professionalism. A special thank to Jorke, Peter and Philip for having taught me so many new things and helping me in critical moments; Prof. Dr. Juliett Legler and Marjo for letting me work and teach me about the zebrafish embryos; and very special thanks to Ana, Willem and Kees for the friendship and companionship. Thank you all for the hospitality and for making me feel at home!

I would like to express my sincere gratitude to many people of the ECOTOX group (Laboratory of Ecotoxicology of ICBAS and Research Group of Ecotoxicology, Stress Ecology and Environmental Health of CIIMAR) that helped me in this journey: especially Patricia, Joana, Sónia, Daniel and Luis. Really (really) special thank to Pedro Vilares who accompanied me in all field trips, in all the laboratorial and field saga, all stresses and all achievements, with whom I cried and laughed and blurted and heard. Thank you fellow!

Thanks to all that helped in the revision of this Thesis, and to all the journal editors and reviewers that helped to improve the paper with their pertinent comments.

A very special (from the heart) thank to Ju and Lie.

To my family and, especially, to my little princess that gave me the bravery to end this endless saga.

I would also like to acknowledge the institutions that provided conditions for this Thesis, namely the Institute of Biomedical Sciences Abel Salazar (ICBAS) and the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR) of the University of Porto, the Research Centre for Experimental Marine Biology and Biotechnology from the University of the Basque Country, the Institute for Environmental studies in the University of Amsterdam, and the Portuguese Foundation for Science and Technology (FCT) for the financial support through a PhD grant (SFRH/BD/68423/2010) during the last four years.

Author's declaration

In agreement with the Portuguese law though the article 4th of the "Regulamento Geral dos Terceiros Ciclos de Estudos na Universidade do Porto" of January 14th (GR.04/01/2014)", the author states devotion in a major contribution to the conceptual design and technical execution of the work, interpretation of the results and manuscript preparation of the published, submitted or under preparation publications corresponding to sections of the present Thesis.

Publications:

From the experimental research work carried out in the scope of the present Thesis, one article was published and 2 manuscripts are in preparation, as further indicated:

Oliveira, C., Vilares, P., Guilhermino, L. (2015). Integrated biomarker responses of the invasive species *Corbicula fluminea* in relation to environmental abiotic conditions: a potential indicator of the likelihood of clam's summer mortality syndrome. *Comparative Biochemistry and Physiology, Part A* 182: 27–37.

(Corresponds to the chapter II, included in this Thesis with permission of Elsevier, see Annex I)

Oliveira, C., Vilares P., Soler, F., Marigómez, I., Guilhermino, L. Factors influencing the accumulation of metals by *Corbicula fluminea* and its health status in estuarine tidal freshwater areas (*In preparation*).

Oliveira C., Vilares, P., Guilhermino, L., Marigoméz, I. Histological study of the gonadal development cycle of *Corbicula fluminea* and its relationship with spatial and temporal variation of environmental parameters. (*In preparation*)

Contents index

May pollution restrict the invasive behaviour of the non- indigenous species *Corbicula fluminea*?

Abstract

The Asian Clam, *Corbicula fluminea*, is one of the worst 100 non-indigenous invasive species in Europe. This bivalve is well known for its high capacity of new habitats colonization, by the rapid and extensive dispersion that its populations in general have in colonized habitats and by the ecological and economic damage that its invasions often cause. Despite the several studies that have been made in recent decades, the factors influencing the invasive behaviour of *C. fluminea* are not yet understood. This knowledge is of utmost importance to prevent new invasions, and to control and mitigate the impacts of populations already established. To contribute to the progression of knowledge in the area, the central goal of this Thesis was to investigate if long-term exposure to pollution may restrict the invasive behaviour of *C. fluminea*. Long-term exposure to pollution may restrict the invasive behaviour of *C. fluminea* populations mainly through an increase of the population mortality rate and a decrease of the population health status with negative effects on its fitness, and/or direct negative effects on reproduction. These potential effects were investigated in the present study through a field approach taking advantage of *C. fluminea* populations of the tidal freshwater areas (TFA) of Minho (M-est) and Lima (L-est) Rivers (NW Iberian Peninsula). Such populations were selected because they have been showing a distinct invasive behaviour and M-est and L-est are neighbor estuaries having several comparable hydromorphological characteristics but also some differences including in environmental factors and chemical contamination. Three specific questions, corresponding to the specific objectives of the Thesis, were addressed: (i) May summer environmental conditions influence the health status of *C. fluminea* potentially contributing to the differences related with the summer mortality syndrome observed between M-est and L-est populations?(ii) Do metals concentration increase *C. fluminea* stress levels contributing to the invasive behaviour differences observed between M-est and L-est populations? (iii) Do *C. fluminea* populations from M-est and L-est have differences in their gonadal development cycle possibly contributing to the differences observed between their invasive behaviour?

To answer the first specific question, a monitoring study was carried out monthly from July to October in the TFA of M-est and L-est (*Chapter II*). In the M-est, three sampling sites along an upstream => downstream gradient were selected. In the L-est, due to the sparse distribution of the *C. fluminea* population, only one site was sampled, corresponding approximately to the most downstream site of the M-est. In each sampling site, twenty *C. fluminea* specimens were collected per month. Seven biological parameters, hereafter indicated as biomarkers, were determined in each individual, namely: the activities of the enzymes isocitrate dehydrogenase (IDH) and octopine dehydrogenase (ODH) involved in cellular energy production; esterases (EST) involved in biotransformation; glutathione S-transferases (GST) involved in biotransformation and anti-oxidant defences; glutathione reductase (GR) and catalase (CAT), involved in anti-oxidant defences; and lipid peroxidation levels (LPO) as indicative of oxidative damage. Fifteen water abiotic and sediment physico-chemical parameters were also determined per month and sampling site: water temperature, conductivity, pH, hardness and turbidity and dissolved oxygen, nitrates, nitrites, ammonium, phosphates, silica, phenol and iron concentrations and sediment chlorophylls and organic matter concentrations. Biomarkers data were integrated using the integrated biomarker response index (IBR) that indicates the stress levels of the animals, and biological and abiotic parameters data were integrated through a redundancy analysis. The main conclusions were: July and August are particularly stressful months for *C. fluminea*, especially in the most downstream sampling site of the M-est and in the L-est sampling site; the water concentration of ammonia and nutrients, temperature and conductivity were the parameters contributing most to *C. fluminea* increased stress; moreover, in July/August, *C. fluminea* is probably exposed to oxidative stress inducers, environmental contaminants biotransformed by EST and GST enzymes, and additional energy to cope with the stress induced by temperature and/or contaminants is required. Therefore, summer environmental conditions increase *C. fluminea* stress decreasing the populations health condition likely contributing to summer massive mortality events that have been described in several *C. fluminea* populations, including in the M-est one. However, the increased stress was also observed in the L-est population for which summer massive mortality events were not described so far. *C. fluminea* density likely plays a determinant role since, in areas with higher densities, oxygen and food consumption and production of ammonia would be higher which might be lethal to individuals who are already under thermal stress. Therefore, in the L-est population that has a low density, the phenomena, if occurring, may be of small magnitude.

Thus, although the summer increased stress leading to massive mortality events may influence the invasive behaviour of *C. fluminea* populations, it seems not be a determinant factor regarding the invasive behaviour differences between the M-est and L-est populations.

The second question was investigated through a monitoring study carried out seasonally over one year in the sampling sites previously indicated (*Chapter III*). In addition to the biomarkers and abiotic parameters used in the previous study, the concentrations of 13 metals were determined in the whole soft body of *C. fluminea* and in sediments. The main conclusions were: the L-est sampling site showed the highest total concentration of metals, followed by the most downstream site in the M-est; *C. fluminea* populations from the M-est and L-est accumulated seven metals (Cr, Cu, Zn, Se, As, Cd, Pb); the health condition of both *C. fluminea* populations shows variation along the seasons, with the higher stress levels being recorded in summer/autumn and the lowest in spring; high Mn, Al and Se concentrations in sediments were associated with higher stress levels in *C. fluminea,* especially in the L-est site. Overall, the results indicate that the relatively high concentrations of some metals in L-est sediments are negatively influencing the heath status of the *C. fluminea* population in this estuary. Therefore, the reduced health condition of the L-est *C. fluminea* relatively to the heath condition of the M-est population, may contribute, at least partially, to the invasive behaviour differences between the two populations.

The third specific question was addressed by comparing the gonadal development cycle of *C. fluminea* populations of M-est and L-est in relation to water and sediments parameters, and the concentration of metals in sediments and in the whole softy body of *C. fluminea* specimens. From October 2011 to November 2012, monthly, ten specimens of *C. fluminea* were collected at each sampling site for the gonadal development study. The visceral mass of each animal that contains the gonad tissue was separated, fixed, dehydrated and embedded in paraffin. Longitudinal sections with 5 µm thick (2 per visceral mass sample) were cut and stained with hematoxyline and eosine. Slides were observed by optical microscopy to determine the sex and the gonadal phase. The main conclusions were: in both estuaries, *C. fluminea* shows hermaphroditism all over the year but with higher percentages in spring which are associated with a higher quantity of food; It was observed a seasonal pattern in the relative percentage of completely filled follicles with both oocytes and sperm, which is higher in autumn and spring and indicates, most likely, two stronger reproductive periods; the follicles filled with only oocytes were associated with the concentration of nickel in sediments which could be impairing the

development of sperm; and no significant differences between the populations of M-est and L-est in relation to gonadal development cycle were found. Overall, the findings of *Chapter IV* revealed that, despite pollution may impair male gonad development, the gonadal development cycle does not seem to be contributing to the observed differences in the invasive behaviour between populations of M-est and L-est.

Overall, the findings of the present Thesis increase the knowledge on the factors contributing to the invasive behaviour of *C. fluminea* in TDF areas of temperate regions indicating that pollution may be restricting the invasive behaviour of *C. fluminea* by decreasing their health status. In addition, the present Thesis contributes to the knowledge necessary to achieve the goals posed by The European Union Convention on Biodiversity Strategy for 2020, to control or eradicate the priority species, by discussing the use of eradication and/or control measures in upstream sampling sites where there is, probably, higher reproductive output.

Poderá a poluição limitar o comportamento invasor da espécie não-indígena *Corbicula fluminea*?

Resumo

A amêijoa asiática, *Corbicula fluminea,* é uma das 100 piores espécies invasoras na Europa. Este bivalve é bem conhecido pela sua elevada capacidade de colonização de novos habitats, pela sua dispersão, em geral, rápida e extensiva nos habitats colonizados e pelos danos ecológicos e económicos que as suas invasões frequentemente causam. Apesar dos estudos que têm sido efetuados nas últimas décadas, os fatores que influenciam o comportamento invasor de *C. fluminea* ainda não são totalmente conhecidos. Este conhecimento é da maior importância para tentar evitar novas invasões, bem como para controlar e mitigar os impactos de populações já estabelecidas. Para contribuir para o avanço do conhecimento na área, o objetivo central da presente Tese foi investigar se a exposição a longo termo a poluição poderá estar a limitar o comportamento invasor de *C. fluminea*. Exposições longas a poluição podem limitar o comportamento invasor das populações de *C. fluminea* maioritariamente através um aumento da taxa de mortalidade e uma diminuição do estado de saúde da população com efeitos negativos na sua boa condição física e/ou na reprodução, seja direta ou indiretamente. Estes efeitos foram investigados nesta Tese através de uma abordagem de campo tirando partido de populações de *C. fluminea* de áreas de água doce de zonas estuarinas (TFA) dos Rios Minho (M-est) e Lima (L-est) (Noroeste da Península Ibérica). Estas populações foram escolhidas uma vez que têm vido a mostrar diferentes comportamentos invasores e o M-est e o L-est são estuários vizinhos com várias características hidromorfológicas comparáveis mas também com algumas diferenças, incluindo factores ambientais e contaminação. Três questões específicas, correspondentes aos objectivos específicos da Tese, foram abordadas: a) Poderão as condições ambientais associadas ao verão influenciar o estado de saúde de *C. fluminea* contribuindo para as diferenças relacionadas com a mortalidade em massa observada no verão entre as populações do M-est e o L-est; b) poderá a concentração de metais aumentar os níveis de stress de *C. fluminea* contribuindo para as diferenças observadas entre os comportamentos invasores das populações do M-est e do L-est?; c) haverá diferenças entre o ciclo de desenvolvimento

gonadal das populações do M-est e L-est que poderão estar a contribuir para as diferenças observadas entre os seus comportamentos invasores?

Para responder á primeira questão, um estudo de monitorização foi levado a cabo mensalmente de Julho a Outubro nos TFA de M-est e L-est (*Capítulo II*). No M-est, foram selecionados 3 locais de amostragem ao longo de montante para jusante gradiente. No L-est, devido á sua distribuição esparsa da população de *C. fluminea*, apenas um local foi amostrado, correspondendo, aproximadamente, ao local mais a jusante do M-est. Em cada local de amostragem, vinte indivíduos foram recolhidos por mês. Sete parâmetros biológicos, doravante indicados como biomarcadores, foram determinados em cada indivíduo, nomeadamente: as atividades das enzimas isocitrato desidrogenase (IDH) e octopina desidrogenase (ODH) envolvidas na produção celular de energia; esterases (EST) envolvidas na biotransformação; glutationa S-transferases (GST) envolvidas na biotransformação e defesas anti-oxidantes; glutationa reductase (GR) e catalase (CAT) envolvidas nas defesas anti-oxidantes; e níveis de peroxidação lipídica (LPO) indicativos de danos oxidativos. Quinze parâmetros abióticos da água e físico-químicos dos sediments foram também determinados por mês e local de amostragem: temperatura, conductividade, pH, dureza, e turbidez e as concentrações de oxigénio dissolvido, nitratos, nitritos, amónia, fosfatos, sílica, fenos e ferro da água e a concentração de clorofilas, matéria orgânica e granulometria dos sedimentos. Os dados dos biomarcadores foram integrados usando o índice de respostas integradas dos biomarcadores (IBR) que indica o nível de stress dos animais, e os parâmetros biológicos e ambientais foram integrados através de uma análise de redundância. As principais conclusões foram: Julho e Agosto são meses particularmente stressantes para *C. fluminea*, especialmente no local mais a jusante do M-est e no local selecionado em L-est; as concentrações de amónia e nutrientes na água, e a temperatura e condutividade da água foram os parâmetros que mais contribuíram para o aumento de stress de *C. fluminea*; ademais, em Julho/Agosto C. flumínea está, provavelmente, exposta indutores de stress oxidativo, contaminantes ambientais biotransformados pelas enzimas EST e GST, e necessita de energia adicional de forma a lidar com o stress induzido pela temperatura e/ou contaminantes. Portanto, as condições ambientais do verão aumentam o stress de *C. fluminea* diminuindo a condição de saúde da população e provavelmente contribuindo para os eventos de mortalidade em massa que ocorrem no verão em várias populações de *C. fluminea*, incluindo a de M-est. No entanto, os elevados níveis de stress foram também observados na população de L-est para a qual eventos de mortalidade

em massa no verão não foram descritos até ao momento. A densidade de *C. fluminea* desempenha um papel importante, uma vez que, em áreas com maior densidade, o consumo de oxigénio e alimento e a produção de amónia será maior o que poderá ser letal para os indivíduos que já estão sob stress termal. Portanto, na população de L-est que tem baixa densidade, este fenómeno, se ocorrer, poderá ser de uma magnitude pequena. Deste modo, apesar de o stress induzido pelo verão que leva a eventos de mortalidade em massa poder influenciar o comportamento invasor das populações de *C. fluminea*, parece que não será um factor determinante em relação ás diferenças encontradas entre os comportamentos invasores das populações de M-est e L-est.

A segunda questão foi investigada através de uma monitorização sazonal durante um ano nos locais de amostragem indicados anteriormente (*Capítulo III*). Além dos biomarcadores e dos parêmtros abióticos da água e físico-químicos dos sedimentos avaliados no estudo anterior, a concentraçãoo de 13 metais foi determinada no corpo mole total de *C. fluminea* e nos sediementos de cada local amostrado. As conclusões principais foram: o local de amostragem do L-est mostrou maior concentração total de metais, seguido pelo local mais a jusante do M-est; as populações de *C. fluminea* de M-est e L-est acumularam sete metais (Cr, Cu, Zn, Se, As, Cd, Pb); a condição de saúde de ambas as populações de *C. fluminea* mostraram variação ao longo das estações do ano, com os maiores níveis de stress sendo observados no verão/outono e os menores na primavera; concentrações altas de Mn, Al e Se nos sedimentos foram associadas com maiores níveis de stress em *C. fluminea*, especialmente no local amostrado em L-est. No geral, os resultados indicam que concentrações relativamente altas de aguns metais nos sedimentos de L-est estão a influenciar negativamente a condição de saúde de *C. fluminea* neste estuário. Portanto, uma diminuição da condição de saúde da população de L-est relativamente á condição de saúde da população de M-est pode contribuir, pelo menos parcialmente, para as diferenças encontradas entre os comportamentos invasores das duas populações.

A terceira questão específica foi abordada comparando o ciclo de desenvolvimento gonadal das populações de C. flumínea de M-est e L-est em relação a parâmetros da água e dos sedimentos e á concentração de metais nos sedimentos no corpo mole de *C. fluminea*. De Outubro de 2011 a Novembro de 2012, mensalmente, dez indivíduos de *C. fluminea* foram recolhidos em cada local de amostragem para o estudo do desenvolvimento gonadal. A massa visceral de cada animal, contendo o tecido gonadal, foi separada, fixada, desidratada e incluída

em parafina. Secções longitudinais com 5 µm de espessura (2 por amostra de massa virceral) foram cortados e corados com hematoxilina e eosina. As lâminas foram observadas num microscópio óptico para determinar o sexo e a fase gonadal. As conclusões principais foram: em ambos os estuários, C. flumínea mostra hermafroditismo durante todo o ano mas com uma maior percentagem na primavera associada a uma maior quantidade de alimento; foi observado um padrão sazonal na percentagem relativa de folículos completamente preenchidos com ambos oócitos e espermatozoides, que foi maior no outono e na primavera e indica, muito provavelmente, dois períodos reprodutores fortes; os folículos preenchidos apenas com oócitos foram associados com a concentração de níquel nos sedimentos o que poderá estar a diminuir a produção de espermatozoides; e não foram encontradas diferenças significativas entre as populações do M-est e do L-est no que respeita o ciclo de desenvolvimento gonadal de *C. fluminea*. No geral, os descobertas do Capítulo IV revelam que, apesar da poluição poder diminuir o desenvolvimento de gónadas masculinas, o ciclo de desenvolvimento gonadal não parece estar a contribui para as diferenças observadas entre os comportamentos invasores das populações de M-est e L-est.

No geral, os descobertas desta Tese aumentam o conhecimento sobre os factores que contribuem para o comportamento invasor de C. fluminea em TFAs de regions temperadas indicando que a poluição pode limitar o comportamento invasor de C. fluminea afectando o seu estado de saúde. Além disso, a presente Tese contribui para o conhecimento necessário para alcançar os objectivos propostos pela Convenção da União Europeia na estratétia de Biodiversidade para 2020, de controlar ou erradicar as espécies invasoras prioritáris, discutindo o uso de medidas de erradicação e/ou control em locais mais a montante onde se observa maior capacidade reproductora.

Figures index

Tables index

Acronyms and abbreviations

Ach	Acetylcholine
AChE	Acetylcholinesterase
AhR	Aryl Hydrocarbon Receptor
ANOVA	Analysis of Variance
ASE	Accelerated Solvent Extraction
BCh	Butyrylcholine
BHT	Butylhydroxytoluene
BSAF	Biota-sediment accumulation factors
CALUX	Chemical-activated luciferase expression
CAT	Catalase
CDNB	1-chloro-2,4-dinitrobenzene
ChE	Cholinesterase
Chla	Chlorophyll *a*
Chlb	Chlorophyll *b*
Chlc	Chlorophyll *c*
Cond	Conductivity
CS	Coarse sand
d.w.	Dry weight
DMSO	Dimethyl sulfoxide
DO	Water dissolved oxygen
DTT	(*2S,3S*)-1,4-bis(sulfanyl)butane-2,3-diol
ECHA	European Chemicals Agency
Eds	Endocrine disruptors
EDTA	Ethylenediaminetetraacetic acid
EEQs	E_2 equivalent values
ER	Estrogen receptor
EROD	Ethoxyresorufin-*O*-deethylase
EST	Esterase
FS	Fine sand
GPC	Gel permeation chromatography
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GST	Glutathione S-transferases
H_2O_2	Hydrogen peroxide

IARC	International Agency for Research on Cancer
IBR	Integrated biomarker response index
IDH	Isocitrate dehydrogenase
IPMA	Instituto Português do Mar e da Atmosfera
L-est	Lima River estuary
LPO	Lipid peroxidation
LUC	Luciferase
M-est	Minho River estuary
MS	Medium sand
MSFD	Marine Strategy Framework Directive
MT	Metallothioneins
NAD^+	Nicotinamide adenine dinucleotide oxidized
NADH	Nicotinamide adenine dinucleotide reduced
$NADP^+$	Nicotinamide adenine dinucleotide phosphate oxidized
NADPH	Nicotinamide adenine dinucleotide phosphate reduced
NIS	Non-indigenous species
NP	Net peroxidation
NW	Northwest
O_2^-	Superoxide anion
ODH	Octopine dehydrogenase
OH^-	Hydroxyl groups
OM	Organic matter
OP	Organophosphates
P418	Cytochrome P418
P450	Cytochrome P450
PAHs	Polycyclic aromatic hydrocarbons
PBDDs	Dibenzo-*p*-dioxins
PBDEs	Polybrominated diphenyl ethers
PBDFs	Polybrominated dibenzofurans
PCAOB	Polychlorinated azoxybenzenes
PCBs	Polychlorinated biphenyls
PCDDs	Polychlorinated dibenzo-*p*-dioxins
PCDFs	Dibenzo-furans
PChE	Pseudocholinesterase
PCNs	Polychlorinated naphthalenes
PCTs	Polychlorinated terphenyls
PHAHs	Polyhalogenated aromatic hydrocarbons
PL	Stimulated peroxidation

PLI	Basal peroxidation
POPs	Persistent organic pollutants
RDA	Redundancy analysis
ROS	Reactive oxygen species
S+C	Silt and clay
SD	Standard deviation
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
TCDD	2,3,7,8-tetrachlorodibenzodioxin
TEQs	TCDD equivalent values
TFA	Tidal freshwater areas
TOC	Total organic carbon
Turb	Turbidity
UNEP	United Nations Environment Programme
USEFA	United States Environmental Protection Agency
USFDA	United States Food and Drug Administration
VCS	Very coarse sand
VFS	Very fine sand

Chapter I

General Introduction

1. The problem of bioinvasions

Invasions of non-indigenous species (NIS) are recognized as one of the major threats to natural ecosystems, having ecological, economic and social consequences (European Commission 2013; Karatayev *et al.* 2007; Kolar and Lodge 2001; Larson *et al.* 2011; Ojaveer *et al.* 2014; Sakai *et al.* 2001). NIS are identified as harmful to native species and they can drastically impact the physical environment, the ecosystem functioning, the biodiversity, recreational activities, and human and animal health (Simberloff *et al.* 2013). Consequently, the focus of researchers and policy makers in bioinvasions has been growing progressively, being the subject considered as one of the most important concerning global environmental changes (Rahel and Olden 2008). The European Union Convention on Biodiversity Strategy (European Commission 2011) established that, by 2020, the NIS and their pathways should be identified and prioritized, being the priority species controlled or eradicated, and the pathways should be managed to prevent the introduction and establishment of new invasive species. The European Union, as a party to the Convention on Biological Diversity, decided that, to manage bioinvasions, a three-way approach should be applied (European Commission 2014): prevention, eradication, and control (Figure I.1). Prevention requires measures that should reduce the probability of invasions before the beginning of the invasion process, such as borders control, risk analysis and application of directives defined by specialized legislation and regulation (Lockwood *et al.* 2007; European Commission 2014). Prevention measures are generally environmentally sustainable but more cost-effective than reactive strategies (Perrings 2005; Simberloff *et al.* 2013). Eradication measures are applied when an invasive species has already been introduced. To prevent future consequences, the early detection and the rapid eradication are the most cost-effective measures (Zavaleta *et al.* 2001). The early detection efforts may include an efficient communication system either to inform other areas that are also at risk of invasion and/or to exchange information on potential eradication strategies (European Commission 2008) or, to detect dispersal trends, the regularly monitoring of a specific site (Lockwood *et al.* 2007). And control measures are applied in cases that

prevention, early detection and eradication measures fail or are not feasible (European Commission 2014). Control methods, generally, include integrated approaches using combinations of different methods, such as physical, mechanical, chemical and biological methods or the use of them individually (Mackie and Claudi 2010; Sousa *et al.* 2012). When control measures result in high mortality or removal, it is important to monitor the ecosystem complemented by preventive measures against reintroductions (Sousa *et al.* 2012).

The term “invasive” refers to a species whose population is propagated quickly out of their geographical area and who present an impact on the receiver environment (Occhipinti-Ambrogi and Galil 2004). The invasive process is usually divided into three stages: introduction, establishment and spread (Figure I.1) (Engel *et al.* 2011; Hellmann *et al.* 2008; Lockwood *et al.* 2007). Introduction corresponds to the arrival of individuals to a given area outside of its native range and is dependent on the dispersal vector(s), the process duration, the conditions faced along the way, and the conditions of the individuals (Colautti and MacIsaac 2004; Davis 2009). After being introduced in the new habitat, the species needs to survive and reproduce (establishment stage). So it can succeed in the establishment stage, the invasive species needs to find conditions within its tolerance range, to be able to get the necessary energy for maintenance, growth, and reproduction from the available resources, and to be able to reproduce (Brockerhoff *et al.* 2014). Finally, to be a successful invader, the introduced NIS must persist for subsequent generations, grow in abundance and disperse well beyond the original point of entry (Brockerhoff *et al.* 2014; Lockwood 2010). About half of all non-indigenous species documented, to date, have established self-sustaining populations (Gollasch 2006). More than 1,000 non-indigenous aquatic species have been found in European waters (Nentwig 2007). The bivalve *Corbicula fluminea* is one of the most “efficient” worldwide freshwater invaders, listed among the 100 worst invasive alien species (both aquatic and terrestrial) in Europe (DAISIE 2015).

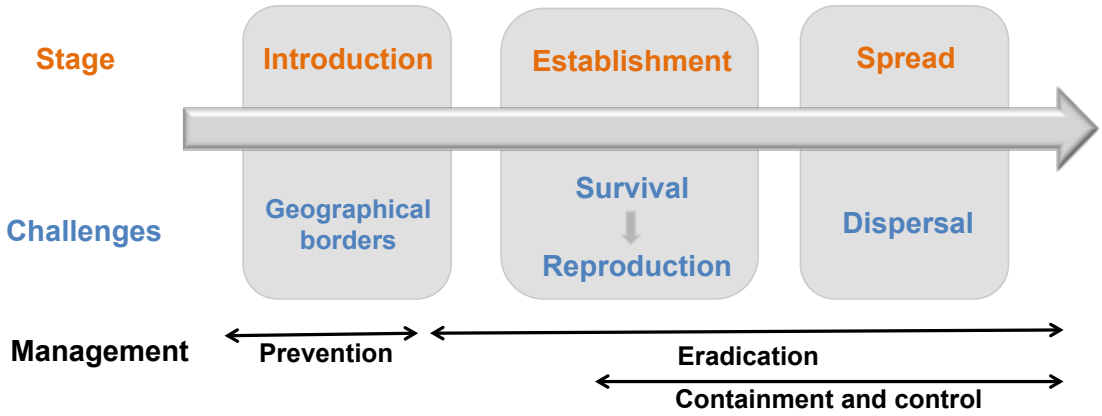


Figure I. 1. Invasion process illustrating the main stages of the invasion process, some limitations they have to overcome, and the management approaches that can be applied in each stage.

2. Bioinvasions by *Corbicula fluminea*

2.1. Occurrence

C. fluminea is believed to be native from West to Southern Asia, Africa and some areas in eastern Australia (Morton *et al.* 1986). The global spreading of *C. fluminea* started in the 20th century (McMahon 1999; Karatayev *et al.* 2007). The first publish record of *C. fluminea* invasion was in 1938, in the Columbia River, Washington (USA) and since then, this NIS spread throughout 36 continental states of this country, Hawaii, and Northern and Central Mexico (reviewed in McMahon 1999). Considering the dates of the first studies reporting the presence of *C. fluminea* in some European ecosystems, its introduction may have occurred in the late 1970s, in the Dordogne River estuary (France) and the estuary of Tagus River in 1978 (Mouthon 1981). Currently is widely distributed in continental Europe: in Austria, Belgium, the Czech Republic, France, Germany, Hungary, Luxembourg, Netherlands, Poland, Portugal, Serbia, Spain, Switzerland and Ukraine (reviewed in Minchin 2014 and Hubenov *et al.* 2013). After the first successful colonization in a region, in general the invasion and colonization of other ecosystems occurs rapidly likely through several dispersion ways, including anthropogenic activities (e.g. ballast water transport) and natural ones (e.g. transport by birds) (McMahon 2000). For example, after the first report of the

species in the Tagus River estuary (Mouthon 1981), *C. fluminea* was reported to occur in the Minho River estuary (M-est) in 1981 (Araújo *et al.* 1993), and more recently in several other ecosystems such as the Lima River estuary (L-est) (Sousa *et al.* 2005), among others. At the present, in Portugal, *C. fluminea* populations occur in all the main hydrological basins except in those of Cávado, Ave, Leça and Lis Rivers (reviewed in Rosa *et al.* 2011).

2.2. Habitat requirements of *C. fluminea*

In general, *C. fluminea* shows preference for habitats with well-oxygenated sandier sediments containing high percentages of organic matter (Hakenkamp and Palmer 1999; Sousa *et al.* 2008b; Vaughn and Hakenkamp 2001). However, in invaded areas, it has been found in several types of ecosystems including oligotrophic and eutrophic streams, rivers including estuaries, and lakes, with different types of sediments such as oxygenated muddy, sandy, gravel and cobble ones (McMahon 2002; Minchin 2009). As other invasive species, *C. fluminea* is tolerant to a considerable variation of several environmental factors other than sediment type. For example, its tolerance regarding temperature variation is believed to be from 2ºC (Karatayev *et al.* 2005; Werner and Rothhaupt 2008) to 37ºC (McMahon and Williams 1986). However, it has a limited tolerance to high salinity, especially juveniles (Byrne and McMahon 1994; Ilarri *et al.* 2010; Ilarri and Johnson and McMahon 1998; McMahon 1999; Sousa *et al.* 2008, 2011; Xiao *et al.* 2014). High nutrient concentrations (Oliveira *et al.* 2015a) and low oxygen levels in the water are also not favorable conditions for *C. fluminea*, with respiration becoming impaired at water dissolved oxygen concentrations of 1 to 3 mg/L (Belanger *et al.* 1991; Johnson and McMahon 1998; Matthews and McMahon 1999). *C. fluminea* is also sensitive to waters with pH below 5 and hardness below 3 mg $CaCO_3$/L (Mackie and Claudi 2010). Because the sensitivity of *C. fluminea* to several environmental factors is higher than the sensitivity of several of its native competitors (Reviewed in Sousa *et al.* 2008a), the high invasive success of this species, in general, relies more on its biological characteristics (e.g. high fecundity, rapid growth, small juvenile size, and the capacity for downstream dispersal) than on its physiological tolerance (Fureder and Pockl 2007; McMahon 2002; Sousa *et al.* 2006a, 2008a; Vohmann *et al.* 2009).

2.3. *C. fluminea* reproduction

C. fluminea is generally classified as a hermaphroditic (Figure I. 2) species (Park and Chung 2004) with a high reproductive capability estimated to be around 70, 000 juveniles per adult per year (Aldridge and McMahon 1978).

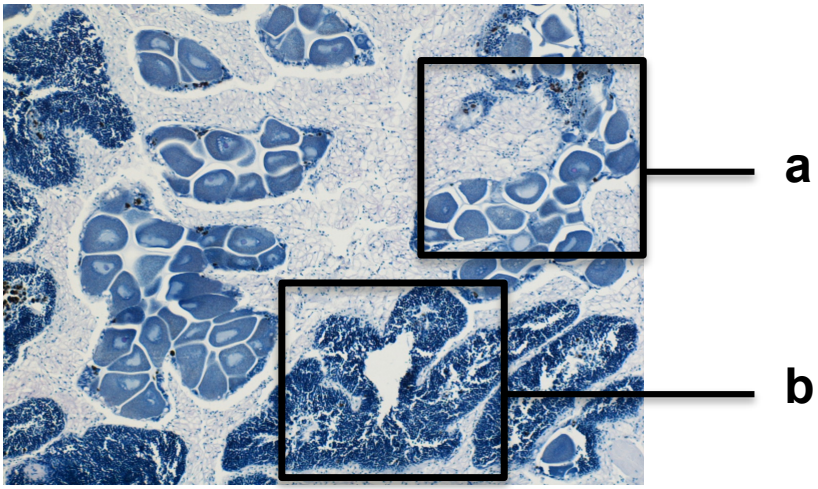


Figure I. 2. Histological section of *C. fluminea* gonads showing a) female and b) male follicles (hermaphroditism).

In several ecosystems of temperate regions, *C. fluminea* has two main reproduction periods, one in the spring and the other in the autumn (McMahon 1983; McMahon and Williams 1986; Morton 1977; Sousa *et al.* 2008a). However, the number of reproductive events may be different and seems to be influenced by water temperature (Hornbach 1992; Mouthon 2001b; Rajagopal *et al.* 2000) and the availability of food resources (Cataldo and Boltovskoy 1999; Mouthon 2001a,b). The gametogenesis is continuous (Byrne *et al.* 2000). The fertilization occurs inside the paleal cavity (Sousa *et al.* 2008a) and the incubation of embryos occurs in the inner demibranchs (Figure I. 3 a) (Ilarri and Sousa 2011). The juveniles, have a D-shapped configuration, measure around 250 µm (anterior-posterior shell length) (Figure I. 3 b) and are released to the water column (McMahon 2002).

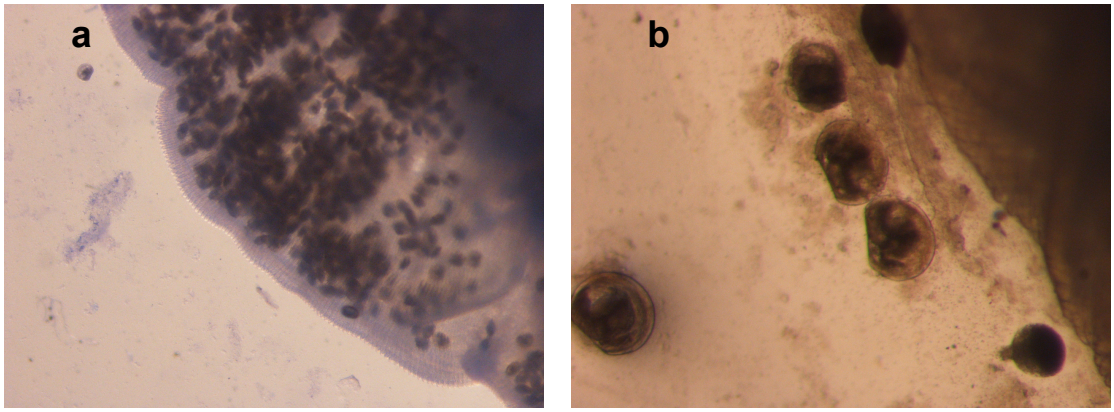


Figure I. 3. *C. fluminea* a) inner demibranch with larvae and b) small juveniles recently released (completely formed with the common D-shaped configuration).

Around four days of being released, juveniles attached to the sediments (Mackie and Claudi 2010) where they further develop. However, they can be released from sediments back to the water column by strong hydrodynamic events (McMahon 1999). When juveniles are in the water column, especially in streams, rivers and estuaries, they can be dispersed for long distances, mainly downstream (McMahon 1999). In general, the maturation period occurs within the first three to six months when the shell length reaches about 6 to 10 mm (anterior-posterior shell length) (Ilarri and Sousa 2011).

2.4. *C. fluminea* feeding

Filter feeding (Figure I. 4 a) clearly is the main process of food uptake by the Asian clam, complemented with by 'pedal feeding' in adults (Figure I. 4 b) (Cummings and Graf 2010; Hakenkamp and Palmer 1999; Hakenkamp *et al.* 2001; Sousa *et al.* 2008a; Vaughn and Hakenkamp 2001; Yeager and Cherry 1994). *C. fluminea* is considered a non-selective suspension feeder and it can effectively remove detritus, bacteria and algae from the water column (Boltovskoy *et al.* 1995; Lauritsen 1986; Way *et al.* 1990).

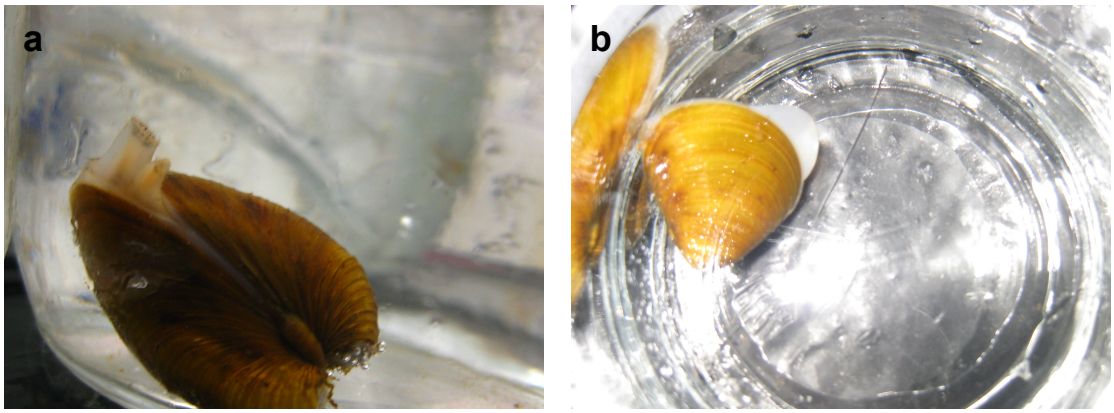


Figure I. 4. *C. fluminea* feeding structures: a) inhalant and exhalant opening (used for filter feeding) and b) foot (used for 'pedal feeding').

2.5. Impacts of *C. fluminea* invasions

The invasions by *C. fluminea* are well known by the negative ecological and economic impacts that they in general cause (Reviewed in Karatayev *et al.* 2007 and Sousa *et al.* 2008a). The major ecological negative impacts that have been described are: reductions in the native bivalve abundance and biodiversity by reducing the available habitats (Vaughn and Hakenkamp 2001); competition, advantageously, for food resources and changes in the nutrient cycling because of their high filtration rates (Hakenkamp and Palmer 1999; McMahon 1991; Vaughn and Hakenkamp 2001); several impacts on habitat and benthic and planktonic structure, in biomineralization and oxygenation (Reviwed in Karatayev et al 2007); degradation of water quality by *C. fluminea* massive mortalities in highly invaded areas (Cherry *et al.* 2005; Cooper *et al.* 2005; Johnson and McMahon 1998; Sousa *et al.* 2012; Strayer 1999): and *C. fluminea* may also be a vector of parasites and pathogens (Sousa *et al.* 2008a). Besides ecological impacts, *C. fluminea* may also have negative economic impacts mainly related to their biofouling activity that usually results in damages on man-made structures (Minchin *et al.* 2002; Pimentel *et al.* 2005). *C. fluminea* impair and damage underwater structures and equipment by growing and establishing dense populations on it which will block pipes and equipment, reduce efficiency of water cooling systems, increase corrosion, impair safety hazards and disturb the industry operation because of the need for biofouling removal (reviewed in Rosa *et al.* 2011).

Despite several negative ecological and economic impacts, positive impacts may also occur in systems invaded by this NIS (Reviewed in Karatayev *et al.* 2007 and Sousa *et al.* 2008a): *C. fluminea* shells may serve as shelter and substrate for other species (Crooks 2002; Gutiérrez *et al.* 2003); it can be used as food resource (Cantanhêde *et al.* 2008; Fried and Emili 1987; McMahon 1991); it may reduce euthrophication processes and increase water clarity due to the high filtration rates (McMahon 2002; Phelps 1994); and it can be used as a bioindicator species for ecotoxicological studies (Cataldo *et al.* 2001; Doherty 1990; Inza *et al.* 1997; Takabe *et al.* 2011).

2.6. Factors affecting the invasive behaviour of *C. fluminea*

The introduction and dispersion of *C. fluminea* in aquatic ecosystems is, most likely, a result of human activities such as their use as food resource or as fish bait, aquarium releases, transport of juveniles and/or adults as a tourist curiosity, their transport in ballast water, or the juvenile byssal attachment to boats (Darrigran 2002; Lee *et al.* 2005; McMahon 2000, 2002). *C. fluminea* has also great capacities for natural dispersion. The occurrence of both pelagic and benthic life stages enables *C. fluminea* to spread over long distances on the feet or feathers of waterfowl and/or by fluvial or tidal currents (Figuerola and Green 2002; McMahon 2000, 2002). In addition, the production of long mucous threads secreted in response to water current stimuli indicate that flotation assisted by mucous seems to be an important mechanism of dispersal (Prezant and Chalermwat 1984).

Three main types of factors have been considered to have a major influence on the success of a bioinvasion: genetic variability and phenotypic plasticity of the NIS, its tolerance to environmental conditions of the new habitat (Byers 2002) and competitive capability of the NIS *versus* native species (Byers 2002; Facon *et al.* 2006; Lee 2002). Despite this knowledge and the considerable amount of studies on *C. fluminea*, the main individual factors influencing its invasive behaviour and their mode of action are not known (Sousa *et al.* 2008a). Low genetic variability may lead to altered fitness over time and decrease the capability of populations to adapt to the environmental conditions of the new habitat (Byers 2002). Introduced individuals of *C. fluminea* show low levels of genetic diversity (Schmidlin *et al.*

2012; Simard *et al.* 2012) because of a relatively rare mode of asexual reproduction called androgenesis (Hedtke *et al.* 2008; Komaru *et al.* 1998). However, despite low genetic diversity, asexual reproduction is a common mean to become invasive (Roman and Darling 2007) and androgenesis play an important role in the invasive success of *C. fluminea* (Pigneur *et al.* 2011). Regardless their low genetic diversity, *C. fluminea* show high phenotypic plasticity (Lee *et al.* 2005; Pfenninger *et al.* 2002; Renard *et al.* 2000) that may play an important role in the adaptation of new environments (Pigneur *et al.* 2011; Sousa *et al.* 2007b).

C. fluminea is very sensitive to several environmental factors (Reviewed in Sousa *et al.* 2008a). For example, *C. fluminea* population density and size structure were influenced by abiotic conditions such as salinity fluctuations, nutrients and environmental contaminants in L-est located in the NW of Portugal (Sousa *et al.,* 2006a) however salinity, temperature, calcium and oxygen concentration and pH do not seem to limit the spread of the species in the lake Maggiore in Italy (Kamburska *et al.* 2013). Therefore, environmental contaminants seem to be an important factor limiting the invasive behaviour of *C. fluminea*.

2.7. Effects of environmental contaminants on *C. fluminea*

Several studies have demonstrated the adverse effects of various environmental contaminants on *C. fluminea* (reviewed in Doherty and Cherry 1988, Doherty 1990). Inadequate water quality induced by strong sources of industrial and sewage effluents was observed to be responsible for 100% of mortality among *C. fluminea* newborns, for dwarfed adult clams, and for the lack of discernible cohorts in the delta of the Paraná River in Argentina (Boltovskoy *et al.* 1997). Aquatic contamination was also found to be responsible for dwarfed *C. fluminea* individuals (e.g., Belanger *et al.* 1991; Britton and Morton 1982; Fritz and Lutz 1986), low population densities (Belanger *et al.* 1990, 1991), decreasing growth rates and poor condition index (Cataldo *et al.* 2001). Juveniles of *C. fluminea* were shown to be extremely sensitive to cooper exhibiting growth inhibitions when exposed to concentrations as low as 0.0084 mg/L and a high mortality rate at 0.0139 mg Cu/L (Belanger *et al.* 1990). Zinc was also found to be strongly harmful

for *C. fluminea*'s survival, osmoregulation, cellulolytic activity and growth (Belanger *et al.* 1986; Farris *et al.* 1988). Size-dependent *C. fluminea* metabolic changes related to a perturbed amino acid and energy metabolism were observed after short term exposure to environmentally relevant concentrations of a cadmium- zinc mixture (1.5 mg/kg of cadmium and 350 mg/kg of zinc) in the sediment but no mortality neither reduced condition index were observed (Spann *et al.* 2011). A 3-week exposure to 0.025 mg/L of cadmium in water was found to induce mortality and significant reductions in cellulase activity preceded mortality, whereas significant reductions of DNA strand lengths in *C. fluminea* individuals exposed to lower cadmium treatments (0.003 and 0.006 mg/Kg) preceded of cellulolytic enzyme activity (Barfield *et al.* 2001). Histopathological alterations were also observed after *C. fluminea* exposure to pollutants such as Aroclor 1260, a polychlorinated biphenyl compound, that caused significant gonadal atrophy, accumulation of brown cells, and inflammation and necrosis in digestive glands and foot tissues (Lehmann *et al.* 2007) and diamond nanoparticles that induce vacuolization and thickening of digestive gland cells (Cid *et al.* 2015). Several changes on biochemical biomarkers related to oxidative stress, detoxification mechanisms, neurotransmission and energetic metabolism were also observed on *C. fluminea* exposed to pollutants (See Table I. 1). Oxidative stress is known to have a severe impact in cell's metabolism and viability with significant consequences at the organ and organism levels and will likely result in population wide effects, including reduced fecundity, organ injury and chronic maladies (Lehmann *et al.* 2007;) which might affect the invasive behaviour of *C. fluminea*. A decreased health status related with increased oxidative stress and damage, detoxification mechanisms and neurotransmission impairments may have negative effects on *C. fluminea* fitness, and/or direct negative effects on reproduction that will probably reduce the ability of C. fluminea to establish in new environments and/or decreased their dispersion rates.

Table I. 1. Responses of biochemical biomarkers obtained after *Corbicula fluminea* exposure to contaminants. + indicate significant inductions, - indicate significant inhibitions and = indicate no significant differences from respectively controls. Abbreviations are corresponding to cholinesterase (ChE), octopine dehydrogenase (ODH), isocitrate dehydrogenase (IDH), glutathione reductase (GR), glutathione S-transferase (GST) pi-class glutathione S-transferase(pi-GST) and glutathione S-transferase using ethacrynic acid (GST/EA), catalase (CAT), glutathione peroxidase (GPx), lipid peroxidation (LPO), metallothioneins (MT), multi-xenobiotic resistance protein (MXR), selinium dependent glutathione peroxidase (Se-GPx), peroxided lipids (PL), peroxidizable lipids (PLI), net peroxidation (NP), cytrochrome P450 (P450), cytrochrome P418 (P418), NADH-cytrochrome P450 reductase (NADH-red), NADPH- cytrochrome P450 reductase (NADPH-red), ethoxyresorufin-*O*-deethylase (EROD), oxidized glutathione and reduced and oxidized glutathione ration (GSH/GSSG).

Exposure	Tissue	Time of exposure	Concentration	Biomarker response											Reference
				ChE	ODH	IDH	GR	GST	CAT	GPx	LPO	MT	MXR	Se-GPx	
Mercury (µg/L)	**Gills (Except for ChE (adductor muscle) and ODH and IDH (foot muscle))**	**96h**	31	=	=	=	=	=	=	=	=	n.a	n.a	n.a	(Oliveira *et al.* 2015b)
			63	=	=	=	=	+	=	=	=	n.a	n.a	n.a	
			125	=	=	=	=	+	=	=	+	n.a	n.a	n.a	
			250	=	=	=	=	+	=	=	+	n.a	n.a	n.a	
			500	=	=	-	-	=	+	-	+	n.a	n.a	n.a	
Cadmium (µg/L)	**Gills**	**3d**	30	n.a	n.a	n.a	=	n.a	=	=	=	n.a	+	-	Legeay *et al.* 2005
		7d		n.a	n.a	n.a	-	n.a	=	=	=	+	+	=	
		14d		n.a	n.a	n.a	=	n.a	=	=	=	+	+	-	
Cadmium (µg/L) + Hypoxia		**3d**		n.a	n.a	n.a	=	n.a	=	-	+	n.a	+	-	
		7d		n.a	n.a	n.a	-	n.a	=	=	+	+	+	=	
		14d		n.a	n.a	n.a	+	n.a	=	=	+	+	+	-	

Table I. 1. (Continued…)

Exposure	Tissue	Time of exposure	Concentration	Biomarker response					Reference
				CAT	GR	GST	LPO	SOD	
Paracetamol (mg/L)	**Body**	**96h**	0.05	=	=	=	=	n.a.	(Brandão *et al.* 2011)
			0.48	=	-	-	=	n.a.	
			4.82	=	-	-	+	n.a.	
			532.78	=	=	-	+	n.a.	
Paracetamol (µg/L)		**28d**	3.88	=	=	=	-	n.a.	
			7.74	=	=	=	-	n.a.	
			15.49	=	=	=	=	n.a.	
			30.98	=	=	-	=	n.a.	
			61.95	=	=	-	+	n.a	
Cooper (mg/L)	**Gills**	**5d**	0.05	=	=	n.a.	n.a.	+	(Netpae *et al.* 2012)
			0.1	=	+	n.a.	n.a.	+	
			0.5	=	+	n.a.	n.a.	+	
		10d	0.05	=	=	n.a.	n.a.	+	
			0.1	=	+	n.a.	n.a.	+	
			0.5	+	+	n.a.	n.a.	+	
		20d	0.05	+	=	n.a.	n.a.	+	
			0.1	+	+	n.a.	n.a.	+	
			0.5	+	+	n.a	n.a	+	
		30d	0.05	+	=	n.a.	n.a.	+	
			0.1	+	+	n.a.	n.a.	+	
			0.5	+	+	n.a	n.a	+	

Table I. 1. (Continued…)

Exposure	Tissue	Time of exposure	Concentration	Biomarker response															Reference
				CAT	PL	PLI	NP	P450	P418	NADH-red	ChE	GST	GST/EA	NADPH-red	EROD	SOD	Se-GPX	Pi-GST	MT	
Trichloro-ethylene (mg/L)	Digestive gland except for EROD, P450 and P418 (Body)	5d	1.2	+	+	=	=	=	=	=	=	=	=	=	=	n.a.	n.a.	n.a.	n.a.	(Vidal *et al.* 2001)
			3.6	+	=	=	=	+	+	=	=	=	=	=	=	n.a.	n.a.	n.a.	n.a.	
			14	=	=	=	=	+	=	=	=	=	=	=	=	n.a.	n.a.	n.a.	n.a.	
			69.4	=	=	=	=	+	=	=	=	=	=	=	=	n.a.	n.a.	n.a.	n.a.	
Toluene (mg/L)			4.3	+	+	+	+	=	=	=	=	=	=	=	=	n.a.	n.a.	n.a.	n.a.	
			8.2	=	=	+	+	=	=	=	=	=	=	=	=	n.a.	n.a.	n.a.	n.a.	
			28.2	=	=	=	=	+	+	=	=	=	=	=	=	n.a.	n.a.	n.a.	n.a.	
			36.4	=	=	=	=	=	=	=	=	=	=	=	=	n.a.	n.a.	n.a.	n.a.	
Cooper (µg/L)	Gills	12h	10	-	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	=	+	-	+	(Bigot *et al.* 2011)
			50	-	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	-	=	=	-	
	Digestive gland		10	-	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	-	-	+	-	
			50	=	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	+	=	+	+	
Cadmium (µg/L)	Gills		2	-	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	-	+	-	=	
			10	-	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	=	+	-	=	
			50	-	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	-	+	-	-	
	Digestive gland		2	=	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	=	=	=	+	
			10	-	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	+	=	+	+	
			50	-	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	=	=	=	+	
Cooper+ Cadmium (µg/L)	Gills		10	-	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	=	+	-	-	
			50	-	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	-	+	-	-	
	Digestive gland		10	=	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	=	=	+	+	
			50	-	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	-	-	+	+	

Table I. 1. (Continued…)

Exposure	Tissue	Time of exposure	Concentration	Biomarker response								Reference
				EROD	GST	LPO	MT	SOD	CAT	GSSG	GSH/GSSG	
Domestic landfill leachate (%)	Gills	5d	2	-	=	+	=	n.a.	n.a.	n.a.	n.a.	(Oliveira *et al.* 2014)
			3	-	=	=	=	n.a.	n.a.	n.a.	n.a.	
			6	-	=	=	=	n.a.	n.a.	n.a.	n.a.	
			10	-	+	+	=	n.a.	n.a.	n.a.	n.a.	
		15d	2	=	=	=	=	n.a.	n.a.	n.a.	n.a.	
			3	-	=	=	+	n.a.	n.a.	n.a.	n.a.	
			6	=	=	=	=	n.a.	n.a.	n.a.	n.a.	
			10	=	=	=	=	n.a.	n.a.	n.a.	n.a.	
	Digestive gland	5d	2	=	=	+	n.a.	n.a	n.a	n.a	n.a	
			3	=	=	+	n.a.	n.a.	n.a.	n.a.	n.a.	
			6	=	=	=	n.a.	n.a.	n.a.	n.a.	n.a.	
			10	=	=	=	n.a.	n.a	n.a	n.a	n.a	
		15d	2	=	=	+	n.a.	n.a	n.a	n.a	n.a	
			3	=	=	+	n.a.	n.a.	n.a.	n.a.	n.a.	
			6	=	=	=	n.a.	n.a.	n.a.	n.a.	n.a.	
			10	+	=	n.a.	n.a.	n.a	n.a	n.a	n.a	
Cadmium (mg Kg/ sediments d.w.)	Digestive gland	28d	0.72	n.a.	n.a	=	=	=	=	=	=	(Ren et al. 2013)
			0.91	n.a.	n.a.	=	=	-	=	=	=	
			1.62	n.a.	n.a.	=	=	-	=	+	=	
			2.59	n.a.	n.a	+	=	-	+	+	-	
			11.2	n.a.	n.a	+	+	=	=	+	-	
			20.4	n.a.	n.a.	+	+	-	=	=	=	
			40.6	n.a.	n.a.	=	=	-	-	=	=	

3. *Corbicula fluminea* invasive behaviour: A case study in Minho and Lima estuaries

The invasive bivalves of the genus *Corbicula* are one of the most widespread species in terms of both abundance and biomass (Byrne et al. 2000; Pérez-Quintero 2008). There are several studies about the life-history traits of *C. fluminea* in invaded areas (North-America: Aldridge and McMahon 1978; South-America: Cataldo and Boltovskoy 1998; Europe: Rajagopal et al. 2000; Sousa et al. 2008) and all authors agree that the relatively short lifespan, early sexual maturity (at a shell length of 6–10 mm), rapid growth, high fecundity, small juvenile size, and the capacity for downstream dispersal of C. fluminea make it highly invasive (McMahon 2002; Sousa et al. 2006a; Fureder and Pockl 2007; Vohmann et al. 2009). However, in the north of Portugal there are two invaded rivers, Minho and Lima, where *C. fluminea* population invasive behaviour differ substantially, despite the relatively short geographical distance between them and the hydrological and geological similarities (Sousa et al. 2006a; Sousa et al. 2008d). Araujo et al. (1993) observed for the first time, in Minho estuary in 1989, a few juveniles of *C. fluminea* that less than one year after increased in number and area occupied. In 1991, C. fluminea already reached 8 to 24 Km upstream. Since then the Asian clam has become a major benthic component in terms of abundance and biomass (contributing with more than 90% of the macrobenthic biomass in the estuarine area (Sousa et al. 2008d) having more than 4000 individuals m-2 and more than 400 g AFDW m-2, respectively (Sousa et al. 2005). In Lima estuary, *C. fluminea* was observed for the first time in 2002 in upper estuarine areas (Sousa et al. 2006b) and since then their occurrence has been constant but at low densities in a limited intertidal area of the Lima estuary and was rarely found in the subtidal zone (Sousa et al. 2006a). In the Lima estuary the abundance and biomass per site never exceeded 60 individuals m-2 and 26 g AFDW m-2, respectively (Sousa et al. 2006a; Sousa et al. 2006b). Sousa et al. (2006a; 2008d) studied the factors influencing the occurrence and distribution of this invasive species in these two estuaries. In Minho estuary (Sousa et al. 2008d) the C. fluminea higher biomasses are

supported by higher values of redox potential, water hardness, organic matter and very coarse and fine sands and lower values of nutrient concentrations (namely nitrates and ammonia). In Lima Estuary (Sousa et al. 2006a) the author suggests a lag time phase due to its more recent introduction and, since they did not find individuals with a shell length less than 13 mm, due to a deficient recruitment (reduced spawning and/or high mortality rate of larvae and/or juveniles). Other parameter that seems to be highly influencing the success and velocity of the invasion in Lima estuary is the salinity that has increased throughout the first years of the invasion. The authors further pose alternative hypothesis such as nutrient enrichment, higher heavy metals concentration and other contaminants and genetic characteristics. However, in a later study, the authors show that there are no genetic differences between the two populations and state that both populations belong to the species *C. fluminea* (Sousa et al. 2007). However their studies were not sufficient to get a definitive answer about the factors contributing to the distinct invasive behaviour presented.

Given the great environmental similarities between these two adjacent Portuguese estuaries it was expected a higher invasive behaviour in the Lima estuary. One of the hypotheses raised by Sousa et al. (2006a) is that heavy metals and other contaminants concentration might be limiting the invasive behaviour of the Asian clam in Lima estuary.

Minho and Lima are international rivers draining hydrological basins of 10,080 km2 and 2,250 km2, respectively (Bettencourt et al. 2004).

The Minho River is located on both sides of the border between northern Portugal and Spain. The estuary is considered as one of the least contaminated along the Portuguese coast, having relatively low levels of environmental contamination and it was used as a reference site in previous studies (Cairrão et al. 2004; Moreira et al. 2006; Quintaneiro et al. 2006; Monteiro et al. 2007; Sousa et al. 2008; Gravato et al. 2010; Guimarães et al. 2012). However, in since the last decade, several contaminants with dioxin- and estrogen-like properties, including PAHs, organochlorine pesticides and natural estrogens, have been quantified in the river (Table I. 2) associated with industrial and agricultural activities in both upstream and downstream

and with boat traffic downstream in the river (Cunha et al. 2005; Rodrigues et al. 2006a; Vioque-Fernández et al. 2007; Carvalho et al. 2009; Reis et al. 2009; Gravato et al. 2010; Mil-Homens et al. 2013b). Lima River, also located in the northwest of Portugal, in comparison with other Portuguese rivers, receives low levels of urban and industrial effluent inputs, but the amount of total suspended solids, phosphorus and nitrogen reaching the river basin is 3-fold higher compared to that reaching the Minho River basin (Guerreiro and Pereira, 2002). Additionally, the river system is considerably impacted by anthropogenic activities, mainly due to the harbour of Viana do Castelo, one of the largest cities in northern Portugal (47,000 inhabitants) located on the left bank of the river, a paper mill and discharges of urban origin that are released without prior treatment (INAG, 2000). Several contaminants were since the last decade also quantified in this river basin (Table I. 2) (Cunha et al. 2005; Rodrigues et al. 2006a; Carvalho et al. 2009; Gravato et al. 2010).

Despite the amount of agricultural activity in the hydrographical basin of the river, Minho estuary exhibited relatively low levels of different organochlorine pesticides, however, upstream, sediments revealed concentrations of DDT, DDD, dieldrin, endrin and lindane (Carvalho et al., 2009) that can induce adverse estrogenic effects (Shanle and Xu 2010). Concentrations of organochlorine pesticides are generally lower in Lima estuary, except for αHCH and heptachlor, that are in the same range and aldrin and heptachlor epoxide that showed higher levels in Lima as compared to Minho estuary (Carvalho et al. 2009). Higher concentrations upstream in Minho estuary may be due to inputs from the Louro River, since, generally, Spanish tributaries have a significant effect in Minho River physicochemical water quality (Santos et al. 2013). Louro River is the most polluted tributary of Minho (Table I. 3), since it receives untreated or insufficiently treated industrial and municipal wastewaters from the Tui (a highly industrialized city in Spain) industrial area containing essentially metals but also high levels of organic matter and high concentrations of pesticides (Filgueiras et al. 2004; Concha-Graña et al. 2006; Lavilla et al. 2010; Planelló et al. 2013; Santos et al. 2013). Concerning PAHs and metals, concentrations are usually lower in Minho estuary as compared to Lima (Gravato et al. 2010). Yet, the concentrations of major contaminants considerably increase, in summer as compared to winter, to the same levels

as reported in Lima or sometimes to even higher levels (Table I. 2) (Reis et al. 2009; Gravato et al. 2010; Mil-Homens et al. 2013b), suggesting a moderate contamination in this river system.

Table I. 2. Concentrations of several groups of contaminants in different matrices that have been quantified in Minho and Lima rivers by other research groups.

Contaminants	River			Matrices	Season	Group of compounds	References
	Minho Upstream	Minho Downstream	Lima				
αHCH (ng/g)	1.9 ± 0.2	2.9 ± 0.4	2.1 ± 0.2	Sediments	Spring 2007-2008	Organochlorine pesticides	Carvalho et al. 2009
Lindane (ng/g)	2.3 ± 0.3	2.3 ± 0.1	1.1 ± 0.2				
Heptachlor (ng/g)	3.7 ± 0.4	1.9 ± 0.1	2.9 ± 0.4				
Aldrin (ng/g)	0.29 ± 0.01	0.08 ± 0.01	0.42 ± 0.02				
Heptachlor epoxide (ng/g)	0.18 ± 0.02	0.23 ± 0.04	0.25 ± 0.02				
Endosulfan I (ng/g)	1.26 ± 0.03	1.1 ± 0.2	0.68 ± 0.04				
DDE (ng/g)	1.25 ± 0.06	0.35 ± 0.05	0.25 ± 0.03				
Dieldrin (ng/g)	0.9 ± 0.1	0.73 ± 0.07	0.26 ± 0.05				
Endrin (ng/g)	1.5 ± 0.3	1.2 ± 0.2	0.63 ± 0.03				
DDD (ng/g)	2.6 ± 0.2	0.42 ± 0.04	0.29 ± 0.05				
DDT (ng/g)	2 ± 0.3	1.44 ± 0.07	0.27 ± 0.03				
Methoxydolor (ng/g)	2 ± 0.2	1.15 ± 0.04	1 ± 0.1				
PFOS (ng/g WW)	77.24 ± 21.4		76.98 ± 14.81	Mussels	----------	Fluorosurfactant	Cunha et al. 2005
17β-estradiol (pg/L)	130		160	Water	Autumn 2005	Natural estrogens	Rodrigues et al. 2006
Estrone (pg/L)	110		183				
Acenaphthylene (ng/g)	0.21 ± 0.05		0.28 ± 0.03	Sediments	Winter 2006	PAHs	Gravato et al. 2010
Fluorene (ng/g)	0.57 ± 0.06		0.82 ± 0.20				
Phenantrene (ng/g)	0.99 ± 0.19		2.21 ± 0.60				
Anthracene (ng/g)	0.14 ± 0.02		0.27 ± 0.06				
Fluoranthene (ng/g)	0.78 ± 0.04		2.3 ± 0.87				
Pyrene (ng/g)	0.77 ± 0.10		2.1 ± 0.72				
Benzo[a]anthracene (ng/g)	0.06 ± 0.06		0.65 ± 0.35				
Chrysene (ng/g)	0.21 ± 0.13		0.75 ± 0.28				
Benzo[b]fluoranthrene (ng/g)	<0.4		1.36 ± 0.67				
Benzo[k]fluoranthrene (ng/g)	<0.4		1.66 ± 0.86				
Benzo[e]pyrene (ng/g)	<0.4		1.44 ± 0.99				
Benzo[a]pyrene (ng/g)	<0.6		0.6 ± 0.64				
Perylene (ng/g)	<0.5		3.09 ± 0.42				
Indenol[1,2,3-cd]pyrene (ng/g)	<0.5		0.19 ± 0.33				
Dibenzo[a,h]anthracene (ng/g)	<0.5		<0.5				
Benzo[g,h,l]perylene (ng/g)	<0.7		<0.7				

Table I. 2. (continued)

Contaminants	Rivers			Matrices	Season	Group of compounds	References
	Minho Upstream	Minho Downstream	River Lima				
Cd (µg/g)	0.01 ± 0.01		0.04 ± 0.01	Sediments	Winter 2006	Metals	Gravato et al. 2010
Cr (µg/g)	1.57 ± 0.12		6.43 ± 0.51				
Cu (µg/g)	1.73 ± 1.1		3.63 ± 0.55				
Hg (µg/g)	<0.036		<0.036				
Ni (µg/g)	2.7 ± 0.3		5.63 ± 0.35				
Pb (µg/g)	1.8 ± 0.35		5.27 ± 0.31				
Zn (µg/g)	<6.7		22 ± 2.65				
V (µg/g)	<2.4		6.87 ± 0.81				
2,4,6-trichlorophenol (TCP) (freq[a] (%); ng/L)	45; 0.4-3.8		----------	Water	March 2003	Phenolic derivatives	Almeida et al. 2007
pentachlorophenol (PCP) (freq[a] (%); ng/L)	55; 0.5-0.7^d		----------				
Chlorothalonil (freq[a] (%); ng/L)	41; <0.1		----------				
Biphenyl(freq[a] (%); ng/L)	68; <0.1		----------			PAHs	
Naphtalene(freq[a] (%); ng/L)	23; <0.1		----------				
Bisphenol A (freq[a] (%); ng/g, dry wt)	77; 0.1-0.6		----------	Sediments		Phenolic derivatives	
TCP (freq[a] (%); ng/g, dry wt)	100; 0.2-0.4		----------				
2,3,4,5-tetrachlorophenol (TeCP) (freq[a] (%); ng/g, dry wt)	100; 22.2-34.7		----------				
PCP (freq[a] (%); ng/g, dry wt)	92; 51.0-78.6		----------				
Al (%)	0.82 ± 0.06	0.9 ± 0.2	----------	Sediments	September 2005	Metals	Reis et al. 2009
Cr (µg/g)	7 ± 2	9 ± 3	----------				
Cu (µg/g)	8 ± 2	6.7 ± 0.7	----------				
Fe (%)	1.4 ± 0.4	1.6 ± 0.4	----------				
Mn (µg/g)	198 ± 11	149 ± 26	----------				
Ni (µg/g)	3.6 ± 0.4	4 ± 1	----------				
Pb (µg/g)	4.8 ± 0.2	5 ± 2	----------				
Zn (µg/g)	41 ± 6	47 ± 2	----------				
Al (%)	4.63±0.13		----------	Surface Sediments	August 2009	Metals	Mil-Homens et al. 2013b
Li (µg/g)	47.04±1.88		----------				
As (µg/g)	10.72±1.52		----------				
Cr (µg/g)	23.5±1.63		----------				
Cu (µg/g)	5.95±0.70		----------				
Hg (µg/g)	0.01±0.00		----------				
Pb (µg/g)	14.91±0.21		----------				
Sn (µg/g)	4.53±0.12		----------				
Zn (µg/g)	43.86±3.23		----------				

Table I. 3. Concentrations of several groups of contaminants that have been quantified in Louro River, a highly contaminated affluent of Minho River, in different matrices, by chemical analysis in different studies

Contaminants	Louro River		Group of compounds	Matrices	Month	References
	Middle	Downstream				
Cd (mg/Kg)	0.550±0.027	1.09±0.11	Metals	Sediments	May-July 2001	Filgueiras et al. 2004
Cr (mg/Kg)	78.1±2.9	103±4				
Cu (mg/Kg)	56.8±2.5	54.8±1.2				
Ni (mg/Kg)	32.5±2.2	45.6±0.9				
Pb (mg/Kg)	73.3±8.7	65.8±3.1				
SiO2 (%)	61.4±0.2	61.3±0.1				
Al2O3 (%)	18.6 ±0.1	16.1±0.3				
Fe2O3 (%)	5.50±0.06	4.66±0.06				
MnO (mg/Kg)	913±1	827±25				
TiO2 (%)	1.31±0.01	1.53±0.01				
CaO (mg/Kg)	6980±14	1.26±0.04				
MgO (%)	1.58±0.01	1.18±0.08				
Na2O (%)	1.93±0.16	2.90±0.03				
K2O (%)	3.60±0.01	3.44±0.06				
P2O3 (mg/Kg)	1920±184	3995±86				
As (mg/Kg)	4.25±0.12	6.77±1.06	Metals	Insect larvae tissues*	May 2007	Lavilla et al. 2010
Cd (mg/Kg)	0.268±0.013	0.35±0.05				
Cr (mg/Kg)	1.28±0.05	1.90±0.79				
Cu (mg/Kg)	19.8±1.0	27.63±9.29				
Fe (mg/Kg)	1560±70	2286.67±618.64				
Mn (mg/Kg)	169±8	40.57±7.08				
Ni (mg/Kg)	2.77±0.11	3.91±2.73				
Pb (mg/Kg)	1.19±0.05	1.95±0.30				
Zn (mg/Kg)	80±4	112.23±43.24				
αHCH (ng/g)		1.26	Organochlorine Pesticides	Sediments	October 2010	Planelló et al. 2013
βHCH (ng/g)		0.32				
γHCH (ng/g)		<0.10				
δHCH (ng/g)		1.85				
Heptachlor (ng/g)		0.11				
Aldrin (ng/g)		<0.10				
ptachlor epoxide (ng/g)		<0.10				
γ-Chlordane (ng/g)		0.37				
α-Chlordane (ng/g)		0.37				
α-Endosulfan (ng/g)		<0.10				
p.p'-DDE (ng/g)		0.76				
Dieldrin (ng/g)		<0.10				
Endrin (ng/g)		1.18				
β-Endosulfan (ng/g)		18.25				
p.p'DDD (ng/g)		1.7				
ndrin aldehyde (ng/g)		<0.10				
dosulfan sulfate (ng/g)		4.13				
p.p'-DDT (ng/g)		3.21				
Endrin Ketone (ng/g)		6.41				
Methoxychlor (ng/g)		1.16				
Bisphenol A (ng/g)		7.44	Phenolic derivatives			
Noniphenol (ng/g)		0.31				
Ibuprofen (ng/g)		14.41	Pharmaceutical Products			
Diclofenac (ng/g)		0.86				
arbamazepine (ng/g)		0.23				
Atenolol (ng/g)		8.36				
Caffeine (ng/g)		0.5				
Enrofloxacin (ng/g)		<0.10				
Galaxolide (ng/g)		3.02	Fragrances			
Tonalide (ng/g)		6.33				
Cd (mg/Kg)		<0.10	Metals			
Pb (mg/Kg)		5.96				
Cr (mg/Kg)		5.02				
Ni (mg/Kg)		2.99				
Cu (mg/Kg)		3.13				
As (mg/Kg)		3.01				
Hg (mg/Kg)		<0.05				
Mn (mg/Kg)		128.46				
Zn (mg/Kg)		31.47				
Al (mg/g)		6.47				
Fe (mg/g)		8.03				

4. Objectives and outline of the Thesis

The main goal of the present Thesis was to answer the central question: may long-term exposure to pollution restrict the invasive behaviour of the non-indigenous invasive species *C. fluminea* in estuarine freshwater tidal areas? Long-term exposure to pollution may restrict the invasive behaviour of *C. fluminea* populations mainly through an increase of the population mortality rate, a decrease of the population health status with negative effects on its fitness, and/or direct negative effects on reproduction. These potential effects were investigated in the present study through a field approach taking advantage of *C. fluminea* populations of the tidal freshwater areas (TFA) of Minho (M-est) and Lima (L-est) Rivers (NW Iberian Peninsula). Such populations were selected because they have been showing a distinct invasive behaviour (Sousa *et al.* 2006b, 2007a), and M-est and L-est are neighbour estuaries having several comparable hydromorphological characteristics but also some differences including in environmental factors and chemical contamination (Cairrão *et al.*, 2004; Guimarães *et al.*, 2012; INAG, 2000; Reis *et al.*, 2009; Sousa *et al.*, 2006a).

To attain the central goal of this study, the following specific questions (SQ) that correspond to the specific objectives of the Thesis were investigated:

SQ1 – May summer environmental conditions influence the health status of *C. fluminea* potentially contributing to the differences related with the summer mortality syndrome observed between M-est and L-est populations?

SQ2 – Do metals concentration increase *C. fluminea* stress levels contributing to the invasive behaviour differences observed between M-est and L-est populations?

SQ3 – Do *C. fluminea* populations from M-est and L-est have differences in their gonadal development cycle possibly contributing to the differences observed between their invasive behaviour?

The present Thesis is organized in six Chapters: *Chapter I*, corresponding to the general introduction; *Chapters II to IV*, corresponding to the presentation and discussion of the experimental work carried out to answer SQ1 to SQ3; *Chapter V*, corresponding to the general discussion and main conclusions; and *Chapter VI* corresponding to the list of references cited.

In the General Introduction (*Chapter I*) the paradigm of biological invasions is introduced, a min-review covering several aspects related with the invasions by *C. fluminea* is made, and the objectives and the outline of the Thesis are presented.

The main objective of *Chapter II*, entitled "*Integrated biomarker responses of the invasive species Corbicula fluminea in relation to environmental abiotic conditions: a potential indicator of the likelihood of clam's summer mortality syndrome*", was to answer SQ1. The rational for SQ1 was that summer massive mortality events might influence the invasive behaviour of *C. fluminea* by several ways. For example, they might reduce the population density by causing stress inducing high mortality. Thus, considering that massive mortality events were reported for the M-est *C. fluminea* population but not for the L-est one, the investigation of biological and abiotic factors potentially leading to them may indicate if they are contributing to the distinct invasive behaviour of *C. fluminea* in the two estuaries.

The main objective of *Chapter III*, entitled "*Factors influencing the accumulation of metals by Corbicula fluminea and its health status in estuarine tidal freshwater areas"*, was to answer SQ2. The hypothesis behind SQ2 was that relatively high concentrations of metals and other environmental contaminants are decreasing the health status of the L-est *C. fluminea*

population and because of this the population has a limited invasive behaviour.

The main objective of *Chapter IV*, entitled "*Histological study of the gonadal development cycle of Corbicula fluminea and its relationship with spatial and temporal variation of environmental parameters*" was to answer SQ4. The rationale behind the question was: a sucesseful reproduction is crucial for population growth rate and thus for the invasive behaviour of NIS; the gonadal developmental cycle is determinant for reproduction and it may be affected by pollution and other adverse environmental conditions; therefore, potential differences in the gonadal developmental cycle between M-est and L-est *C. fluminea* populations may be contributing for their distinct invasive behaviours.

General Discussion and Final Conclusions (*Chapter V*), comprises an integrative discussion of the previous chapters in a global perspective, focusing the main contributions and some of the remaining challenges in this research field.

Chapter II

Integrated biomarker responses of the invasive species *Corbicula fluminea* in relation to environmental abiotic conditions: a potential indicator of the likelihood of clam's summer mortality syndrome

This Chapter is published in the form of a scientific article: Oliveira, C., Vilares, P., Guilhermino, L. (2015). Integrated biomarker responses of the invasive species Corbicula fluminea in relation to environmental abiotic conditions: a potential indicator of the likelihood of clam's summer mortality syndrome. *Comparative Biochemistry and Physiology Part A 182*, 27-37.

Abstract

The goal of the present study was to investigate the variation of a set of biomarkers in wild populations of *C. fluminea* in relation to abiotic conditions changes and to identify environmental factors associated with increased stress in this species potentially leading to massive mortality events. The study was carried out from July to October in the TFAs of the estuaries of Minho and Lima Rivers (NW Iberian Peninsula). Monthly, 7 biomarkers (biotransformation, energy production, anti-oxidant defences and lipid peroxidation damage) were determined in *C. fluminea* and 17 abiotic parameters were determined in water or sediments in 5 sampling sites: M1, M2 and M3 in the M-est (up => downstream); L in the L-est. The results of biomarkers were integrated using the Integrated Biomarker Responses' Index (IBR), and also analysed in relation to environmental parameters by Redundancy Analysis (RDA). Overall, the findings of the present study indicate that July/August are particularly stressful months for the studied *C. fluminea* populations, especially at downstream sites; the increase of nutrients and ammonium water concentrations, water temperature and conductivity were major contributors for this increased stress; the biomarkers indicated that in July/August *C. fluminea* is exposed to oxidative stress inducers, environmental chemical contaminants biotransformed by esterases and glutathione S-transferase enzymes, and that organisms need additional energy to cope with the chemical and/or thermally-induced stress. The

findings of the present study stress the importance of biomonitoring the health condition of *C. fluminea* because it may allow determining the likelihood of summer/post summer mortality syndrome in this species.

Key words: invasive species; biomarkers; bivalve massive mortality; summer; abiotic variation.

1. Introduction

The summer/post-summer mortality syndrome in bivalves is a periodical event in temperate regions that may have significant adverse ecological and economic impacts (Ilarri *et al.*, 2010; Mouthon and Daufresne, 2006; Tomaru *et al.*, 2001; Vohmann *et al.*, 2009; Weitere *et al.*, 2009; Werner and Rothhaupt, 2008). In wild ecosystems, the sudden input of high amounts of organic matter from dead bivalves and their degradation often leads to considerable changes in nutrient cycles, energy fluxes, a deep reduction of water dissolved oxygen and massive mortalities of several other species, with important changes in ecosystem functioning and potential biodiversity losses (Baur and Schmidlin, 2007; Cooper *et al.*, 2005; Sousa *et al.*, 2012, 2008b,c). The dimension and severity of such events are especially high if the primary affected bivalve is a keystone species of the ecosystem and/or if it has a considerable biomass (Power *et al.*, 1996). Furthermore, in ecosystems invaded by non-native invasive species (NIS), massive mortality events may have a decisive influence on the competition between the bioinvasor and its native competitors, often acting in favour of the NIS. This is believed to occur because NIS generally recovers faster from such adverse events than their native competitors (McMahon, 2002).

Several factors and conditions have been pointed as possible causes for the summer/post-summer mortality syndrome in bivalves, including extreme events such as draughts, temperature increase, decrease of oxygen concentration in the water, increase of pollution, post-spawning stress among several others (Cooper *et al.*, 2005; Cotter *et al.*, 2010; Dégremont *et al.*, 2007; García-esquivel *et al.*, 2001; Huvet *et al.*, 2010; Ilarri *et al.*, 2010; Morgan *et al.* 2003; Mouthon and Daufresne 2006; Tremblay *et al.*, 1998; Urrutia *et al.*, 1999; Vohmann *et al.*, 2009; Weitere *et al.*, 2009). However, the phenomenon is not yet completely understood (Rosa *et al.*, 2011; Vohmann *et al.*, 2009) and more research is needed.

The goal of the present study was to investigate the variation of a set of biomarkers in wild populations of *C. fluminea* in relation to abiotic conditions changes to identify environmental factors associated with increased stress in this species potentially leading to massive mortality events. This species was

selected for this study mainly because it is one of the 100 worst invasive species in Europe (DAISIE, 2014), massive mortality events in their populations have been having considerable economic and ecological negative impacts (Cherry *et al.*, 2005; Cooper *et al.*, 2005; Ilarri *et al.*, 2010; Sousa *et al.*, 2008b, 2011), and these events have being pointed as decisive contributors to the decline of several native bivalve competitors.

2. Material and Methods

2.1. Chemicals

All the chemicals used were of analytical grade and purchased from Sigma-Aldrich (Germany), Merck (Germany) or Bio-Rad (Germany).

2.2. Sampling estuaries, sites and *C. fluminea* populations

The populations of the tidal freshwater area (TFA) of two neighbour estuaries, those of Minho and Lima Rivers (NW Iberian coast), were selected for this study because summer massive mortality events have been reported for the former (Ilarri *et al.*, 2010; Sousa *et al.*, 2007a) but not for the latter. *C. fluminea* is present in the Minho estuary (M-est) at least since 1989 (Araújo *et al.*, 1993). At the present, the population is spread over all the TFA and has a very high density (more than 4000 individuals per m^2 in some sites) and biomass (more than 400g of AFDW per m^2) (Sousa *et al.*, 2005). In the TFA of the M-est, 3 sampling sites were selected (Figure II. 1) based on Sousa *et al.* (2008d) identification of three main areas with distinct environmental characteristics (upstream to downstream): M1 (N42º03'09.37'' W8º33'42.73''), a relatively low impacted area with low organic matter (OM) and environmental contamination, and residual tidal influence; M2 (N42º01'25.12'' W8º39'24.49''), located about 3 Km (downstream) far from the mouth of the Louro River, a tributary of the Minho River that is one of the most contaminated rivers in Galicia (Concha-Graña *et al.*, 2006; Farkas *et al.*, 2007; Filgueiras *et al.*, 2004; Lavilla *et al.*, 2010), with a high content of OM; and M3 (N41º54'41.25'' W8º47'36.59''), with a greater tidal influence. *C.*

fluminea was recorded for the first time in the L-est in 2002 and has a relatively low density in the TFA (mean of 60 individuals per m2 and 26 g AFDW per m^2) (Sousa *et al.*, 2006 a,b). This estuary has been considered more contaminated than the M-est, with impacts from a paper mill industry, urban settlement, agriculture crops, and harbour activities (Cairrão *et al.*, 2004; Guimarães *et al.*, 2012; INAG, 2000; Reis *et al.*, 2009; Sousa *et al.*, 2006a). Due to the low density of *C. fluminea* in the TFA, only one sampling site was selected (Figure II. 1), hereafter indicated as L, in a restricted area about 15 km upstream from the estuary mouth (N41º42'07.03'' W8º44'37.05'').

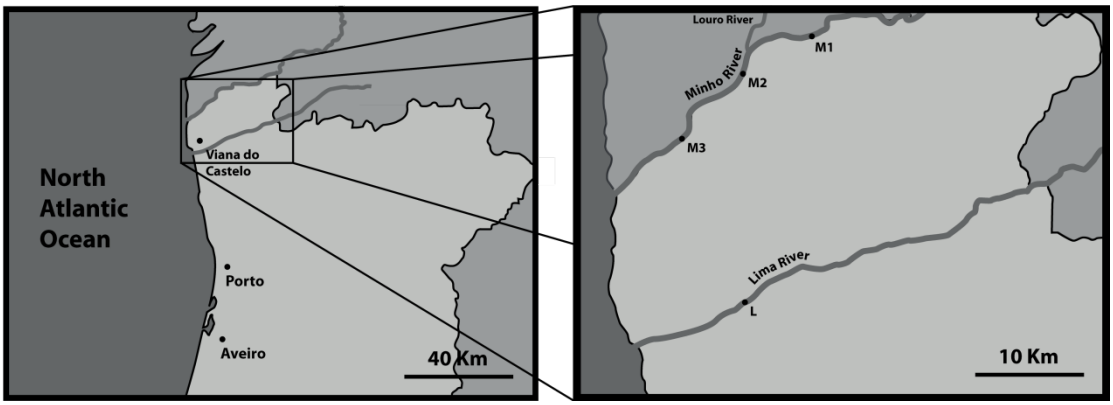


Figure II. 1. Map of the estuaries of Minho and Lima Rivers showing the four sampling sites location.

2.3. *C. fluminea* sampling

C. fluminea specimens were collected monthly from July to October 2011 in the subtidal area (about 40 – 90 cm deep), at low tide using a hand rake with a net. Clams were transported to the laboratory within 2h after their collection, being maintained in water from the local where they were collected in thermally isolated boxes regularly open to allow water oxygenation. These conditions were found adequate for the transport of the clams and did not influence significantly the biomarkers' determinations in preliminary studies (data not shown).

2.4. Abiotic parameters determination

Monthly, at each sampling site and simultaneously to *C. fluminea* collection, water temperature, conductivity, dissolved oxygen and pH were measured in triplicate using a multi-parameter probe (WTW 340i). In addition, water samples were collected, in triplicate, and maintained in appropriate bottles on ice until arrival to the laboratory where the following parameters were determined using colorimetric methods following the Photometer Systems for Water Analysis protocols (Palintest 7000 interface photometer): hardness ($CaCO_3$), turbidity (Turb) and the concentration of ammonium (NH_4^+), nitrates (NO_3^-), nitrites (NO_2^-), phosphates (PO_4^-), iron (Fe), phenol (C_6H_5OH), and silica (SiO_2).

Sediment samples were collected in triplicate with a 100 ml syringe to a 50 ml polyethylene centrifuge tube. Thirty ml of acetone (90%) and 0.2 ml of magnesium carbonate (1 g 100 ml ultra-pure water) were added to each tube, and the mixture was shaked and maintained on ice in a thermally isolated box until arrival to the laboratory. Once in the laboratory, samples were kept at 4ºC for 24 h. After 24h, samples were centrifuged (in a Kubota 5400 centrifuge) for 3 min at 2330 g, mixed and centrifuged again for 10 min in similar conditions; 3 ml of each sample supernatant were carefully collected, put in a glass spectrophotometer cuvette and its absorbance was read in a spectrophotometer (Jenway 6405 UV/VIS) at 480, 630, 645, 647 664, 665 and 750 nm; after addition of 0.2 ml of a HCl 1 N solution directly to the cuvette, agitation and 4 min waiting, the absorbance was read again at 665 and 750 nm; the concentrations of chlorophyll *a*, chlorophyll *b* and chlorophyll *c* were calculated according to Jeffrey and Humphrey (1975).

The quantification of organic matter was obtained after combusting for 24h at 550°C in a muffle furnace (Fisher Scientific, Isotemp Muffle Furnace). Values were expressed in percentage relatively to the weight loss on ignition of each sample analysed.

2.5. Determination of biomarkers

In the laboratory, the anterior-posterior shell length of the clams was measured with a calliper (0-150 mm). Animals (20) with a shell length between 28 and 31 mm were selected for the study. Clams were open immediately after being measured and gills and foot tissues were isolated on ice for biomarkers' determination.

The biomarkers included in this study were: the activity of esterases (EST) and glutathione-S transferases (GST) that are important enzymes involved in the biotransformation of several environmental contaminants; the activity of octopine dehydrogenase (ODH) and isocitrate dehydrogenase (IDH) that are important enzymes of the anaerobic and aerobic pathways of energy production in molluscs; the activity of the anti-oxidant enzymes catalase (CAT) and glutathione reductase (GR) (GST is also involved in this system), and lipid peroxidation levels (LPO) as indicative of oxidative stress and damage.

Foot tissue was used for the determination of EST, IDH and ODH activities; gill tissue was used for LPO levels, and GST, CAT and GR activity determinations. Biomarkers were determined individually per animal. A piece of muscle tissue was put in 1 ml of cold K-phosphate buffer (pH = 7.2; 0.1 M), homogenized for 1 min on ice (Ystral GmbH d-7801 Dottingen homogenizer) and centrifuged (in a Sigma, 3K30 centrifuge) at 3300 g for 3 min at 4°C; the supernatant was recovered and its protein content was determined by the Bradford method (Bradford, 1976) adapted to microplate (Frasco and Guilhermino, 2002), using bovine γ-globulin as protein standard and absorbance readings at 600 nm (in a Spectra Max M2e spectrophotometer). After standardization of the protein content to 1 mg/ml, the supernatant was used to determine the activity of EST activity following the production of thiocholine as acetylthiocholine is hydrolysed at 412 nm, by the Ellman's method (Ellman *et al.* 1961), adapted to microplate (Guilhermino *et al.*, 1996). Because preliminary results on the characterization of the esterase enzymes present in *C. fluminea* foot tissue suggest that non-specific esterases may be responsible for a considerable degradation of the substrate acetylthiocholine

used in the Ellman's technique (data not shown), EST activity will be used to indicate the overall esterase activityresponsible for acetylthiocholine degradation present in this tissue.

Another piece of foot tissue was placed in 1 ml of K-phosphate buffer tris(hydroxymethyl)-aminomethan buffer (pH = 7.8; 0.5 M), homogenized for 1 min on ice (Ystral GmbH d-7801 Dottingen homogenizer) and centrifuged (in a Sigma, 3K30 centrifuge) at 3300 g for 3 min at 4 °C; the supernatant was recovered and used to determine IDH activity following the increase in NADPH at 340 nm (in a Spectra Max M2e spectrophotometer), according to Ellis and Goldberg (1971) adapted to microplate (Lima *et al.*, 2007), after standardization of the protein content of each sample to 1 mg/ml as explained above.

The last piece of muscle tissue was placed in 1 ml tris buffer (pH = 7.5; 0.2 M), with 0.1 M ethylenediaminetetraacetic acid (EDTA) and 0.1 M DL-dithiothreitol (DTT) homogenized for 5 s on ice (Ystral GmbH d-7801 Dottingen homogenizer) and centrifuged (in a Sigma, 3K30 centrifuge) at 3300 g for 3 min at 4 °C; the supernatant was recovered and used to determine ODH activity by the measure of the amount of pyruvate consumed due to NADH oxidation at 340 nm (in a Spectra Max M2e spectrophotometer) according to Livingstone *et al.* (1990), after standardization of the protein content of each sample to 1 mg/ml as explained above.

The gills were isolated on ice and homogenized (1:10 g wt/v) in K-phosphate buffer (pH = 7.4; 0.1M). Part of the homogenate was used to determine the amount of endogenous LPO (in a Spectra Max M2e spectrophotometer) by measuring the thiobarbituric acid reactive substances at 535 nm, according to Ohkawa (1979) and Bird and Draper (1984), preventing lipid oxidation by adding 0.2 mM butylhydroxytoluene (BHT) (Torres *et al.*, 2002). The remaining gills homogenate was centrifuged (in a Sigma, 3K30 centrifuge) for 20 min at 10000 g (4ºC), to obtain the post-mitochondrial supernatant used for the determination of GST, CAT and GR activities, after standardization of the protein content of each sample to 4 mg/ml as explained above. GST activity was determined (in a Spectra Max M2e spectrophotometer) measuring the conjugation of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm (Habig *et al.* 1974)

adapted to microplate (Frasco and Guilhermino, 2002). CAT activity was determined by measuring the H_2O_2 consumption at 240nm (in a Spectra Max M2e spectrophotometer) according to Clairborne (1985). GR activity was measured by following the decrease of NADPH levels, at 340 nm (in a Spectra Max M2e spectrophotometer), according to Cribb *et al.* (1989).

The protein content of each sample used for enzymatic analysis was checked again at the end of enzymatic determinations in triplicate and the mean of these determinations was used to express the enzymatic activities per mg of protein.

All biochemical analyses were performed at a constant temperature of 25ºC. All enzymatic activities expressed as nmol/min/mg protein, except catalase, which was expressed as µmol min/mg protein.

2.6. Data analysis

2.6.1. Analysis of individual parameters

For each biological and abiotic parameter, measurements or determinations corresponding to different sites and months were checked for normality of distribution using the Shapiro-Wilk test and for homogeneity of variance using the Levene's test. When required, appropriate data transformations were made to achieve the assumptions of the Analysis of Variance (ANOVA). Then, a two-way ANOVA (main factors: site and month) with interaction was used to compare data of each parameter collected in different sites and months. The Statistics 18.0 package was used and the significance level was 0.05.

2.6.2. Integrated biomarker responses

The integration of the information given by the different biomarkers was performed applying the Integrated Biomarker Response index (IBR) described by Beliaeff and Burgeot (2002). Briefly, for each parameter, the mean and standard deviation (SD) per site and month were calculated; then, a standardization of mean values was made using the formula $y = (x - m) / SD$,

where *y* is the standardized value of the biomarker for each sampling site and month; *x* is the mean value of the biomarker per month at each sampling site; *m* is the mean of the biomarker calculated for all the sampling sites and months, and *SD* is the standard deviation calculated per month and sampling site; using standardized data, *Z* is computed as equal to *y* or –*y* depending if the biological effect of the correspondent biomarker is activation or inhibition, respectively. The minimum value for each biomarker (min) was determined and its absolute value was added to *Z* values. Finally, the index calculation is based in the triangular areas of star plots. The area (A) connecting two consecutive coordinates was calculated for each biomarker result in star plots, being B_i and B_{i+1} two consecutive biomarker scores and *n* the number of biomarkers considered:

Where:

$$A = \frac{B_i}{2} \sin\beta \left(B_i \cos\beta + B_{i+1} \sin\beta\right)$$

$$\beta = \arctan\left(\frac{B_{i+1} \sin\alpha}{B_i - B_{i+1} \cos\alpha}\right) \quad \text{and} \quad \alpha = \frac{2\pi}{n}$$

The IBR was then calculated through the sum of the star plots triangular areas represented by two consecutive biomarker scores for a given sampling site and month. Biomarkers used for the IBR calculation were ranged clockwise as follows: LPO, GST, CAT, GR, ODH, IDH and EST. The higher the IBR value more stressed is the organism (Beliaeff and Burgeot, 2002).

2.6.3. Relating biological and environmental parameters

The integrated analysis of biological and environmental parameters data was carried out through a Redundancy Analysis (RDA), using model-based type of Monte Carlo permutation test (ter Braak and Prentice 1986; ter Braak and Šmilauer, 2002). Quantitative environmental variables were: water temperature, dissolved oxygen, conductivity, pH, hardness, turbidity, ammonium, nitrates, nitrites, phosphates, iron, phenol and silica; and sediment chlorophyll *a*, *b* and *c* and organic matter. Multivariate analyses were performed with Canoco for Windows 4.5.

3. Results

3.1. Environmental parameters

The environmental parameters measured monthly in the water and sediments of the 4 sites are indicated in Tables II. 1 and 2, respectively. The comparison of the mean values (Table II. 3) indicated: significant differences among sites in all the parameters except in water phenol and turbidity, and in chlorophyll *b* and *c* in sediments; significant differences among months were also found in all the parameters, except turbidity; the interaction between the two factors (site and month) was also significant for all the parameters except water phenol and sediment chlorophylls a and c. In the M-est (Table II. 3), the mean of water temperature increases from upstream (M1) to downstream (M3), and L had a mean of water temperature significantly different from all the other sites being in about the middle range of water temperatures recorded. The mean of water dissolved oxygen; pH, nitrates and silica were significantly higher in M1 than in all the other sites. The highest mean values of water conductivity and hardness, and chlorophyll a in sediments were recorded in M3 and L. The mean of nitrites in the water was significantly higher in all the M-est sites than in L-est site. The highest mean concentration of ammonium in the water was found in M2, whereas the highest mean concentrations of phosphate and iron in the water were found in M3, and the highest means of organic matter in sediments were recorded in M2 and M3. The highest mean of water temperature was recorded in August and the lowest in October, while the corresponding values for water dissolved oxygen were found in July and August/September, respectively. Regarding the nutrients, the highest mean of nitrates, nitrites and ammonium were found in July, whereas the highest mean of phosphates was observed in August. The mean of sediment organic matter increased from July to August and remained high in September and October. The lowest means of sediment chlorophylls a, b and c were found in July and the highest ones in September and/or October.

Table II. 1. Abiotic water variables values determined for each sampling site and month in the tidal freshwater area (TFA) of M-est and L-est, from July to October 2011. Values are mean±standard error of temperature (T, ºC), dissolved oxygen (DO, mg/L), conductivity (Cond, µS/cm), pH (pH units), nitrites (NO_2^-, mg/L), nitrates (NO_3^-, mg/L), ammonium (NH_4^+, mg/L), phosphates (PO_4^{3-}, mg/L), silica (SiO_2, mg/L), phenol (C_6H_5OH, mg/L), iron (Fe, mg/L), hardness ($CaCO_3$, mg/L) and turbidity (Turb, FTU).

Sampling site	Month	Abiotic water variables												
		T	DO	Cond	pH	NO_2^-	NO_3^-	NH_4^+	PO_4^{3-}	SiO_2	C_6H_5OH	Fe	$CaCO_3$	Turb
M1	July	18.27±0.09	9.17±0.22	14.43±0.03	7.28±0.02	0.13±0.00	4.48±0.07	0.04±0.01	0.01±0.00	6.32±0.21	0.07±0.01	0.03±0.00	27.22±1.11	0.00±0.00
	August	25.27±0.12	10.11±0.12	88.23±2.40	7.77±0.10	0.05±0.02	0.41±0.03	0.29±0.10	0.09±0.01	6.02±0.74	0.07±0.01	0.04±0.01	36.11±0.56	3.33±2.69
	September	19.57±0.15	8.47±0.03	79.67±0.33	7.28±0.07	0.01±0.00	0.65±0.06	0.14±0.02	0.11±0.01	6.96±0.38	0.07±0.01	0.02±0.00	33.33±2.55	7.33±0.38
	October	19.03±0.27	10.21±1.79	85.67±0.12	9.90±0.12	0.00±0.00	0.40±0.05	0.13±0.06	0.04±0.01	7.24±0.10	0.06±0.00	0.02±0.01	31.67±0.96	6.67±3.42
M2	July	22.33±0.03	10.10±0.22	17.40±0.02	7.33±0.02	0.13±0.00	1.96±0.09	0.94±0.06	0.01±0.00	1.38±0.06	0.08±0.00	0.08±0.01	40.00±2.54	8.00±2.00
	August	25.73±0.24	8.01±0.04	34.00±1.36	7.69±0.12	0.01±0.00	0.56±0.04	0.13±0.01	0.15±0.02	0.98±0.03	0.07±0.00	0.05±0.01	42.22±0.56	5.78±0.22
	September	22.50±0.17	8.55±0.05	117.33±0.33	7.46±0.02	0.01±0.00	0.60±0.02	0.09±0.01	0.09±0.01	1.98±0.05	0.11±0.01	0.05±0.02	43.89±2.22	3.56±1.74
	October	20.50±0.23	8.35±0.05	122.67±0.33	7.47±0.02	0.01±0.00	0.26±0.02	0.08±0.05	0.02±0.01	1.65±0.06	0.09±0.02	0.03±0.01	54.44±2.42	6.67±2.69
M3	July	23.50±0.00	9.10±0.04	37.67±1.45	6.96±0.03	0.12±0.03	2.16±0.10	0.33±0.09	0.00±0.00	1.04±0.01	0.04±0.02	0.04±0.00	263.33±14.24	3.11±1.94
	August	25.87±0.12	8.25±0.03	29.4±2.56	7.49±0.04	0.00±0.00	0.61±0.04	0.08±0.02	0.60±0.05	1.15±0.04	0.08±0.02	0.05±0.02	257.22±3.09	12.67±1.39
	September	23.20±0.31	8.22±0.18	1477.00±3.51	7.82±0.04	0.01±0.00	0.41±0.00	0.17±0.03	0.16±0.03	2.16±0.01	0.10±0.01	0.16±0.05	228.89±10.82	5.33±1.54
	October	20.87±0.09	9.67±0.07	987.00±7.21	7.93±0.05	0.01±0.00	0.19±0.02	0.14±0.08	0.11±0.03	2.09±0.03	0.06±0.01	0.02±0.00	155.00±8.66	1.33±0.67
L	July	21.33±0.19	10.52±0.04	32.00±0.58	7.49±0.04	0.00±0.00	1.84±0.07	0.31±0.05	0.05±0.01	2.17±0.16	0.07±0.01	0.10±0.02	21.11±1.11	6.67±2.52
	August	26.13±0.15	7.84±0.04	29.30±3.37	7.46±0.06	0.00±0.00	0.72±0.06	0.08±0.01	0.05±0.01	4.28±0.12	0.08±0.02	0.03±0.00	132.78±4.75	2.89±1.35
	September	22.20±0.15	8.53±0.00	1566.67±0.88	7.26±0.12	0.00±0.00	0.49±0.07	0.12±0.04	0.06±0.01	2.51±0.10	0.09±0.01	0.04±0.01	149.44±10.56	1.33±1.33
	October	19.13±0.12	8.25±0.23	824.33±28.59	8.08±0.15	0.00±0.00	0.21±0.01	0.22±0.06	0.07±0.01	2.13±0.08	0.09±0.01	0.03±0.00	90.56±5.47	5.33±2.40

Table II. 2. Sediments chlorophyll a,b and c values and organic matter percentage for each sampling site and month in the tidal freshwater area of M-est and L-est, from July to October 2011. Values are mean±standard error of chlorophyll a, b and c (Chl a, Chl b and Chl c, respectively, units are µg/L), very coarse sand (VCS, %), coarse sand (CS, %), medium sand (MS, %), fine sand (FS, %), very fine sand (VFS %) and silt + clay (S+C, %) for granulometry and organic matter (%).

Sampling site	Month	Sediment chlorophyll (µg/L)			Organic matter (%)
		Chl *a*	Chl *b*	Chl *c*	
M1	July	1.20±0.15	0.84±0.03	2.04±0.07	0.99±0.23
	August	1.88±0.58	1.06±0.18	2.13±0.14	1.40±0.40
	September	1.85±0.30	0.95±0.04	2.09±0.09	0.86±0.07
	October	2.16±0.36	1.39±015	2.41±0.19	1.60±0.09
M2	July	0.75±0.01	0.69±0.01	1.85±0.02	1.35±0.17
	August	0.91±0.05	0.77±0.00	1.98±0.05	2.40±0.16
	September	2.11±0.26	1.18±0.11	2.25±0.11	2.75±0.74
	October	1.31±0.18	0.98±0.12	2.15±0.09	4.58±0.82
M3	July	1.32±0.10	0.79±0.02	1.95±0.03	0.72±0.05
	August	1.78±0.25	0.97±0.06	2.25±0.06	3.74±0.32
	September	3.14±0.54	1.20±0.10	2.64±0.09	4.65±0.45
	October	1.77±0.28	0.87±0.08	2.24±0.14	0.73±0.05
L	July	2.46±0.58	0.87±0.06	2.10±0.11	1.76±0.12
	August	2.03±0.40	0.99±0.16	2.55±0.45	1.62±0.06
	September	1.93±0.64	0.82±0.08	2.12±0.09	1.44±0.08
	October	3.08±0.58	0.96±0.04	2.58±0.27	1.39±0.03

September and October showed the highest means of water conductivity and silica. The highest mean of water pH was found in October and the lowest in July. The highest values of water iron, phenol and hardness were found in July/September, September and August/September, respectively.

3.2. *C. fluminea* biomarkers variation

Biomarker responses are given in Figure II. 2. Significant differences among the means calculated for different months and sites were found for all the parameters, except GST and EST activities among sites (Table II. 4); a significant interaction between months and sites was also found, except in the CAT activity (Table II. 4).

Table II. 3. Results of the one-way analysis of variance (one-way ANOVA) and post-hoc test (Tukey) of abiotic water and sediment variables performed to investigate significant differences among sampling sites and among months. Small letters represents significant differences between sampling sites and capital letters represents significant differences between sampling months. Temperature (T), dissolved oxygen (DO), conductivity (Cond), pH (pH), nitrites (NO_2^-), nitrates (NO_3^-), ammonium (NH_4^+), phosphates (PO_4^{3-}), silica (SiO_2), phenol (C_6H_5OH), iron (Fe), hardness ($CaCO_3$), turbidity (Turb) chlorophylls a, b and c (Chl a, Chl b and Chl c, respectively) and organic matter (OM).

Parameters	Factor	df	F	p	Tukey groups			
					M1 July	M2 August	M3 September	L October
T	site	3	210.999	<0.001	20.53^{a}	22.77^{c}	23.36^{d}	22.20^{b}
	month	3	892.900	<0.001	21.36^{B}	25.75^{D}	21.87^{C}	19.88^{A}
	site x month	9	31.461	<0.001				
DO	Site	3	12.543	<0.001	9.49^{b}	8.75^{a}	8.81^{a}	8.78^{a}
	Month	3	34.832	<0.001	9.72^{C}	8.55^{A}	8.44^{A}	9.12^{B}
	site x month	9	17.202	<0.001				
Cond	Site	3	7150.701	<0.001	67.00^{a}	72.85^{a}	632.77^{c}	613.08^{b}
	Month	3	10140.528	<0.001	25.38^{A}	45.23^{B}	810.17^{D}	504.92^{C}
	site x month	9	2854.141	<0.001				
pH	Site	3	46.174	<0.001	8.06^{b}	7.49^{a}	7.55^{a}	7.57^{a}
	Month	3	148.979	<0.001	7.27^{A}	7.60^{C}	7.45^{B}	8.35^{D}
	site x month	9	55.685	<0.001				
NO^{2-}	Site	3	21.653	<0.001	0.05^{b}	0.04^{b}	0.03^{b}	0.00^{a}
	Month	3	93.474	<0.001	0.09^{B}	0.01^{A}	0.01^{A}	0.01^{A}
	site x month	9	12.196	<0.001				
NO^{3-}	Site	3	148.372	<0.001	1.49^{b}	0.84^{a}	0.84^{a}	0.81^{a}
	Month	3	1625.051	<0.001	2.61^{C}	0.58^{B}	0.54^{B}	0.26^{B}
	site x month	9	136.232	<0.001				
NH_4^+	Site	3	7.923	<0.001	0.15^{a}	0.31^{b}	0.18^{a}	0.18^{a}
	Month	3	27.217	<0.001	0.41^{B}	0.15^{A}	0.13^{A}	0.14^{A}
	site x month	9	17.529	<0.001				
PO_4	Site	3	71.300	<0.001	0.06^{a}	0.07^{a}	0.22^{b}	0.06^{a}
	Month	3	89.124	<0.001	0.02^{A}	0.22^{D}	0.11^{C}	0.06^{B}
	site x month	9	43.242	<0.001				
SiO_2	Site	3	458.692	<0.001	6.64^{c}	1.50^{a}	1.61^{a}	2.77^{b}
	Month	3	6.811	0.001	2.73^{A}	3.11^{AB}	3.40^{B}	3.28^{B}
	site x month	9	10.219	<0.001				
C_6H_5OH	Site	3	2.710	0.061	0.07^{a}	0.09^{a}	0.07^{a}	0.08^{a}
	Month	3	3.960	0.017	0.07^{A}	0.08^{AB}	0.09^{B}	0.07^{AB}
	site x month	9	1.695	0.131				
Fe	Site	3	3.933	0.017	0.03^{a}	0.05^{ab}	0.07^{b}	0.05^{ab}
	Month	3	5.508	0.004	0.06^{B}	0.04^{AB}	0.07^{B}	0.03^{A}
	site x month	9	4.975	<0.001				
$CaCO_3$	Site	3	842.181	<0.001	32.08^{a}	45.14^{b}	226.11^{d}	98.47^{c}
	Month	3	32.995	<0.001	87.92^{A}	117.08^{B}	113.89^{B}	82.92^{A}
	site x month	9	40.919	<0.001				
Turb	Site	3	1.021	0.396	4.33^{a}	6.00^{a}	5.61^{a}	4.06^{a}
	Month	3	0.770	0.520	4.45^{A}	6.17^{A}	4.39^{A}	5.00^{A}
	site x month	9	4.022	0.002				
Chl *a*	site	3	5.758	0.003	1.78^{ab}	1.27^{a}	2.00^{b}	2.38^{b}
	month	3	3.853	0.019	1.44^{A}	1.65^{AB}	2.26^{B}	2.08^{AB}
	site x month	9	1.732	0.125				
Chl *b*	Site	3	2.427	0.085	1.07^{a}	0.90^{a}	0.96^{a}	0.91^{a}
	Month	3	5.802	0.003	0.80^{A}	0.95^{AB}	1.04^{B}	1.05^{B}
	site x month	9	2.984	0.012				
Chl *c*	Site	3	2.094	0.122	2.17^{a}	2.06^{a}	2.27^{a}	2.33^{a}
	Month	3	3.487	0.028	1.99^{A}	2.23^{AB}	2.27^{AB}	2.34^{B}
	site x month	9	1.199	0.332				
Organic matter	Site	3	18.991	<0.001	1.21^{a}	2.77^{b}	2.46^{b}	1.55^{a}
	Month	3	10.575	<0.001	1.20^{A}	2.29^{B}	2.43^{B}	2.07^{B}
	site x month	9	14.322	<0.001				

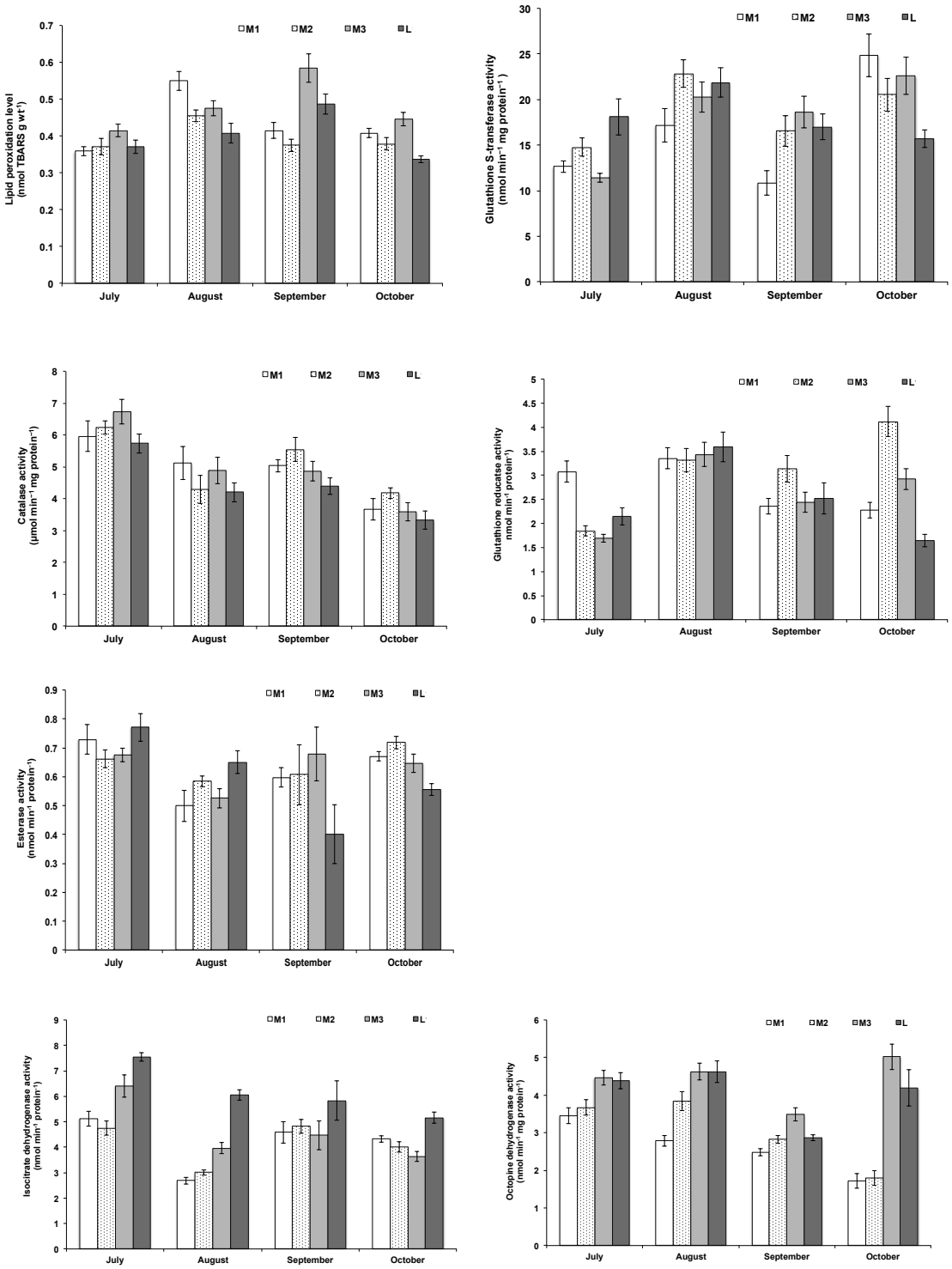


Figure II. 2. Gills lipid peroxidation (LPO) levels, and glutathione S-transferase (GST), catalase (CAT) and glutathione reductase (GR) activities and foot muscle esterase (EST), isocitrate dehydrogenase (IDH) and octopine dehydrogenase (ODH) activities determined for each sampling site and month in the tidal freshwater area of M-est and L-est, from July to October 2011. Values are the mean of 20 clams per sampling site per month with the corresponding standard error bars.

Comparing the mean of biomarkers among sampling months, the highest LPO levels were found in August/September and the lowest in July/October. The highest mean of CAT, IDH, ODH and EST activities were determined in July, and the lowest in October, August, September/October and August/September, respectively. The highest GR activity was found in August and the lowest in July. Clams from M3 had the highest means of LPO levels and ODH activity, while those from M2 had the highest GR activity. The L-est site had the lowest levels of LPO, CAT and GR, and the highest IDH activity.

Table II. 4. Results of the two-way analysis of variance (one-way ANOVA) and post-hoc test (Tukey) of biomarkers values performed to investigate significant differences among sampling sites, among months, and the respective interaction. Small letters represents significant differences between sampling sites and capital letters represents significant differences between sampling months. Lipid peroxidation (LPO), glutathione S-transferase (GST), catalase (CAT), glutathione reductase (GR), isocitrate dehydrogenase (IDH), octopine dehydrogenase (ODH) and esterase (EST).

Parameters	Factor	df	F	p	Tukey groups			
					M1	M2	M3	L
					July	August	September	October
LPO	site	3	12.716	<0.001	0.43^{a}	0.39^{a}	0.48^{b}	0.40^{a}
	month	3	18.195	<0.001	0.38^{A}	0.47^{B}	0.47^{B}	0.39^{A}
	site x month	9	6.063	<0.001				
GST	Site	3	0.953	0.416	16.38^{a}	18.68^{a}	18.22^{a}	18.17^{a}
	Month	3	12.837	<0.001	14.24^{A}	20.53^{B}	15.76^{A}	20.92^{B}
	site x month	9	4.151	<0.001				
CAT	Site	3	2.323	0.075	4.94^{b}	5.06^{b}	5.02^{b}	4.42^{a}
	Month	3	30.640	<0.001	6.16^{C}	4.63^{B}	4.96^{B}	3.69^{A}
	site x month	9	1.026	0.419				
GR	Site	3	5.147	0.002	2.76^{ab}	3.10^{b}	2.62^{a}	2.47^{a}
	Month	3	17.746	<0.001	2.19^{A}	3.42^{C}	2.61^{AB}	2.74^{B}
	site x month	9	8.673	<0.001				
IDH	Site	3	23.666	<0.001	4.18^{a}	4.15^{a}	4.62^{a}	6.15^{b}
	Month	3	22.796	<0.001	5.96^{C}	3.93^{A}	4.92^{B}	4.28^{AB}
	site x month	9	3.105	0.001				
ODH	Site	3	49.984	<0.001	2.61^{a}	3.03^{a}	4.40^{c}	4.01^{b}
	Month	3	20.585	<0.001	3.99^{B}	3.97^{B}	2.91^{A}	3.18^{A}
	site x month	9	7.641	<0.001				
EST	Site	3	0.482	0.695	0.62^{a}	0.64^{a}	0.63^{a}	0.59^{a}
	Month	3	5.803	0.001	0.71^{B}	0.56^{A}	0.57^{A}	0.65^{AB}
	site x month	9	2.351	0.014				

The results of the integrated analysis of biomarkers through the IBR index are indicated in Table II. 5. For all the sites, the highest values of IBR were found in July/August with, in general, higher contributions of ODH and IDH (Figure II. 3), whereas the lowest ones were calculated for October. In the M-est, the increasing order of IBR is M1< M2 < M3, except in July where the lowest value was determined for M2. Clams from L, in the L-est, had the highest IBR values in all months (contributions mainly from IDH and ODH), except in August where the highest value was found in M3 (higher contributions of EST, ODH and GR) (Figure II. 3).

Table II. 5. IBR index values calculated for clams from each sampling site and month in the tidal freshwater area of M-est L-est, from July to October 2011. Values are computed using the following biomarkers responses: lipid peroxidation (LPO), glutathione S-transferase (GST), catalase (CAT), glutathione reductase (GR), isocitrate dehydrogenase (IDH), octopine dehydrogenase (ODH) and esterase (EST). Higher values indicate higher stressed condition.

Sampling site	Sampling month			
	July	August	September	October
M1	6.21	3.28	1.65	0.47
M2	4.08	5.33	3.95	1.15
M3	6.25	11.95	4.25	3.95
L	10.57	8.92	5.74	4.23

3.3. Integrated data analysis of biological and abiotic parameters

Ten variables (hardness, turbidity, phosphates, iron, phenol, silica, chlorophylls a, b and c and organic matter) had to be removed from the final analysis because they were strongly correlated with one or more of the remaining environmental variables, producing collinearity problems. Therefore, these variables were causal variables explaining the biological responses variation weakly than the other abiotic variables included in the final RDA (Nally, 2000).

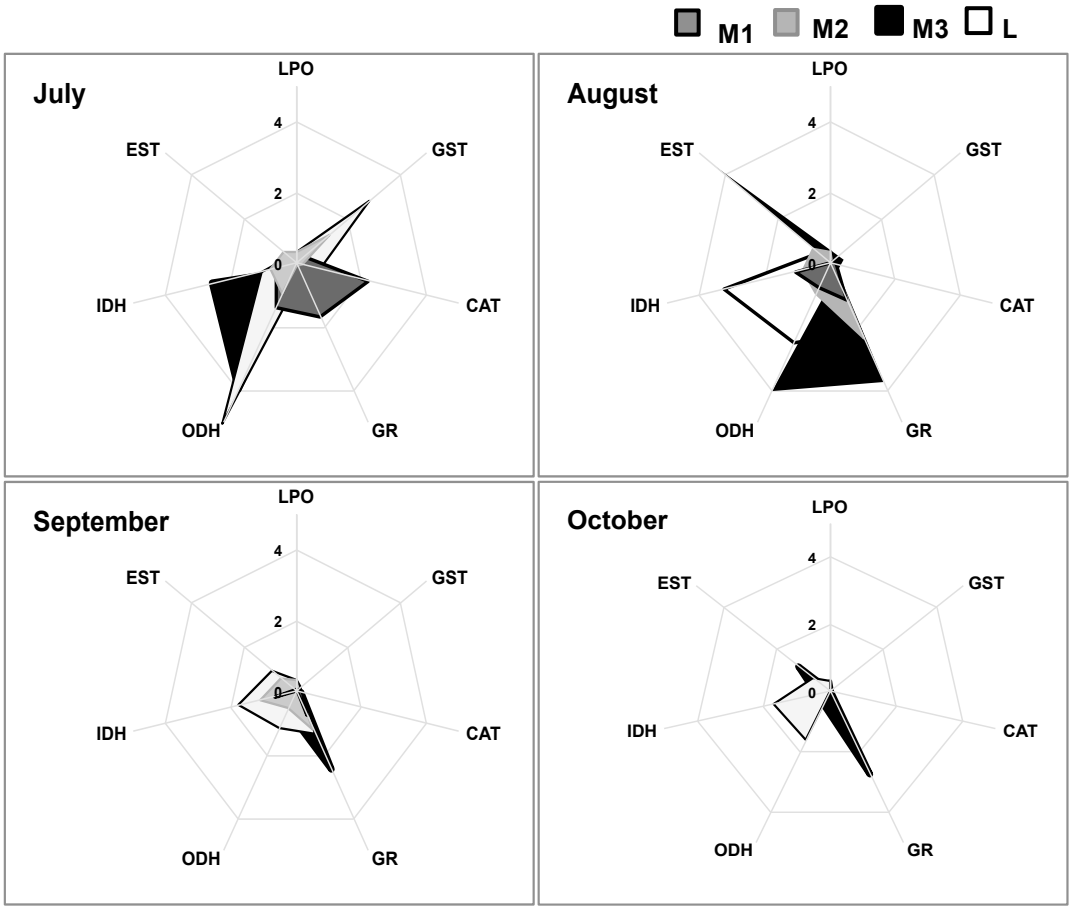


Figure II. 3. Star plots representing the contribution of each biomarker (gills lipid peroxidation (LPO) levels, glutathione S-transferase (GST), catalase (CAT) and glutathione reductase (GR) activities and foot muscle esterase (EST), isocitrate dehydrogenase (IDH) and octopine dehydrogenase (ODH) activities), used to compute the IBR index calculated for each sampling site and month in the tidal freshwater area of M-est and L-est, from July to October 2011.

The results of RDA are indicated in Figure II. 4. The first two axes explain 93.4% of the total variance (F-ratio = 1666.665, p = 0.0220, Monte Carlo permutation) with the first canonical axis (horizontal) explaining 84% of the relation between biological and environmental parameters while the second canonical axis (vertical) explains 10.5%. Regarding sites, the first axis separates M1 from M2, M3 and L; M1 and M2 are further separated by the second axis from M3 and L. M2 is associated with high levels of GR activity and LPO levels, pH values and phenol concentration. Increased ODH activity, conductivity and temperature are associated to M3 and L. Concerning months, July and September are separated from August and October by the

first axis; the second axis further separates July from September and August from October. Higher levels of nitrites, nitrates, ammonium, and CAT, EST and IDH activities are positively associated to July. High GST and GR activities, temperature and pH are associated to August. The same parameters except temperature are also associated to October.

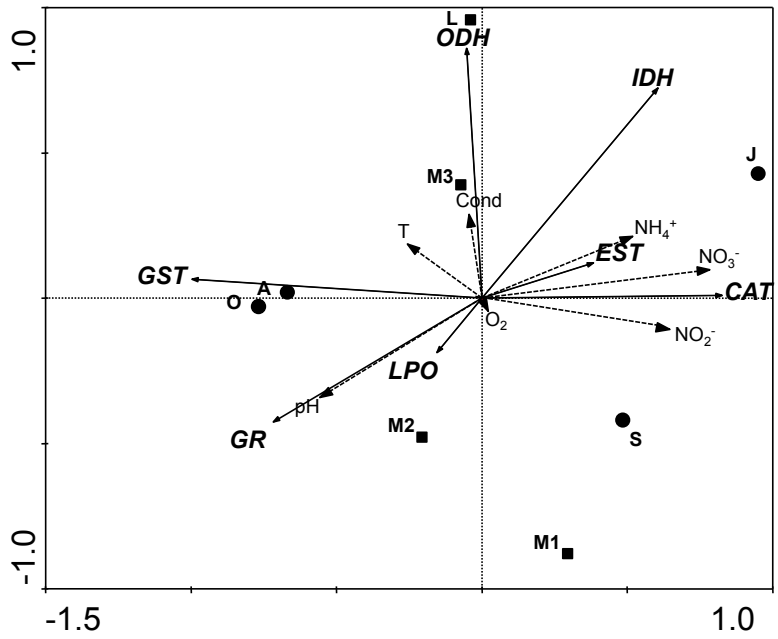


Figure II. 4. Redundancy analysis ordination diagram displaying the scores for biological variables (represented by straight arrows) showing correlations with quantitative environmental variables (represented by dotted arrows): the first axis (horizontal) significantly explained 84.0% and second (vertical) 10.5% of the variability. The biological variables are: Gills lipid peroxidation (LPO) levels, and glutathione S-transferase (GST), catalase (CAT) and glutathione reductase (GR) activities and foot muscle esterase (EST), isocitrate dehydrogenase (IDH) and octopine dehydrogenase (ODH) activities. The quantitative environmental variables are: temperature (T), dissolved oxygen (DO), pH, NH_4^+ (Ammonium), NO_2^- (nitrites) and NO_3^- (nitrates). Centroids of the sampling sites are represented by black triangles and centroids of months by black circles.

4. Discussion

4.1. Abiotic parameters variation along sites and time

The results of abiotic parameters indicate important spatial and temporal variability in environmental conditions (Table II. 1). The high levels of nitrites and nitrates found in July in M1 (Table II. 1) reflect an environmental condition previously associated with summer massive mortality of *C. fluminea* in this area (Sousa *et al.*, 2007a).

Nevertheless, M1 is located in a sort of 'bay' relatively to the main river flow and in the vicinity of the mouth of a Minho River tributary (PBH 2001) that crosses agriculture fields. Due to these conditions and the accumulation of sediments, part of the tributary discharge stays in the 'bay' instead of entering directly into the main river flow. Aquatic plants and green algae are very abundant and eutrophication conditions regularly occur which may be adverse for bivalves' physiological status (Farcy *et al.*, 2013). The analysis of the abiotic parameters considering the overall means per site (Table II. 3) reveals an increasing gradient (upstream to downstream) of temperature, hardness, and conductivity in the M-est. Furthermore, downstream sites (M2 and M3) had higher levels of organic matter and lower levels of dissolved oxygen than those recorded in M1; M3 had the highest phosphate concentrations, and M2 the highest ammonium concentrations. As a whole, these results suggest that in downstream sites, especially in M3, *C. fluminea* is exposed to more stressful conditions than in M1.

The highest means of water conductivity observed at M3, the most downstream site of the M-est, and at L, in the L-est (Table II. 3) associated to their location relatively to the mouth of M-est and L-est suggest that these sites are under a higher tidal influence than M1 and M2, in good agreement with findings of previous studies (Ilarri *et al.*, 2012; Sousa *et al.*, 2008a,d).

Considering now the comparison of means per month (Table II. 3), the highest mean of water temperature recorded in August combined with a relatively low water dissolved oxygen should be highlighted since the combination of these two factors is particularly stressful to *C. fluminea* and bivalves in general (Cherry *et al.*, 2005; Cooper *et al.*, 2005; Johnson and

McMahon, 1998; Vohmann *et al.*, 2009; Weitere *et al.*, 2009; Werner and Rothhaupt, 2008). August had also the highest phosphates mean (Table II. 3), and these water contaminants were especially high in M3 at this time (Table II. 1). Concentrations of phosphates in the water above 0.25 mg/L were associated with zebra mussel extinction (Karatayev *et al.*, 2003).

4.2. *C. fluminea* health status variation

The highest IBR values were found in L in July (10.57) and in M3 in August (11.95), respectively. At this time, in L, the highest water concentrations of nitrates (1.84 mg/L) and ammonia (0.31 mg/L), and turbidity (6.67) were recorded (Table II. 1), whereas at M3, the highest water temperature (25.87 ºC), phosphate concentration (0.60 mg/L) and turbidity (12.67) were found (Table II. 1). These findings suggest that increased water nutrients, ammonia, turbidity and/or temperature contributed to the high levels of stress found in *C. fuminea* collected in July (L) and August (L and M3). The highest IBR values calculated for M1 (6.21) and M2 (5.33) were observed in July and August, respectively, with a similar IBR value in M3 in July. In July, high water concentrations of nitrates and nitrites were recorded in all these sites, whereas the water temperature and phosphates were particularly high in August in M2. This finding also suggests, and supports the previous findings that increased water nutrients; ammonium, temperature and/or turbidity are important factors in the summer-induced stress in *C. fluminea*, likely acting in combination. This is in good agreement with the outcomes and hypotheses raised in previous studies with *C. fluminea* (Cherry *et al.*, 2005; Cooper *et al.*, 2005; Ilarri *et al.*, 2010; Sousa *et al.*, 2007a, 2008b; Vohmann *et al.*, 2009; Weitere *et al.*, 2009) and other bivalves (Cotter *et al.*, 2010; Samain *et al.*, 2007; Samain and McCombie, 2008; Soletchnik *et al.*, 2005). Moreover, our findings also mean that monitoring along the year the stress levels of *C. fluminea* populations will make possible to determine the likelihood of summer/post summer mortality syndrome in this species.

The biomarkers most contributing to the high IBR value found in clams from M3 in August were the enzymes EST (decreased), ODH (induced) and GR (induced) (Figures II. 2 and 3). EST inhibition indicates exposure to

inhibitors of these enzymes that may be anticholinesterase pesticides used in neighbour crop fields that may be at increased concentrations in the water due to a reduced river flow in the summer. The induction of GR activity, an anti-oxidant enzyme, indicates exposure to oxidative stress inducers. These may be the increased water temperature observed at this time (Table II. 1) and/or chemical stressors present in the water as environmental contaminants. The increase of ODH activity suggests increased energetic demands that may be need to face thermal and/or chemically induced stress. In fact, ODH is an enzyme involved in the anaerobic metabolism of several invertebrate species (Gerd *et al.*, 1978) and its induction is often a response to cope with respiratory deficit and to supply extra ATP (Lima *et al.*, 2007). In clams collected in July in L that had the second highest IBR value (Table II. 5), increased activity of the energy related enzymes ODH and IDH, and of the biotransformation GST enzymes were found. The increase of IDH and ODH suggest increased energy demands probably to cope with the stress caused by the presence of environmental contaminants (no increase of water temperature was found at this time in L), while the increase of GST activity indicates exposure to chemicals that are biotransformed by these enzymes and are known to be present in L-est (Guimarães *et al.*, 2012). Other biological factors that may have contributed to the highest levels of stress found in *C. fluminea* in July/August are those related with the reproductive cycle. In bivalves, important physiological alterations occur during the gonads development and spawning, which are very demanding processes (Cotter *et al.*, 2010; Dégremont *et al.*, 2007; Huvet *et al.*, 2010; Myrand *et al.*, 2000; Soletchnik *et al.*, 2005). In *C. fluminea*, an additional stress factor may be the embryos incubation process because it occurs in the adults' gills (Byrne *et al.*, 2000). A study of the gonadal development of *C. fluminea* in the studied sites has been carried out. The preliminary evidences indicate the presence of individuals with mature gametes almost all the year (data not shown). Thus, the influence of factors related with the gonads development, spawning, and embryo incubation is unlikely to be an important factor in the variability of IBR found, despite potentially having some influence.

Overall, the IBR index provided an integrated view of *C. fluminea* stress levels allowing the discrimination of the more stressful months and sites.

However, attention should be taken when using IBR index (Cravo *et al.*, 2012) since is a dynamic index that varies with the type and number of biomarkers used, and the kind of response recorded (e.g. activation/inhibition) (Beliaeff and Burgeot, 2002; Tsangaris et al., 2011).

4.3. Relating biological and environmental parameters

The association between high ODH activity and high water conductivity and in a less extend with water temperature in M3 and L, suggests that clams from these sites are under energetic stress caused by these two abiotic variables. Because these are downstream sites (in M-est and L-est) the tidal influence is likely to significantly contribute to these high conductivity values as previously discussed (section 3.1). Although *C. fluminea* can tolerate some salinity for relatively short periods (Sousa, 2006a), this is a freshwater species, and it seems to be sensitive to salinity fluctuations (Xiao *et al.*, 2014). Therefore, the increased ODH activity shown by clams from M3 and L may be at least in part due to the need of getting more energy to face salinity-induced stress. *C. fluminea* is also sensitive to increased water temperature (Cooper *et al.*, 2005; Sousa *et al.*, 2008d; Weitere *et al.*, 2009). In *C. fluminea*, thermal stress also requires additional energy to activate molecular defences against its effects, increase tissue repair mechanisms, and other processes (Weitere *et al.*, 2009). M2 appears separated from the other sites in the RDA ordination diagram mainly because the high GR activity that is positively associated with water pH values. In fact, M2 clams showed increased GR activity relatively to clams from all the other sites in September and October (Figure II. 2), indicating increased exposure to oxidative stress inducers.

The positive association between high water concentrations of nitrites, nitrates, ammonium, and increased activities of the enzymes CAT, EST and IDH contributing to the separation of July from all the other months (Figure II. 2), suggests that these biological responses are, at least in part, due to these abiotic factors. A recent study also observed that the fast proliferation of algal cells could influence negatively the biomarker responses in *Mytilus edulis L.* (Farcy *et al.*, 2013). Such enzymatic inductions indicate exposure to oxidative stress inducers (CAT), environmental contaminants hydrolysed by EST, and

that additional energy is required to cope with chemically induced stress resulting in IDH induction. August and October appear separated from the other months in the RDA ordination diagram mainly due to high GST activity. In fact, the clams from all the sampling sites showed high GST activity in August, being reduced in September and increased again in October, except in L where no increase was observed in October (Figure II. 2). The induction of this enzyme is very important to face chemical stress induced by electrophilic agents that are biotransformed through glutathione conjugation. Common environmental contaminants of this type are organochlorine pesticides that may reach the rives through lixiviation from adjacent fields, PAHs, PCBs and several dioxin-like environmental contaminants that may reach the estuaries through contaminated tributaries and/or direct inputs. Because in August the flow of M-est and L-est is reduced due to draw conditions, the water concentration of these chemicals is likely to be high. They are expected to decrease when the river flow comes back to normal values and is expected to further increase if strong rain occurs in the autumn causing the lixiviation of contaminated surrounding crop fields.

4. Conclusions

Important spatial and temporal variation in several of the 17 environmental parameters analysed were found. The most important ones were related with the increase of water temperature, conductivity and nutrients in downstream sites (M3 and L) creating stressfully conditions to *C. fluminea*. The IBR analysis indicated that July/August were more stressful to clams than September/October, especially in downstream sites. The clam's stress in the summer months was mainly due to increased activity of energy-related (IDH and ODH) and biotransformation (EST and GST) enzymes. These findings indicate that in the summer *C. fluminea* populations of M-est and L-est are exposed to chemical environmental contaminants biotransformed by EST and GST enzymes and probably also to thermal stress and a higher tidal influence (especially in downstream sites), and that they need to activate energy relatedenzymes to get the additional energy required to face these types of stress. Finally, the integration of environmental and biological variables

through a RDA analysis provided a separation among sites (M1 ≠ M2 ≠ M3 ≈ L) and among months (J ≠ A ≠ S ≠ O). Increased activities of the energy related enzyme IDH, of the anti-oxidant enzyme CAT and of EST enzymes associated with increased water concentrations of ammonia, nitrate and nitrites contributed to the separation of July from the other months; induction of the GST biotransformation enzyme mainly contributed to separate August and October from the other months. Overall, the findings of the present study indicate that July/August are particularly stressful months for the studied *C. fluminea* populations, especially at downstream sites; the increase of nutrients and ammonia water concentrations, water temperature and conductivity are major contributors for this increased stress; the biomarkers indicated that in July/August *C. fluminea* is exposed to oxidative stress inducers, environmental chemical contaminants biotransformed by EST and GST enzymes, and that organisms need additional energy to cope with the chemical and/or thermally-induced stress. The findings of the present study highlight the importance of biomonitoring the health condition of *C. fluminea* because it may allow determining the likelihood of summer/post summer mortality syndrome in this species.

Acknowledgments

This research was supported by the European Regional Development Fund (ERDF) through the COMPETE - Operational Competitiveness Programme and national funds through FCT – Foundation for Science and Technology, under the projects “FCT Ref.: PTDC/AAC-AMB/102121/2008; FCOMP-01-0124-FEDER-008556”, and “PEst-C/MAR/LA0015/2013. Cristiana Oliveira received a FCT grant (SFRH/BD/68423/2010) from the Portuguese Foundation for the Science and Technology (FCT), with FEDER European funds and European social funds of (POPH-QREN-Tipology 4.2.).

Chapter III

Factors influencing the accumulation of metals by *Corbicula fluminea* and its health status in estuarine tidal freshwater areas

Abstract

The main goal of this study was to identify sediment metal concentrations effects on the health *status* of the non-indigenous invasive species (NIS) *Corbicula fluminea* in estuarine tidal freshwater areas (TFA), and the factors influencing the metals accumulation on their tissues a topic of high interest to increase the knowledge on the specific factors influencing the invasive behaviour of this non-invasive species (NIS). A seasonal monitoring study was carried out on two *C. fluminea* wild populations inhabiting the TFA of two estuaries over one year. The parameters investigated were: 16 water/sediments variables; concentrations of 13 metals in sediments (bioavailable fraction) and *C. fluminea* soft tissues, and 9 biomarkers. *C. fluminea* accumulated 7 metals (Cr, Cu, Zn, Se, As, Cd, Pb) with bioconcentration factors (BSAF) ranging from ≈ 2 (Cr) to ≈ 36 (Cu). The redundancy analysis integrating abiotic variables and BSAF indicated that sediment granulometry and organic matter, and water temperature and turbidity were the main factors influencing the accumulation of metals by *C. fluminea*. The Integrated Biomarker Response index (IBR) integrating the biomarkers' responses indicated that *C fluminea* individuals from downstream sampling sites had a decreased health *status* relatively to those from upstream sites. The main factors found to influence the health *status* of *C. fluminea* were: the coarser sands and silt/clay fraction and the concentrations of Mn, Al, Se and organic matter in sediments, and water temperature. Higher stress levels were extremely associated with Mn, Al, Cr, As and Se mostly in L-est, however, the concentration in the tissues was lower suggesting that *C. fluminea* inhabiting in L-est can efficiently eliminate, detoxificate and/or avoid excessive concentrations of these particular metals. The mechanisms of metal elimination and/or avoidance are probably inducing higher stress levels

that may be limiting the invasive behaviour of this population. In addition the energy expended to cope with metal pollution might be also contributing for the low dispersion of *C. fluminea* in L-est since the energy required for the establishment and spread of the population may be redirected to cope with metal contamination.

Key words:

Corbicula fluminea; Bioinvasions; Metals bioaccumulation; Health *status*; Biomarkers; Invasive Behaviour.

1. Introduction

Biological invasions are recognized as one of the major threats to natural ecosystems (European Commission 2013; Karatayev *et al.* 2007; Kolar and Lodge 2001; Larson *et al.* 2011; Ojaveer *et al.* 2014; Sakai *et al.* 2001). Consequently, the European Union Convention on Biodiversity Strategy (European Commission 2011) established that, by 2020, the priority non-indigenous invasive species (NIS) should be controlled or eradicated, and their invasive pathways should be managed to prevent the introduction and establishment of new invasive species. Knowing how specific factors influence the invasive behaviour of NIS is a most important issue since it will allow preventing new invasions, controlling already existent ones and mitigating adverse effects that are almost impossible tasks at the present (Occhipinhi-Ambrogi 2007). *Corbicula fluminea* is one of the worst 100 invasive species (DAISIE 2015) and the bioinvasions by this species are of high concern because they can cause adverse economical and ecological impacts (Rosa *et al.* 2011) and is considered a major treat to biodiversity conservation (Sousa *et al.* 2008d). Despite the several studies that have been made in recent decades, the factors influencing the invasive behaviour of *C. fluminea* are not yet completely understood. For example, in the Northwest of Portugal there are two Rivers estuaries (Minho and Lima estuaries hereafter indicated as M-est and L-est respectively) where *C. fluminea* populations have substantially different invasive behaviours (Sousa *et al.* 2006a, 2008b,d). Several hypothesis were already raised to explain these differences, such as that the population inhabiting in Lima may still be in a lag phase, or it may have a reduced spawning and/or high mortality rate of larvae and/or juveniles (Sousa *et al.* 2006a), however the reasons behind it are still unclear. M-est and L-est are neighbour estuaries having several comparable hydromorphological characteristics but also some differences including in environmental factors and chemical contamination (Cairrão *et al.* 2004; Guimarães *et al.* 2012; INAG 2000; Reis *et al.* 2009; Sousa *et al.* 2006a) which might be contributing to the differences observed between the invasive behaviours of *C. fluminea* inhabiting both estuaries. Exposure to contaminants have already be found to impair *C. fluminea* recruitment (Boltovskoy *et al.*

1997), decrease growth rates and the condition index (Cataldo *et al.* 2001) and induce histopathological alterations (Cid *et al.* 2015; Lehmann *et al.* 2007) and several changes on biochemical biomarkers (Oliveira *et al.* 2015b; Legeay *et al.* 2005; Brandão *et al.* 2011; Netpae *et al.* 2012; Bigot *et al.* 2011; Ren et al 2013). Therefore, the main goals of the present study were to investigate the factors influencing the health *status* of natural *C. fluminea* populations in relation to the concentrations of metals in sediments, water quality and other abiotic parameters variation and the accumulation of metals by *C. fluminea* in real scenarios. Metals were selected for this study mainly because *C. fluminea* is known to accumulate metals (Cataldo *et al.* 2001; Sebesvari *et al.* 2005; Shoults-Wilson *et al.* 2009; Reis *et al.* 2014; Spann *et al.* 2011; Takabe *et al.* 2011), however the factors influencing the process and the effects of these environmental contaminants on the health *status* of native populations are not totally understood. Furthermore, among the diverse types of environmental contaminants that may be accumulated by *C. fluminea*, metals deserve special attention since several metals are ubiquitous pollutants able to cause toxic effects on the biota and humans after exposure to ecologically relevant concentrations, have a long environmental persistence (Nicolodi *et al.* 2011; Hill *et al.* 2011) and a long half-life in the body of several species including humans (Vasanthi *et al.* 2012). Moreover, some forms of metals (*e.g*. arsenic, cadmium, chromium, nickel, mercury) are carcinogenic (IARC 1993, 2009), and the organic forms of some metals (*e.g*. mercury) are biomagnified in trophic chains (Lee *et al.* 2015; Vasanthi *et al.* 2012; Zaza *et al.* 2015). Thus, metals are considered chemicals of specially concern (UNEP, USEPA, ECHA), and their concentrations in the environment and species used for human consumption, such as *C. fluminea* (Fried and Emili 1987; Graczyk and Fried 1998; Phelps 1994), should be monitored under the scope of national and international regulations, such as the European Marine Strategy Framework Directive (MSFD 2008/56/EC).

2. Material and Methods

2.1. Chemicals

All the chemicals used were of analytical and trace metal grade and purchased from Sigma-Aldrich (Germany), Merck (Germany), Bio-Rad (Germany), Panreac (Spain) or Theta (Portugal).

2.2. Sampling estuaries and sites, and *C. fluminea* collection

C. fluminea populations of the estuaries of Minho (M-est) and Lima (L-est) Rivers (NW Iberian Peninsula) were selected for this study mainly because they have been studied for decades (*e.g*. Araujo *et al.* 1993; Sousa *et al.* 2006a, 2007b, 2008d,e; Oliveira *et al.* 2015a; Reis *et al.* 2014) and have been showing a distinct invasive behaviour. For instance, the M-est population colonized practically all the TFA reaching a very high density and biomass (up to 4000 ind m^{-2} and 400g m^{-2}, respectively) (Sousa *et al.* 2005), whereas the TFA L-est population has a relatively lower density and biomass population (up to 60 ind m^{-2} and 26 g m^{-2}, respectively), sparsely distributed (Sousa *et al.* 2006b). The M-est and L-est are located in the NW Iberian Peninsula coast, their mouths are separated by ≈ 25 Km, they have several comparable hydromorphological characteristics but also several differences, including in some water parameters (*e.g*. salinity) and concentrations of several environmental contaminants such as metals (Guimarães *et al.* 2012). The M-est, that makes border between Portugal and Spain, is included in NATURA 2000 and is considered a low impacted estuary (Ferreira *et al.* 2003), despite having some punctual sources of contamination, such as input of the Louro river, considered one of the most contaminated Rivers in Galicia (Spain) (Planelló *et al.* 2013; Santos *et al.* 2013). In general, the L-est has been considered as more contaminated than the M-est, especially in its lower part due to the presence of a harbour, oil storage facilities, a paper mill, a marina, among other facilities (Guimarães *et al.* 2012). In the vicinity of both estuaries there are agriculture field crops of small-medium dimension, urban settlements, and industrial facilities (Ribeiro *et al.* 2015).To study the potential effects of tidal influence and the potential impact of the Louro tributary, 3

sampling sites were selected in the M-est (Figure III. 1): M1, M2 and M3 (upstream to downstream). M1 (N42º03'09.37'' W8º33'42.73'') located upstream to Louro River mouth (≈ 6 Km from it); M2 (N42º01'25.12'' W8º39'24.49'') located ≈ 3 Km below Louro River mouth; and M3 (N41º54'41.25'' W8º47'36.59'') located ≈ 20 Km downstream to Louro River mouth and ≈15 Km from the mouth of the estuary. Because only one spot of *C. fluminea* could be found in comparable areas of the L-est, only a sampling site (L) was selected in this estuary (N41º42'07.03'' W8º44'37.05''), ≈ 15 km upstream from the estuary mouth (Figure III. 1). *C. fluminea* specimens were collected seasonally with a hand rake including a net: in summer 2011 (July), autumn 2011 (October), winter 2011 (January) and spring 2012 (April). In each sampling period fifty individuals with ≈ 30 mm (maximal distance from the valve extreme perpendicular to the umbo) were selected for further study, whereas smaller or bigger individuals were released back to the wild. After collection, the specimens were transported to the laboratory in thermally isolated boxes containing water from the location where *C. fluminea* individuals were collected, within the lowest time possible, with aeration (Oliveira *et al.* 2015a). In the laboratory, all the specimens were measured (digital caliper) and weighted (balance). Fifteen to thirty *C. fluminea* individuals per site and season were used for chemical analyses, and twenty for biomarkers determinations.

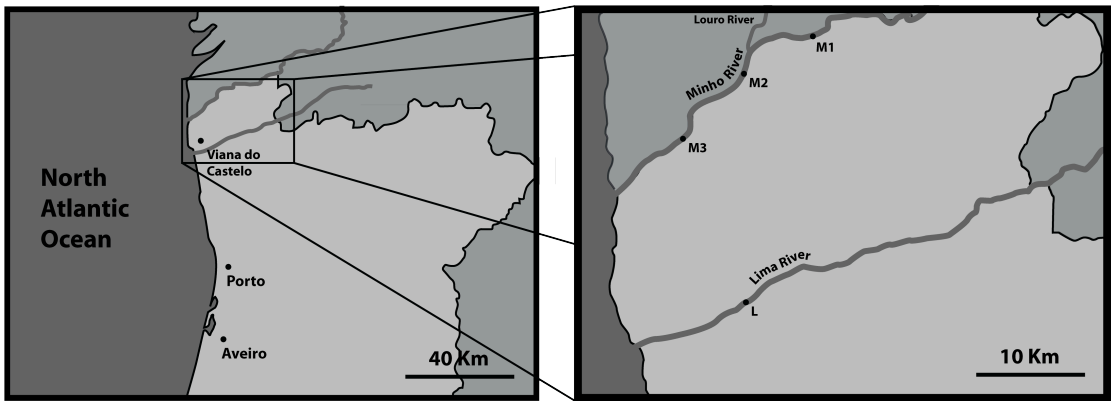


Figure III. 1. Map showing the location of sampling sites in the estuaries of Minho and Lima Rivers.

2.3. Integrated monitoring approach

Following the recommendations included in the MSFD (Descriptor 8), an integrated monitoring approach was used. It included the following parameters: 13 water variables; 3 sediment variables; concentrations of 13 metals in sediment samples and in *C. fluminea* body (soft tissues); and 7 biomarkers. The biomarkers were: the activity of the enzymes isocitrate dehydrogenase (IDH) and octopine dehydrogenase (ODH) that are involved in the pathways of energy production; the activity of esterases (EST) that are involved in the biotransformation of xenobiotics; the activity of glutathione S-transferases (GST) that are involved in biotransformation and anti-oxidant defences; the activity of catalase (CAT) and glutathione reductase (GR) that are part of the antioxidant defences; and the levels of lipid peroxidation (LPO) as indicative of lipid oxidative damage. These particular enzymes were selected as biomarkers for this study due to their crucial role in functions that are determinant for the survival and performance of the organisms in the wild. LPO was selected because the valuable information that it provides in relation to oxidative damage.

2.3.1. Water and sediment parameters

In each season and per sampling site, water and sediments were collected for determination of several parameters, following the procedures described in Oliveira *et al.* (2015a). Briefly, water temperature, dissolved oxygen, conductivity and pH were measured *in situ* in triplicate with a multiparameter probe (WTW 340i). Water samples were collected, in triplicate, to determine the following parameters using colorimetric methods following the Photometer Systems for Water Analysis protocols (Palintest 7000 interface photometer): hardness, turbidity and the concentration of ammonium, nitrates, nitrites, phosphates, iron, phenol, and silica. Sediment samples were collected in triplicate to determine granulometry, organic matter and chlorophylls *a*, *b* and *c*. Briefly, for granulometry, samples were placed into an oven for 72 hours at 60°C. Subsequently, a grain size analysis was performed by sifting with a Ro-Tap agitation, with columns of sieve, according to the grain size scale of

different mesh sizes (1 - 2 mm - very coarse sand; 0.5 - 1mm - coarse sand; 0.25 - 0.5 mm - medium sand; 0.125 - 0.25 mm - fine sand; 0.063 - 0.125 mm - very fine sand; < 0.063 mm - silt + clay fraction) and the frequency of each class was expressed as % of total weight of the sample. Organic matter was determined after combusting the sediments during 24h at 550ºC in a muffle (Fisher Scientific, Isotemp Muffle Furnace). Values are expressed in percentage relatively to the weight loss on ignition of each sample analysed. For chlorophyll determinations 30 ml of acetone (90%) and 0.2 ml of magnesium carbonate solution (1%) were added to the samples *in situ* and the mixture was shaked and maintained on ice in a thermally isolated box until arrival to the laboratory. After 24h at 4ºC, samples were centrifuged and the supernatants were carefully collected, put in a glass spectrophotometer cuvette and its absorbance was read in a spectrophotometer (Jenway 6405 UV/VIS) at 480, 630, 645, 647 664, 665 and 750 nm. The concentrations of chlorophyll *a*, *b* and *c* were calculated according to Jeffrey and Humphrey (1975).

2.3.2 Chemical analysis

Chemical analyses (Fe, Al, Cr, Cu, Mn, Ni, Zn, Se, As, Co, Cd, Hg and Pb) were performed seasonally in *C. fluminea*'s tissues (15-30 animals per determination) and sediments (3 replicates per site) that were collected from summer 2011 to spring 2012. Extractable/acid-exchangeable metals in sediments were analysed using the first step of the three-stage sequential extraction procedure proposed by the European Standards, Measurements and Testing (SM&T) Program, formerly the Community Bureau of Reference (BCR) as described by Pueyo *et al.* (2001). This method was originally developed for the analysis of heavy metals in sediments, and has been widely accepted and applied to metal fractionation in different environmental samples, and the first step is considered the fraction with the higher bioavailability (Reis *et al.* 2009; Rao *et al.* 2008; González-Flores *et al.* 2011). So, in this article we will refer to them as bioavailable metal concentration. Briefly, 40 ml of acetic acid solution (0.11 mol l^{-1}) were added to 1 g of

sediment in a 80–100 mL centrifuge tube and immediately shaked for 16 h at 22±5 ºC (overnight). Then, the extract was separated from the solid residue by centrifugation at 3000 g for 20 min and the supernatant was decanted into a polyethylene container. It was stored in a refrigerator at ≈ 4ºC prior to analysis. Before extraction samples were precisely weighed in porcelain crucibles, and placed in an oven (105±2ºC) until constant weight. In the case of *C. fluminea* samples, 0.5 g of freezed dried *C. fluminea*'s tissue was digested in a microwave oven Ethos 1 (Milestone Sorisole, Italy) with 7 ml of nitric acid and 1ml H_2O_2. After digestion the extract was dissolved with milli-Q water. In both types of extracts, metals concentrations were analysed by using a NexION 300D Inductively Coupled Plasma Mass Spectrometer (ICP-MS) (PerkinElmer, Inc., Shelton, CT). In all batches of analysis, reagents and procedures blanks were included.

All metal concentrations were expressed as μg metal g^{-1} dry weight (d.w.).

2.3.3. Biomarkers

The preparation of the tissues and biomarkers were determined as indicated in more detail in Oliveira *et al.* (2015a). Briefly, immediately after the determination of size and weight, each animal was sacrificed by opening the valves under cold induced anaesthesia. The tissue isolation was carried out on ice, and all the biomarkers were determined per individual. Gills were isolated, washed in phosphate buffer, and used to determine LPO levels, and GST, CAT and GR activity determinations and foot tissue was used for the determination of EST, IDH and ODH activities.

All biochemical analyses were performed at a constant temperature (25±1ºC) and protein content of supernatants was standardized to 4 mg ml^{-1} for CAT and GR activities and for 1 mg ml^{-1} for GST, EST, IDH and ODH activities. LPO levels were determined in the homogenate and no protein standardization was performed. All enzymatic activities were expressed as nmol min^{-1} mg^{-1} protein, except catalase, which was expressed as μmol min^{-1} mg^{-1} protein.

2.4. Data analysis

Data from each parameter was checked for normality of distribution and homogeneity of variances before the Analysis of Variance (ANOVA) (Zar, 1999). When these assumptions could not be full field even after data transformation, the non-parametric Kruskal–Wallis comparison test was used to compare different seasons and sites. When significant differences were found, the Dunn's multicomparison test was used to discriminate statistically significant treatments. The Statistics 18.0 package was used and the significance level was 0.05.

To assess the bioaccumulation of each metal by *C. fluminea*, the biota-sediment accumulation factors (BSAF) (Szefer *et al.*, 1999) were determined per month and sampling site through the following ratio: mean concentration of the metal in the whole body (soft tissues) of *C. fluminea* / mean of the metal concentration in sediment samples. In both cases, the units were µg of metal per g of dry weight (d.w.)

The heath *status* of *C. fluminea* along time and in different sites was evaluated by assessing the levels of stress though the Integrated Biomarker Response Index (IBR) described by Beliaeff and Burgeot (2002). Briefly, the computed scores of each biomarker (standardized data) per season and site were represented in star plots. The IBR was then calculated through the sum of the star plots triangular areas represented by two consecutive biomarker scores for a given sampling site and season. Biomarkers used for the IBR calculation were ranged clockwise as follows: LPO, GST, CAT, GR, ODH, IDH and EST. Higher IBR values indicate higher levels of stress than lower IBR values (Beliaeff and Burgeot 2002), and higher levels of stress indicate decreased health *status* (Oliveira *et al.* 2015a).

Data from biological and abiotic parameters were integrated through a Redundancy Analysis (RDA), using a model-based type of Monte Carlo permutation test (ter Braak and Prentice 1986; ter Braak and Smilauer 2002). The quantitative variables were: IBR values; concentrations of 13 metals in sediments; concentrations of 13 metals in the body of *C. fluminea*; water temperature, dissolved oxygen, conductivity, pH, hardness, turbidity,

ammonium, nitrates, nitrites, phosphates, iron, phenol and silica; and sediment granulometry, chlorophylls (*a*, *b* and *c)* and organic matter. Six water parameters (nitrites, nitrates, phosphates, iron, phenol, silica) had to be removed from the final analysis because they produce collinearity problems by being strongly correlated with one or more of the remaining environmental variables. Therefore these variables were causal variables (Nally 2000) explaining the sediment metal concentration and the *C. fluminea*'s bioaccumulation factors variation weakly than the other abiotic variables included in the final RDA. Multivariate analyses were performed with Canoco for Windows (version 4.5).

3. Results

3.1. Seasonal and spatial variation of water and sediment parameters

The mean concentrations of water and sediments abiotic parameters, per season and sampling site are shown in Table III. 1, and the corresponding results of statistically analysis are shown in the Supplementary material (Table III. 2) Significant differences among the seasonal means were found for all the abiotic parameters, except for water dissolved oxygen, turbidity, phenol, silica and the percentages of sediment very coarse sand, coarse sand and fine sand. The means of water temperature and the concentrations of nutrients (ammonium, nitrates and nitrites) were significantly higher in the summer than in the other seasons. Contrariwise, the seasonal means of water dissolved oxygen, conductivity, pH and phosphates concentrations followed in general the opposite trend, being significantly lower in the summer than in the other seasons. Concerning sediments granulometry, significant differences among seasonal means were only found for the percentage of medium sand, fine sand and very fine sand; medium sand showed a significantly lower mean in the summer than in the other seasons, whereas the means of fine sand and very fine sand were significantly lower in the winter than in the remanding seasons. The highest mean concentration of organic matter in sediments was found in the spring, whereas the highest means of chlorophylls *a*, *b* and *c* concentrations in sediments were found in the autumn and spring.

Significant differences of water conductivity, hardness, nitrites, phosphates and silica, and sediment granulometry, organic matter and chlorophyll *a* were found among sampling sites (Table III. 1). Downstream sampling sites (M3 and L) showed higher means of conductivity, hardness and phosphate concentrations than in upstream sites. The mean concentration of nitrites in the water was significantly higher in all the sites of M-est than in the L-est site. Contrariwise, the mean concentration of chlorophyll *a* in sediments was significantly higher in L-est sampling site than in all the M-est sampling sites. The mean percentages of very coarse sand and coarse sand in sediments were significantly higher in M1 and L than in the other sites. The mean percentage of medium sand in sediments was significantly higher in M1 than in the other sites, whereas the mean percentages of fine sand, very fine sand and silt and clay were significantly lower in M1 than in the other sites.

3.2. Concentration of metals in sediments (bioavailable fraction) and *C. fluminea* body

The mean concentrations of metals in sediments and *C. fluminea* body are shown in Table III. 3, and the results of their statistical analysis are indicated in Table III. 4. All the concentrations of Hg in sediments were bellow the detection limit, as well as those of Cd in winter and spring. Regarding the mean of the total bioavailable concentration of metals in sediments, significant differences among seasons and sites were found. The highest metal concentration was found in the summer (1242.73 μg g^{-1} d.w.), and the lowest in the winter (655.93 μg g^{-1} d.w.). The site with the highest total bioavailable metals concentration was L (1548.68 μg g^{-1} d.w.), and the site with the lowest mean was M2 (527.87 μg g^{-1} d.w.). No significant differences among the seasonal means were found for Al, Mn, Zn, Se, As, Co, and Pb, whereas significant differences were found for Fe, Cr, Cu, Ni and Cd. Significant differences in the mean sediment concentrations of Al, Cr, Cu, Mn, Ni, Zn, Co and Pb were found among sites, while no significant differences were found in relation to Fe, Se, As and Cd.

Table III. 1. Temporal and spatial variation of water and sediment parameters. Values represent the mean and corresponding standard deviation of water temperature (T, ºC), dissolved oxygen (DO, mg l^{-1}), conductivity (Cond, µS cm^{-1}), pH (pH units), turbidity (Turb, FTU), hardness ($CaCO_3$, mg l^{-1}), ammonium ($NH4^+$, mg l^{-1}), nitrates (NO_3^-, mg l^{-1}), nitrites (NO_2^-, mg l^{-1}), phosphates (PO_4^{3-}, mg l^{-1}), phenol (C_6H_5OH, mg l^{-1}), silica (SiO_2, mg l^{-1}) and iron (Fe, mg l^{-1}); and sediment organic matter (OM, %), granulometry (very coarse sand (VCS), coarse sand (CS) medium sand (MS), fine sand (FS), very fine sand (VFS) and silt and clay (S+C), all in %), and chlorophylls a, b and c (Chl_a, Chl_b and Chl_c, respectively, µg ml^{-1}). M1, M2 and M3 are sampling sites located in Minho estuary and L in Lima estuary. Different letters identify significant differences among sampling seasons (capital letters) or sites (small letters), as indicated by the Dunn multiple comparison test.

		Factor	Summer 11	Autumn 11	Winter 11	Spring 12
			M1	M2	M3	L
Water	T	Season	21.36±2.24 ^{C}	19.88±0.94B^{C}	9.04±0.26^{A}	13.56±1.27^{B}
		Site	14.58±4.84	16.33±6.18	16.91±6.64	16.03±6.45
	DO	Season	9.97±1.13	9.62±3.37	10.32±1.26	10.76±1.25
		Site	10.70±2.37	10.72±1.88	9.43±0.29	9.81±2.84
	Cond	Season	25.38±11.23^{A}	504.92±467.73^{B}	233.00±191.28^{B}	1017.67±1547^{B}
		Site	67.19±35.94^{a}	87.02±47.26^{a}	519.25±388.53^{b}	1107.50±408.31^{b}
	pH	Season	7.27±0.22^{A}	8.35±1.07^{C}	7.40±0.54^{AB}	7.50±0.27^{B}
		Site	8.17±1.18	7.53±0.16	7.50±0.40	7.32±0.74
	Turb	Season	4.44±3.61	5.00±2.52	2.44±2.94	4.72±2.28
		Site	3.78±3.22	4.17±3.71	4.17±2.40	4.50±3.40
	$CaCO_3$	Season	87.92±117.21^{A}	82.92±53.83 ^{AB}	46.39±20.62^{A}	124.72±109.77^{B}
		Site	34.72±15.02^{a}	46.81±6.82^{a}	149.58±82.91^{c}	110.83±35.12^{b}
	NH_4^+	Season	0.41±0.38^{C}	0.14±0.06^{B}	0.038±0.01^{A}	0.18±0.03^{BC}
		Site	0.09±0.06	0.31±0.43	0.18±0.12	0.19±0.15
	NO_3^-	Season	2.61±1.25^{C}	0.26±0.10^{A}	0.49±0.14^{B}	0.37±0.12^{AB}
		Site	1.38±2.06	0.84±0.76	0.83±0.90	0.68±0.90
	NO_2^-	Season	0.12±0.09^{B}	0.01±0.00^{A}	0.01±0.00^{A}	0.01±0.00^{A}
		Site	0.04±0.06^{b}	0.06±0.11^{b}	0.03±0.05^{b}	0.00±0.00^{a}
	PO_4	Season	0.02±0.02^{A}	0.06±0.04^{B}	0.07±0.01^{B}	0.24±0.32^{C}
		Site	0.05±2.13^{a}	0.04±0.87^{a}	0.08±0.96^{a}	0.23±0.90^{b}
	C_6H_5OH	Season	0.07±0.02	0.07±0.02	0.07±0.01	0.08±0.02
		Site	0.07±0.03	0.08±0.03	0.07±0.05	0.07±0.01
	SiO_2	Season	2.73±2.44	3.28±2.65	3.63±1.74	3.27±1.21
		Site	5.26±0.01^{b}	2.38±0.01^{a}	2.65±0.02^{a}	3.62±0.01^{a}
	Fe	Season	0.06±0.03^{B}	0.03±0.01^{A}	0.02±0.01^{A}	0.06±0.02^{B}
		Site	0.03±1.91	0.05±1.12	0.04±1.70	0.05±0.59

Table III. 1. *(Continued…)*

		Factor	Summer 11	Autumn 11	Winter 11	Spring 12
			M1	M2	M3	L
Sediment	OM	Season	1.20±0.45^{A}	2.07±1.71^{AB}	1.06±0.27^{A}	3.14±2.13^{B}
		Site	1.24±0.01^{ab}	2.11±0.03^{bc}	1.95±0.03^{a}	2.16±0.05^{c}
	VCS	Season	8.63±12.97	6.12±9.86	15.98±15.11	7.72±9.83
		Site	12.05±0.30^{b}	1.39±1.65^{a}	1.87±2.47^{a}	23.15±0.23^{b}
	CS	Season	13.09±16.96	12.31±14.43	19.15±16.52	11.33±13.92
		Site	18.96±15.01^{b}	0.38±1.29^{a}	3.59±2.92^{a}	32.94±3.61^{b}
	MS	Season	29.84±30.58^{A}	54.31±28.52^{B}	45.69±17.29^{B}	40.13±27.31^{AB}
		Site	64.88±8.94^{b}	27.96±0.18^{a}	41.72±4.73^{a}	35.42±2.10^{a}
	FS	Season	29.40±31.53	21.83±28.18	16.66±23.20	25.77±27.11
		Site	2.42±23.23^{a}	59.04±13.43^{c}	28.66±38.16^{b}	3.54±5.62^{a}
	VFS	Season	15.62±19.52^{B}	3.77±4.75^{AB}	1.57±1.42^{A}	11.73±17.08^{B}
		Site	0.85±1.59^{a}	9.03±7.12^{c}	20.41±16.81^{bc}	2.40±0.47^{b}
	S+C	Season	2.89±2.82^{B}	1.03±0.83^{AB}	0.64±0.41^{A}	2.80±2.79^{B}
		Site	0.45±0.27^{a}	1.47±5.75^{b}	3.35±22.82^{ab}	2.10±0.54^{b}
	Chl_a	Season	1.43±0.73^{A}	2.11±0.75^{B}	1.24±0.42^{A}	2.67±0.92^{B}
		Site	1.52±0.23^{a}	1.63±1.12^{a}	1.66±3.63^{a}	2.62±0.31^{b}
	Chl_b	Season	0.80±0.08^{A}	1.07±0.23^{B}	0.79±0.07^{A}	1.10±0.12^{B}
		Site	1.01±0.43	0.87±1.27	0.91±0.76	0.94±0.63
	Chl_c	Season	1.99±0.11^{A}	2.35±0.19^{B}	2.03±0.07^{A}	2.38±0.21^{B}
		Site	2.16±0.26	2.12±0.19	2.18±0.24	2.28±0.06

All metals were detected in the body of *C. fluminea* in all seasons and sites, except Al that was below the detection limit in the summer and autumn (Table III. 3). Considering the means of total metals concentration in *C. fluminea* body, significant differences among seasons and sites were found (Table III. 4). The highest concentration was found in individuals collected in the winter (1310.87 μg g^{-1} d.w.) and the lowest one in individuals collected in the summer (670.61 μg g^{-1} d.w.). *C. fluminea* individuals from M3 had the highest mean of total metals concentration (1781.52 μg g^{-1} d.w.), while those from L had the lowest one (553.21 μg g^{-1} d.w.) (Table III. 3). Regarding the mean concentrations of individual metals in *C. fluminea* body, in general they were significantly higher in *C. fluminea* individuals from the M-est (M1, M2 and/or M3) than in those from the L-est. The exceptions were: As, and Pb for which the individuals from L had higher mean concentrations than those from M1.

Table III. 2. Results of the Kuskal-Wallis test and significance of temporal and spatial variation of water and sediment parameters. Temperature (T), dissolved oxygen (DO), conductivity (Cond), pH (pH), turbidity (Turb), hardness ($CaCO_3$), ammonium (NH_4^+), nitrates (NO_3^-), nitrites (NO_2^-), phosphates (PO_4^{3-}), phenol (C_6H_5OH), silica (SiO_2) and iron (Fe); and sediment organic matter (OM), granulometry very coarse sand (VCS), coarse sand (CS) medium sand (MS), fine sand (FS), very fine sand (VFS) and silt and clay (S+C), and chlorophylls a, b and c (Chla, Chlb and Chlc). M1, M2 and M3 are sampling sites located in M-est and L in L-est.

		Season		Site	
		X^2	p	X^2	p
Water	T	40.833	0.000	2.092	0.554
	DO	0.452	0.092	5.363	0.147
	Cond	27.767	0.000	15.502	0.001
	pH	18.322	0.000	5.219	0.156
	Turb	3.778	0.289	0.702	0.873
	$CaCO_3$	11.175	0.011	22.265	0.000
	NH_4^+	18.501	0.000	4.433	0.218
	NO_3^-	34.113	0.000	2.865	0.413
	NO_2^-	8.839	0.020	28.496	0.000
	PO_4	25.642	0.000	7.982	0.046
	C_6H_5OH	1.008	0,799	4.266	0.234
	SiO_2	4.486	0.214	16.991	0.001
	Fe	26.187	0.000	3.557	0.313
Sediment	OM	9.314	0.025	9.843	0.020
	VCS	3.829	0.281	30.348	0.000
	CS	1.193	0.755	38.433	0.000
	MS	8.050	0.045	10.928	0.012
	FS	1.822	0.610	40.715	0.000
	VFS	9.902	0.019	20.126	0.000
	S+C	8.872	0.031	10.821	0.013
	Chl_a	12.894	0.005	16.752	0.001
	Chl_b	22.689	0.000	7.754	0.051
	Chl_c	21.062	0.000	5.692	0.128

Table III. 3. Temporal and spatial variation of sediments (bioavailable/extractable fraction) and tissues metal concentrations. Values represent the mean and corresponding standard deviation of Iron (Fe), aluminium (Al), chromium (Cr), copper (Cu), manganese (Mn), nickel (Ni), zinc (Zn), selenium (Se), arsenic (As), cobalt (Co), cadmium (Cd), mercury (Hg) and lead (Pb). All metal concentrations are in μg g^{-1} dry weight. M1, M2 and M3 are sampling sites located in Minho estuary and L in Lima estuary. Different letters identify significant differences among sampling seasons (capital letters) or sites (small letters), as indicated by the Dunn multiple comparison test. <DL means below detection limit.

Metal	Matrix	Factor	Summer M1	Autumn M2	Winter M3	Spring L
Fe	Sediment	Season	274.08±106.21^{B}	214.21±79.68^{AB}	163.14±158.07^{A}	300.89±431.28^{A}
		Site	238.82±117.42	132.22±92.19	372.97±392.28	208.29±165.22
	Tissues	Season	477.13±265.56	761.76±631.50	709.82±453.16	544.54±418.06
		Site	370.24±134.87^{a}	562.79±66.23^{b}	1284.05±352.14^{c}	346.68±104.60^{a}
Al	Sediment	Season	827.75±663.00	530.33±418.80	382.40±217.66	449.76±262.43
		Site	345.30±82.76^{a}	329.03±83.21^{a}	400.96±234.19^{a}	1114.95±490.59^{b}
	Tissues	Season	<DL	<DL	376.06±160.48	442.57±275.32
		Site	419.28±123.44^{b}	442.87±109.46^{b}	582.62±226.78^{b}	139.38±24.68^{a}
Cr	Sediment	Season	1.08±0.36^{C}	0.53±0.24^{B}	0.29±0.08^{A}	0.45±0.25^{AB}
		Site	0.45±0.44^{a}	0.42±0.16^{ab}	0.67±0.38^{b}	0.82±0.47^{b}
	Tissues	Season	0.65±0.32	0.86±0.23	1.48±1.08	0.91±0.53
		Site	1.49±0.89^{b}	0.97±0.30^{b}	1.03±0.32^{b}	0.33±0.16^{a}
Cu	Sediment	Season	4.89±7.74^{B}	0.62±0.22^{A}	1.97±1.06^{B}	2.18±1.68^{B}
		Site	0.90±0.55^{a}	1.17±0.72^{a}	6.29±6.98^{b}	1.29±0.52^{ab}
	Tissues	Season	41.94±9.85^{A}	42.98±13.79^{A}	66.96±14.49^{B}	50.95±11.57^{A}
		Site	51.76±8.37^{b}	62.83±11.72^{b}	53.24±14.50^{b}	34.37±9.24^{a}
Mn	Sediment	Season	113.40±117.83	83.77±81.35	95.11±50.06	83.54±79.07
		Site	72.79±34.31^{a}	49.04±5.12^{a}	42.28±15.84^{a}	211.71±57.10^{b}
	Tissues	Season	27.04±7.20^{AB}	21.29±10.6^{A}	56.84±29.36^{C}	54.12±24.79^{B}
		Site	49.72±36.26^{b}	42.53±30.07^{ab}	44.54±10.70^{b}	19.82±3.16^{a}
Ni	Sediment	Season	3.02±1.13^{B}	2.49±1.41^{AB}	1.78±0.96^{A}	1.62±0.59^{A}
		Site	2.88±0.98^{b}	3.07±0.91^{b}	2.07±0.63^{b}	0.91±0.40^{a}
	Tissues	Season	1.37±0.56^{AB}	0.88±0.31^{A}	1.96±1.33^{B}	2.10±1.30^{B}
		Site	2.36±1.11^{b}	1.94±0.91^{b}	1.54±0.51^{b}	0.34±0.09^{a}
Zn	Sediment	Season	12.47±6.98	7.25±2.84	7.01±1.26	9.27±5.10
		Site	8.31±2.37^{b}	8.95±1.83^{b}	12.71±8.30^{b}	6.03±1.21^{a}
	Tissues	Season	70.07±8.20	72.98±11.55	74.98±12.22	75.38±13.59
		Site	73.71±6.98^{b}	82.49±7.99^{b}	74.31±9.12^{b}	62.51±5.99^{a}
Se	Sediment	Season	0.16±0.13	0.07±0.09	0.14±0.06	0.16±0.14
		Site	0.12±0.03	0.10±0.06	0.08±0.05	0.23±0.15
	Tissues	Season	1.93±0.25	2.08±0.32	2.35±0.25	2.18±0.19
		Site	1.84±0.15^{a}	2.42±0.21^{b}	2.31±0.13^{b}	1.98±0.18^{a}
As	Sediment	Season	1.24±0.64	0.95±0.47	0.66±0.17	1.23±1.20
		Site	0.67±0.16	0.78±0.04	1.42±1.17	1.20±0.71
	Tissues	Season	12.53±2.61	14.77±1.41	16.98±3.75	15.22±2.17
		Site	11.80±1.58^{a}	14.19±1.30^{ab}	18.36±2.76^{c}	15.46±1.14^{bc}
Co	Sediment	Season	1.90±0.53	1.46±0.82	1.39±1.06	1.27±0.4
		Site	2.36±0.56^{c}	1.42±0.46^{b}	1.41±0.63^{b}	0.85±0.25^{a}
	Tissues	Season	1.15±0.29	1.05±0.43	1.52±0.65	1.46±0.77
		Site	1.45±0.48^{b}	1.76±0.47^{b}	1.38±0.18^{b}	0.55±0.18^{a}
Cd	Sediment	Season	0.06±0.01^{C}	0.02±0.01^{B}	0.00±0.00^{A}	0.003±0.00^{AB}
		Site	0.03±0.00	0.01±0.00	0.02±0.02	0.02±0.02
	Tissues	Season	0.72±0.26	0.75±0.23	1.00±0.10	0.83±0.25
		Site	1.00±0.07^{b}	0.74±0.17^{a}	0.67±0.17^{a}	0.84±0.22^{a}
Hg	Sediment	Season				
		Site			<DL	
	Tissues	Season	0.15±0.04^{A}	0.18±0.08^{A}	0.27±0.08^{B}	0.18±0.06^{A}
		Site	0.15±0.04^{a}	0.26±0.07^{b}	0.24±0.08^{b}	0.14±0.05^{a}
Pb	Sediment	Season	2.67±1.04	3.15±2.25	2.02±1.18	2.12±1.40
		Site	1.54±0.24^{a}	1.66±0.25^{a}	4.41±1.35^{b}	2.35±1.26^{a}
	Tissues	Season	0.52±0.26	0.55±0.42	0.66±0.20	0.60±0.37
		Site	0.36±0.17^{a}	0.53±0.18^{b}	0.97±0.21^{c}	0.51±0.10^{b}
Total extractable metals concentration	Sediment	Season	1242.73	844.89	655.93	852.53
		Site	674.22	527.87	845.31	1548.68
	Tissues	Season	670.61	920.11	1310.87	1243.73
		Site	815.70	994.89	1781.52	553.21

Table III. 4. Results of the Kuskal-Wallis test and significance of temporal and spatial variation of body and sediments metal concentrations. Iron (Fe), aluminium (Al), chromium (Cr), copper (Cu), manganese (Mn), nickel (Ni), zinc (Zn), selenium (Se), arsenic (As), cobalt (Co), cadmium (Cd), mercury (Hg) and lead (Pb). M1, M2 and M3 are sampling sites located in M-est and L in L-est.

	Body				Sediments			
	Season		Site		Season		Site	
	X^2	p	X^2	p	X^2	p	X^2	p
Fe	2.255	0.521	32.017	0.000	9.339	0.025	7.554	0.056
Al	0.242	0.622	13.366	0.004	7.552	0.056	25.356	0.000
Cr	7.700	0.053	25.212	0.000	16.883	0.001	11.425	0.010
Cu	15.523	0.001	21.210	0.000	19.340	0.000	11.032	0.012
Mn	17.230	0.001	8.840	0.031	2.512	0.473	27.952	0.000
Ni	8.585	0.035	28.027	0.000	9.270	0.026	23.894	0.000
Zn	0.219	0.975	21.316	0.000	7.658	0.054	9.634	0.022
Se	5.187	0.159	17.953	0.000	3.628	0.305	5.164	0.160
As	6.315	0.097	20.217	0.000	7.299	0.063	4.413	0.220
Co	7.710	0.052	27.189	0.000	6.594	0.086	25.817	0.000
Cd	7.483	0.058	16.086	0.001	32.707	0.000	1.469	0.689
Hg	10.636	0.014	21.283	0.000	0.000	1.000	0.000	1.000
Pb	4.082	0.253	27.585	0.000	3.553	0.314	17.634	0.001

C. fluminea BSAFs calculated for each metal per season and sampling site are shown in Table III. 5. Pb BSAFs were lower than 1 in all seasons and sampling sites. BSAFs higher than 1 were found for Fe, Cu, Zn, Se, As and Cd in all seasons, and for Cr in the autumn, winter and spring; and for Ni and Co in the winter and spring. Concerning sites, BSAFs higher than 1 were found for Fe, Cu, Zn, Se, As and Cd in all seasons; for Al and Cr in all M-est sites; for Mn in M3 and for Co in M2.

Table III. 5. Temporal and spatial variation of biota-sediment accumulation factors (BSAFs). Values represent the BSAFs of Iron (Fe), aluminium (Al), chromium (Cr), copper (Cu), manganese (Mn), nickel (Ni), zinc (Zn), selenium (Se), arsenic (As), cobalt (Co), cadmium (Cd), mercury (Hg) and lead (Pb). M1, M2 and M3 are sampling sites located in Minho estuary and L in Lima estuary.

Metal	Factor	Summer M1	Autumn M2	Winter M3	Spring L	Total average
Fe	Season	1.84	3.56	4.35	1.99	2.84
	Site	1.63	4.26	3.42	1.66	
Al	Season	-	-	0.98	0.98	1.04
	Site	1.28	1.35	1.53	0.13	
Cr	Season	0.62	1.60	5.11	2.07	2.15
	Site	3.51	2.33	1.58	0.40	
Cu	Season	9.29	69.84	33.97	23.35	35.90
	Site	61.95	53.79	8.43	26.59	
Mn	Season	0.22	0.25	0.60	0.65	0.55
	Site	0.68	0.87	1.07	0.09	
Ni	Season	0.41	0.35	1.10	1.30	0.72
	Site	0.80	0.63	0.77	0.38	
Zn	Season	6.03	10.07	10.70	8.07	8.72
	Site	9.53	9.22	5.81	10.37	
Se	Season	11.69	14.91	14.56	10.97	13.69
	Site	13.16	20.54	16.29	7.36	
As	Season	10.95	15.58	25.75	12.60	15.99
	Site	18.95	18.29	12.92	12.87	
Co	Season	0.55	0.72	1.10	1.14	0.87
	Site	0.58	1.24	0.99	0.65	
Cd	Season	13.89	17.30	-	19.99	17.00
	Site	20.71	17.76	12.57	16.76	
Hg	Season	-	-	-	-	-
	Site	-	-	-	-	
Pb	Season	0.17	0.18	0.33	0.30	0.24
	Site	0.19	0.32	0.22	0.23	

3.2.1. Abiotic parameters influencing the concentrations of metals in sediments and *C. fluminea* bioaccumulation factors

The results of the RDA analysis carried out to investigate the relationships between water and sediment parameters and the sediment concentrations of metals is shown in Figure III. 2A. The first two axes explain 99.6% of the total variance (F-ratio = 0.00, p = 0.022, Monte Carlo permutation) with the first canonical axis (horizontal) explaining 92.6%, while the second canonical axis (vertical) explains 7% of the total variance. The first axis separate the summer from the remaining seasons and L from the remaining sites. Fe, Zn, Cu, Pb, As and Cd in sediments are positively associated with very fine sand, the silt and clay fractions, and organic matter content in sediments, and the water hardness in M3 during the spring. The sediments concentration of Co and Ni are associated with sediments medium and fine sand, and water pH and dissolved oxygen, in M1 and M2, and the autumn and winter; Cr, Se, Al and Mn are associated with sediments coarse and very coarse sand, and water ammonium, turbidity, conductivity and temperature, in L and the summer.

The results of the integration of abiotic data and *C. fluminea* BSAFs of different metals through RDA are shown in Figure III. 2B. The first two axes explain 97% of the total variance (F-ratio = 0.00, p = 0.022, Monte Carlo permutation) with the first canonical axis (horizontal) explaining 87.9%, and the second canonical axis (vertical) explaining 9.1% of the total variance. The first axis separates *C. fluminea* individuals collected in M1 and M2 from those collected in M3 and L, and individuals collected in the autumn from those collected in the remaining seasons. The second axis further separates *C. fluminea* individulas collected in winter from those collected in the summer and spring, and individuals collected in M3 from those collected in L. BSAFs of Fe, Cr, As, and in a less extent Se contribute to separate the *C. fluminea* individuals collected in the winter from those collected in the other seasons, and are negatively associated with water ammonium, conductivity and hardness. BSAFs of Pb, Co, Al, Mn and Ni are positively associated with sediment very coarse, coarse and fine sands, and negatively associated with water temperature and organic matter in sediments. These BSAFs also contribute to separate *C. fluminea* individuals collected in the winter from

those collected in the other seasons (although in a less extent than those of Fe, Cr, As). Cu, Zn and in a less extent Se concentrations are positively associated with water pH, medium sand and dissolved oxygen, contributing to separate the autumn from the other seasons, and M1 and M2 from the other sites. Cd BSAF was associated mostly with water turbidity and temperature, and organic matter in sediments.

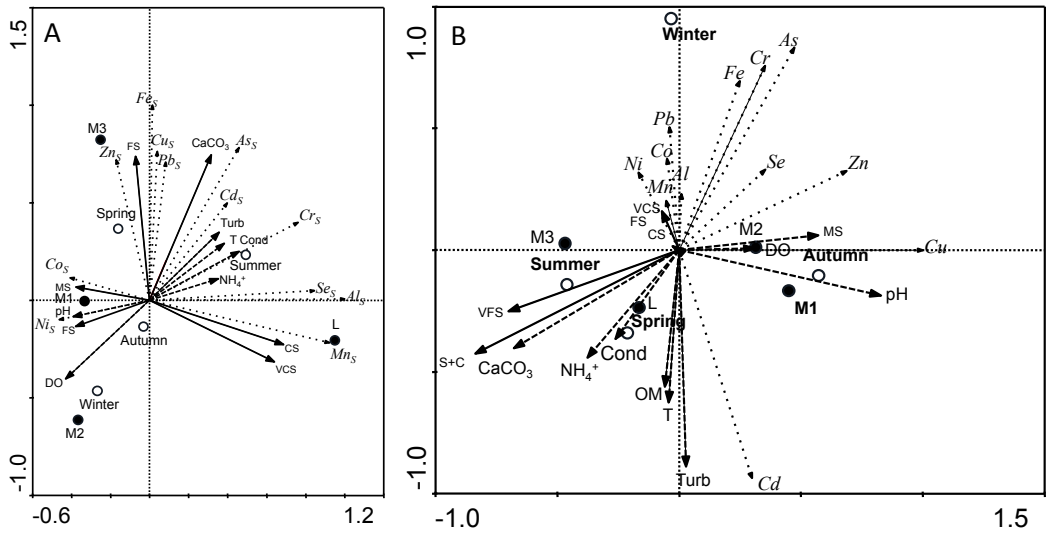


Figure III. 2. Ordination diagrams of RDA analyses showing the relationship between variation in the levels of natural environmental parameters and: (A) the metal concentrations measured in sediments; (B) the biota-sediment accumulation factors (BSAFs). Natural environmental parameters are represented by straight arrows, metal concentrations in sediments and BSFAs are represented by dotted arrows, centroids of the sampling sites are represented by filled circles, and centroids of seasons by open circles. Legend: Iron (Fe), aluminium (Al), chromium (Cr), copper (Cu), manganese (Mn), nickel (Ni), zinc (Zn), selenium (Se), arsenic (As), cobalt (Co), cadmium (Cd), mercury (Hg) and lead (Pb); temperature (T); dissolved oxygen (DO); conductivity (cond); pH; hardness ($CaCO_3$), turbidity (Turb), nitrates (NO_3^-), nitrites (NO_2^-); organic matter (OM) very coarse sand (VCS), coarse sand (CS), medium sand (MS), fine sand (FS), very fine sand (VFS), silt and clay (S+C). M1, M2 and M3 are sampling sites located in Minho estuary and L in Lima estuary.

3.3. Health condition of *C. fluminea* collected in different sites and seasons

The mean and standard deviation of the biomarkers determined along the study are indicated in Table III. 6, and the results of their statistical analysis in Table III. 7.

Table III. 6. Temporal and spatial variation of biochemical biomarkers involved in oxidative stress and damage, detoxification and energy metabolism and condition index. Values represent the mean and corresponding standard deviation of lipid peroxidation levels (LPO), glutathione S-transferase (GST), catalase (CAT), glutathione reductase (GR), esterase (EST), isocitrate dehydrogenase (IDH) and octopine dehydrogenase (ODH) activities and the condition index of 20 *C. fluminea* collected in each sampling season and site. All activities are expressed in nmol min^{-1} mg $protein^{-1}$, except CAT that is expressed in µmol min^{-1} mg $protein^{-1}$. M1, M2 and M3 are sampling sites located in Minho estuary and L in Lima estuary. Different letters identify significant differences among sampling seasons (capital letters) or sites (small letters), as indicated by the Dunn multiple comparison test.

Biomarker	Factor	Summer	Autumn	Winter	Spring
		M1	M2	M3	L
LPO	Season	0.38 ± 0.02^{C}	0.39 ± 0.05^{C}	0.28 ± 0.11^{B}	0.18 ± 0.03^{A}
	Site	0.28 ± 0.12	0.29 ± 0.09	0.33 ± 0.12	0.32 ± 0.06
GST	Season	13.73 ± 2.94^{A}	20.92 ± 3.89^{C}	16.53 ± 2.98^{B}	26.31 ± 4.19^{D}
	Site	21.11 ± 7.26^{b}	21.28 ± 6.17^{b}	17.29 ± 5.14^{a}	18.40 ± 2.23^{a}
CAT	Season	6.22 ± 0.43^{C}	3.69 ± 0.35^{B}	3.39 ± 0.38^{A}	3.37 ± 0.63^{A}
	Site	3.87 ± 1.44	4.29 ± 1.36	4.18 ± 1.71	4.28 ± 1.26
GR	Season	2.20 ± 0.62^{A}	2.75 ± 1.06^{B}	4.86 ± 2.23^{C}	3.09 ± 1.04^{B}
	Site	3.88 ± 1.98^{c}	4.28 ± 2.02^{c}	2.58 ± 0.63^{b}	2.11 ± 0.53^{a}
EST	Season	0.70 ± 0.05^{B}	0.65 ± 0.07^{A}	0.88 ± 0.07^{D}	0.86 ± 0.06^{C}
	Site	0.79 ± 0.11	0.74 ± 0.07	0.77 ± 0.13	0.80 ± 0.21
IDH	Season	5.72 ± 1.28^{B}	4.28 ± 0.65^{A}	5.54 ± 1.16^{B}	4.06 ± 1.34^{A}
	Site	4.86 ± 0.66^{b}	3.91 ± 1.04^{a}	4.72 ± 1.26^{b}	6.38 ± 1.28^{c}
ODH	Season	3.93 ± 0.50^{B}	3.18 ± 1.68^{A}	4.81 ± 0.98^{C}	2.67 ± 1.93^{A}
	Site	2.29 ± 1.42^{a}	3.10 ± 1.84^{b}	4.73 ± 0.27^{c}	4.53 ± 0.74^{c}
Condition index	Season	0.28 ± 0.03^{B}	0.26 ± 0.07^{A}	0.28 ± 0.03^{B}	0.26 ± 0.04^{A}
	Site	0.23 ± 0.01^{a}	0.28 ± 0.03^{b}	0.24 ± 0.03^{a}	0.32 ± 0.03^{c}

For all the biomarkers, significant differences among seasons and sites were found. LPO levels and CAT activity were significantly higher in the

summer and autumn. GST activity was significantly higher in the spring followed by autumn and winter, being significantly lower in the summer. GR activity was significantly lower in the summer and higher in the winter than in the other seasons. EST activity was significantly higher in the winter and spring than in the summer and autumn. Energy related enzymes (IDH and ODH) and the condition index were significantly higher in the summer and winter than in the autumn and spring. Concerning sampling sites, spatial differences were observed for GST, GR, IDH and ODH activities, and the condition index. GST and GR activities were significantly higher in M1 and M2 than in M3 and L. IDH activity and the condition index were significantly higher in L than in M-est sampling sites, whereas the ODH activity was significantly higher in M3 and L than in M1 and M2.

Table III. 7. Results of the Kuskal-Wallis test and significance of temporal and spatial variation of biochemical biomarkers involved in oxidative stress and damage, detoxification and energy metabolism and condition index. Lipid peroxidation levels (LPO), glutathione S-transferase (GST), catalase (CAT), glutathione reductase (GR), esterase (EST), isocitrate dehydrogenase (IDH) and octopine dehydrogenase (ODH) activities and the condition index. M1, M2 and M3 are sampling sites located in M-est and L in L-est.

	Season		Site	
	X^2	p	X^2	p
LPO	173.653	0.000	14.850	0.002
GST	109.181	0.000	42.621	0.000
CAT	127.665	0.000	10.133	0.017
GR	65.103	0.000	116.324	0.000
EST	119.674	0.000	0.165	0.104
IDH	58.953	0.000	78.142	0.000
ODH	62.059	0.000	107.142	0.000
CI	10.273	0.016	158.196	0.000

The results of the integration of biomarkers data through the IBR are shown in Figure III. 3. In all sites, the highest IBR values were found in *C. fluminea* individuals collected in the summer, and in M3 and L. High IBR

values were also found in *C. fluminea* individuals collected in the autumn in M3, and in winter in L. The lowest values per site were recorded in *C. fluminea* individuals collected in spring in M1 and M2, in winter and spring in M3, and in spring in L.

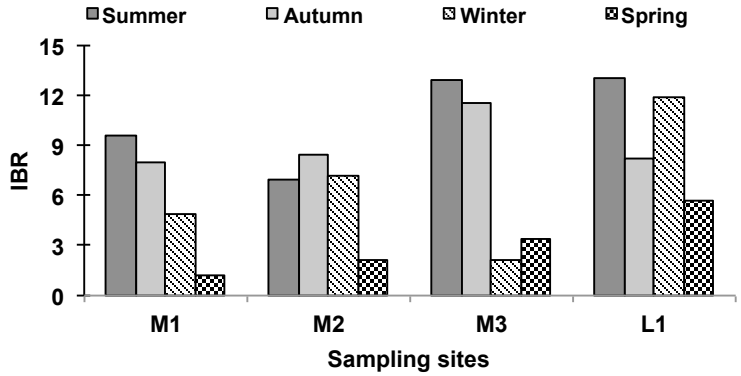


Figure III. 3. Integrated biomarker responses index (IBR) values calculated for clams from each sampling site and season. IBR values were computed using the following biomarkers responses: lipid peroxidation (LPO), glutathione S-transferase (GST), catalase (CAT), glutathione reductase (GR), isocitrate dehydrogenase (IDH), octopine dehydrogenase (ODH) and esterase (EST). Higher values indicate higher stressed condition. M1, M2 and M3 are sampling sites located in Minho estuary and L in Lima estuary.

3.4. Influence of abiotic factors and bioavailable metal concentrations in sediments on biomarkers

The results of RDA analysis integrating the water and sediment parameters, the *C. fluminea* condition index and IBR are indicated in Figure III. 4A. The first canonical axis explains 100% of the total variance (F-ratio = 0.00, p = 0.022, Monte Carlo permutation). It separates M1 and M2 from M3 and L, and the summer and autumn from the spring and winter. M1 was positively associated with fine and very fine sands and pH, M2 with conductivity and organic matter, M3 with temperature and nutrients (nitrites and nitrates) and L with coarse and very coarse sands, ammonium and turbidity. Summer and autumn were positively associated with nutrients

(namely nitrates and nitrites) and temperature; spring was positively associated with dissolved oxygen and organic matter and winter with hardness and conductivity. The IBR was positively associated with nutrients (nitrates, nitrites and ammonium), temperature and turbidity. The condition index was positively associated mainly with coarser sands.

The results of RDA analysis integrating the metal concentrations in sediments and *C. fluminea* body, and the *C. fluminea* condition index and IBR are indicated in Figure III. 4B. The first canonical axis explains 100% of the total variance (F-ratio = 0.00, p = 0.022, Monte Carlo permutation). The first axis separates M1 and M2 sites from L and M3, and the summer and autumn from the spring and winter.

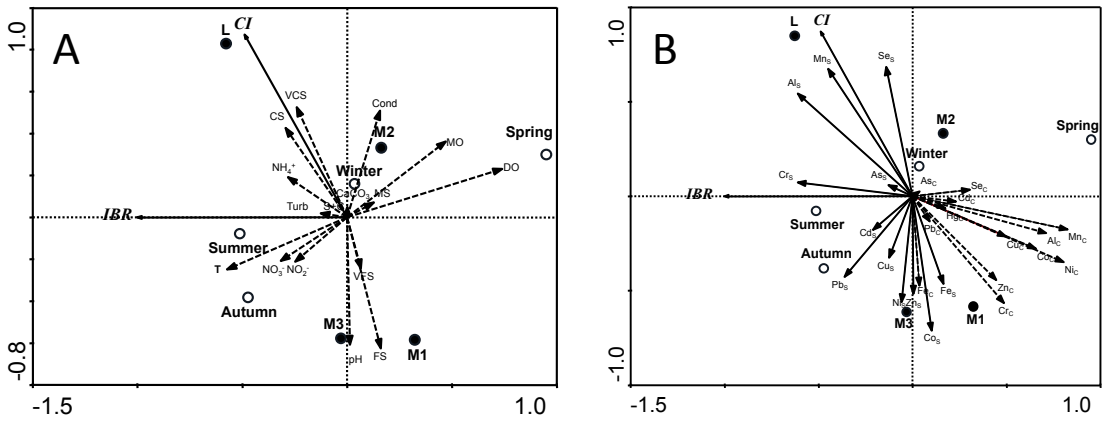


Figure III. 4. Ordination diagrams of RDA analyses showing the relationship between clam's condition index and stress levels (IBR index) and: (A) the natural environmental parameters; (B) the metal concentrations measured in sediments and in tissues. Condition index (CI) and IBR are represented by straight arrows, metal concentrations in clams are represented by dotted arrows and in sediments are represented by straight arrows (bigger than biomarkers), natural environmental parameters are represented by dotted arrows, centroids of the sampling sites are represented by filled circles. Legend: Iron (Fe), aluminium (Al), chromium (Cr), copper (Cu), manganese (Mn), nickel (Ni), zinc (Zn), selenium (Se), arsenic (As), cobalt (Co), cadmium (Cd), mercury (Hg) and lead (Pb); temperature (T); dissolved oxygen (DO); conductivity (cond); pH; hardness ($CaCO_3$), turbidity (Turb), nitrates (NO_3^-), nitrites (NO_2^-); organic matter (OM) very coarse sand (VCS), coarse sand (CS), medium sand (MS), fine sand (FS), very fine sand (VFS), silt and clay (S+C). M1, M2 and M3 are sampling sites located in Minho estuary and L in Lima estuary.

In *C. fluminea* individuals from L sampling sites, the condition index and in a less extent the IBR were positively associated with the concentrations of Se, Al, Mn, Cr and AS in sediments. M3 was positively associated with sediments concentrations of Ni, Zn, Cd, Cu and Pb. M2 was positively associated with the concentrations of As and Se in *C. fluminea* body and M1 with Co and Fe in sediments and Fe, Cr and Zn in *C. fluminea* body. Summer and autumn were positively associated with the concentrations of Cd, Pb and Cu in sediments whereas spring and winter were positively associated with the concentrations of As and Se in the *C. fluminea* body.

4. Discussion

4.1. Brief abiotic characterization of the system over time

The temporal and spatial variation in water and sediments abiotic parameters found in the present study (Table III. 1) are typical of highly dynamic ecosystems such as estuaries (Bettencourt *et al.* 2004) and are in good agreement with the findings of previous studies in M-est and L-est (Ilarri *et al.* 2012; Sousa *et al.* 2008e). Overall, the results of Table III. 1 indicate higher water temperature, conductivity and nutrients concentrations (namely ammonium, nitrites and nitrates) in the summer than in other seasons. These conditions are considered not favourable to *C. fluminea* (Vohman *et al.* 2009; Weitere *et al.* 2009). Moreover, in the summer, the total concentration of metals in sediments was higher than in the other seasons (Table III. 2), in good agreement with the findings of a previous study performed in the M-est (Rubal *et al.* 2009). The results shown in Table III. 3, also indicate a spatial variation of the total metal concentration in sediments, with the following decreasing ranking order of the sampling sites: L (1548.68 μg g^{-1} d.w.) > M3 (845.31 μg g^{-1} d.w.) > M1 (674.22 μg g^{-1} d.w.) > M2 (523.87 μg g^{-1} d.w.). Thus, the highest total metal concentrations in sediments were found in the most downstream sampling sites (L and M3, in L-est and M-est, respectively). Not excluding the possibility of a higher direct input of metals in downstream areas relatively to upstream ones, these findings suggest the accumulation of contaminated sediments dragged way from most upstream areas by the water

flow in L and M3. The retention and accumulation of metals in these sites may be favoured by the relative high contents of very fine sand and silt and clay fractions of local sediments (Table III. 1). For instance, sediments rich in such fractions have a high surface area available for metal adsorption and, in general, are also rich in organic matter, factors that contribute to their high capability to retain metals (Duman *et al.* 2013; Reis *et al.* 2014; Salman *et al.* 2011).

Several studies have been investigating the concentrations of metals in sediments from the estuaries of Minho and Lima Rivers. The mean of total bioavailable metals concentration in sediments determined in the present study are in general lower than those reported in studies performed previously (Gravato *et al.* 2010; Guimarães *et al.* 2012; Reis *et al.* 2009; Reis *et al.* 2014; Rodrigues *et al.* 2014; Rubal *et al.* 2009). However, these studies reported total metals concentrations in Minho basin after strong-acid digestion, and it is known that bioavailable metal concentrations (after weak-acid extraction) are lower (usually <20%) than those of total ones (Reis *et al.* 2009). Sequential extraction schemes are a good compromise that provides a practical method for giving information on environmental contamination risk (Sahuquillo *et al.* 2002). Determinations of broader forms or phases to be measured (*e.g*. "bioavailable" forms of elements, so called ''mobile'' or ''carbonate-bound'' forms, using extraction procedures as step 1 of BCR method) can be a good compromise to give information on environmental contamination risk (Rauret *et al.* 1999; Pueyo *et al.* 2001; Rao *et al.* 2008). This first step is considered the fraction with the higher bioavailability when analysing metals in sediments (Reis *et al.* 2009; Rao *et al.* 2008; González-Flores *et al.* 2011).

4.2. Factors influencing the accumulation of metals by *C. fluminea*

The BSAFs values higher than 1 (Table III. 5) indicate that *C. fluminea* is accumulating several metals in both M-est (Fe, Al, Cr, Cu, Zn, Se, As, Cd and Co in M2 and Mn in M3) and L-est (Fe, Cu, Zn, Se, As and Cd) with the highest BSAF values for Cu, Cd, As and Se. There are several differences between the ranking orders of metal accumulation by *C. fluminea* from all the sampling sites suggesting that metal accumulation strongly depends on the

environmental conditions (Maanan 2008; Shoults-Wilson *et al.* 2009). Nonetheless, in general (except in M3), Cu was the metal with the highest BASF value suggesting that the Asian clam accumulates very efficiently this metal. In fact, a recent study also observed high Cu concentrations bioaccumulated by *C. fluminea* in Minho estuary (Reis *et al.* 2014).

As indicated by the BSAF values lower or near 1 (Table III. 5), *C. fluminea* from the M-est and L-est are not accumulating Al, Mn, Ni, Co and Pb. These results suggest that *C. fluminea* may be able to regulate the concentration of these metals in the body, for example through a reduced absorption and/or efficient elimination among other processes such as the storage of metals in the shells. For instance, Pb was found to be stored in the shell of the Asian clam, *C. fluminea* (Conners *et al.* 1999).

It was observed a spatial and temporal variation of the BSFA values. The positive association between the water dissolved oxygen concentration and the BSAF values (Figure III. 2), suggests that these abiotic factor is influencing the metals accumulation by *C. fluminea*. These findings are in good agreement with the influence of water dissolved oxygen on the accumulation of As by *C. fluminea* found in a previous study (Shoults-Wilson *et al.* 2009). BSAF values were also positively associated with coarser sands and negatively associated with finer sediments (Figure III. 2). Finer sediments have a greater capability of retaining metals (Salman *et al.* 2011), potentially reducing their accumulation by *C. fluminea*.

In summary, *C. fluminea* can accumulate several metals, especially Cu, Cd, As and Se, in its body soft tissues, a characteristics in favour of its use as bioindicator of early contamination of aquatic systems by these metals; the accumulation of several metals by *C. fluminea* showed seasonal as well as spatial variations, increased water dissolved oxygen concentrations, and sediments rich in coarser sands are favourable to the accumulation of metals by *C. fluminea*.

4.3. Heath *status* of *C. fluminea* in relation to abiotic parameters and concentrations of metals

The integrated analysis of biomarkers (IBR index) showed a temporal and spatial variation of *C. fluminea* stress levels (Figure III. 3). In general, higher stress levels were observed in summer/autumn. Environmental conditions observed in summer/autumn have been previously shown to induce a high stress condition that may have led to a possible massive mortality (Oliveira *et al.* 2015a). In fact, LPO levels as well as CAT activity (Table III. 6) were rather higher in summer/autumn decreasing in the following seasons, indicating an attempt to cope with oxidative stress that probably failed since there are higher levels of oxidative damage. Concerning sampling sites, IBR index was generally higher in downstream sampling sites (M3 and L) concomitant with higher sediment metal concentrations, suggesting that a higher exposure to metals induce higher stress levels.

In the integrated analysis, results showed a separation between downstream and upstream sampling sites. The condition index and IBR index were associated with Lima sampling sites and in a less extent with M3 mostly influenced by higher temperatures, organic matter, coarser sands and the silt and clay fraction. When metal concentrations are included in the analysis one can notice that higher stress levels were extremely associated with Mn, Al, Cr, As and Se mostly in L-est. In fact, several of these metals were in much higher concentrations in L-est than in M-est sediments, however the concentration in the tissues was lower. Several detoxification mechanisms, which can range from shell closure and/or reduced filtration rates to induction of metalothionein proteins, may influence the uptake, distribution and elimination of metals (Baudrimont *et al.* 2002; Marigómez *et al.* 2002). Induction of metalothioneins was already observed in *C. fluminea* after exposure to Cd and Zn, which, particularly early in the depuration phase, were shown to decrease nearly 40% (Baudrimont *et al.* 2002). Results suggest that *C. fluminea* inhabiting in L-est can efficiently eliminate and/or avoid excessive concentrations of these particular metals. The mechanisms of metal detoxification and elimination and/or avoidance are known to induce oxidative stress (Valko *et al.* 2005) contributing to higher stress levels that may be

limiting the invasive behaviour of this population, since a strong association was observed between these particular metals and higher IBR values. In addition the energy expended with metal elimination might be also contributing for the low dispersion of *C. fluminea* in L-est since the energy required for the establishment and spread of the population may be redirected to cope with metal contamination.

5. Conclusions

Environmental conditions in summer may be particularly stressful for *C. fluminea*, especially because of an increase in water temperature, conductivity and nutrients concentrations (namely ammonium, nitrites and nitrates) and concentrations of bioavailable metals in sediments. Downstream sampling sites (M3 and L) showed higher concentrations of several metals in sediments (Al, Cr, Cu, Mn, Hg and Pb) that are likely to be more toxic to *C. fluminea* than those found in M1 and M2.

C. fluminea can bioaccumulate concentrations of Cu, Cd, As and Se becoming a good bioindicator of early contamination of aquatic systems by theses metals. Contrariwise, *C. fluminea* seems to efficiently eliminate Al, Mn, Ni, Co and Pb or these metals or may be accumulate them in their shells since no obvious bioaccumulation in the *C. fluminea* body was observed for these metals. Higher metal accumulation was observed in M3 and winter mostly associated to dissolved oxygen concentration and coarser sands indicating that seasonal fluctuations influence the bioaccumulation of metals by *C. fluminea*.

Higher stress levels were observed in summer/autumn mainly induced by an increased water temperature and nutrients concentrations. In winter, higher stress levels were induced by a decreased water quality probably associated to metal resuspension. In addition, IBR index was higher in L-est and the most downstream sampling site in M-est (M3) probably due to the higher concentration of metals that are drawn from upstream. L-est population stress levels were mostly associated with Mn, Al, Cr, As and Se concentrations probably due to the mechanisms of detoxification, elimination or/and avoidance of these metals that may be stressful, since lower

concentrations were observed in their bodies compared with M-est *C. fluminea* population. Mechanisms of metal elimination and/or avoidance seem to be limiting the invasive behaviour of this population. In addition the energy expended with metal elimination might be also contributing for the low dispersion of *C. fluminea* in L-est since the energy required for the establishment and spread of the population may be redirected to cope with metal contamination.

C. fluminea, showed a high ability to bioaccumulate several metals and high tolerance to high metal concentrations in their tissues proving to be useful for bioremediation programs in locations previously invaded by this species, since is spread in several rivers around the world, however, always taking special care not to promote their further dispersion. In addition, the analysis of heavy metals concentrations in *C. fluminea* body inhabiting in M-est and L-est indicated safe levels for human consumption in M-est and L-est.

Acknowledgments

This research was partially supported by the European Regional Development Fund (ERDF) through the COMPETE - Operational Competitiveness Programme and national funds through FCT – Foundation for Science and Technology, under the projects NISTRACKS (PTDC/AAC-AMB/102121; FCOMP-01-0124-FEDER-008556) and PEst-C/MAR/LA0015/2013. C. Oliveira had a PhD fellowship from FCT (SFRH/BD/68423/2010), supported by “Programa Operacional Potencial Humano do QREN Portugal 2007-2013” and national founds from the “Ministério da Ciência e Tecnologia e Ensino Superior” (MCTES – POPH-QREN-Tipologia 4.1).

Chapter IV

Histological study of the gonadal development cycle of *Corbicula fluminea* and its relationship with spatial and temporal variation of environmental parameters

Abstract

The freshwater clam *Corbicula fluminea* is a non-indigenous invasive species in Europe and several other regions of the world. The reproductive outcome of *C. fluminea* populations is determinant for their successful establishment in new areas and likely influences their invasive behaviour in colonized habitats. More knowledge on the reproductive cycle in distinct regions of the world and on the environmental factors affecting it are needed to prevent new invasions and mitigate the adverse impacts in ecosystems already colonized. Thus, the goals of the present study were to investigate the gonads developmental cycle of *C. fluminea* populations inhabiting two neighbour estuaries of the Iberian Peninsula that have been showing distinct invasive behaviours and to identify the abiotic factors influencing the process in estuarine tidal freshwater areas (TFA). From October 2011 to November 2012, monthly, 10 *C. fluminea* specimens were collected in three sampling sites of the Minho River estuary (M-est) and one site in the Lima River estuary (L-est). From each individual, the gonads were isolated and prepared for histological analysis to determine the sex and the gonadal phase. Gonadal development cycle data were analysed in relation to water and sediments parameters variation. Although the gametogenic condition of the follicle did not appear to change over the year, it was observed a seasonal pattern in the relative percentage of hermaphrodites and empty follicles most probably indicative of higher reproductive periods. Results showed higher hermaphrodites percentages among all sampling sites in spring associated with greater availability of food. Because in *C. fluminea*, the fertilization requires the simultaneous occurrence of oocytes and sperm, these findings suggest synchronization between the peaks of fertilization and availability of nutritional resources that are crucial for larval survival. Generally, *C. fluminea* population inhabiting in both estuaries showed higher relative percentage of hermaphrodite individuals, and, therefore, higher reproductive periods, in autumn and spring and no

important differences were observed between M-est and L-est populations indicating that gonadal development cycle do not contribute, most likely, to the differences observed between their invasive behaviours. However, reproductive output also includes the number of juveniles formed and their release and survival, which may be contributing to the invasive behaviour differences observed between M-est and L-est.

The integrated analysis of data indicated an association between high percentages of hermaphroditism and high concentrations of nutrients, organic matter and chlorophylls, suggesting a good synchronism between fertilization/spawning and food availability that is crucial for larvae survival and performance.

Key words: *Corbicula fluminea*; Invasive behaviour; Reproduction; environmental factors; Pollution

1. Introduction

The freshwater clam *Corbicula fluminea*, native in south-eastern Asia, has been introduced globally around the world and is generally considered to be an aquatic non-indigineous invasive species (NIS) of pest *status* in several regions, including in Europe (Denton *et al.* 2012). *C. fluminea* is known to be a highly invasive species mostly due to their reproductive characteristics such as high fecundity, and early sexual maturity (Sousa *et al.* 2008a). The reproductive success is crucial for the establishment of the species in new habitats and their dispersal (Brockerhoff *et al.* 2014; Kamburska et al. 2013). Most studies observed that *C. fluminea* reproduces twice a year in temperate regions: one in the spring and the other starting in late summer (Mouthon 2001; Sousa *et al.* 2008a; Wittmann *et al.* 2008). However, some studies found differences in the number of reproductive periods (Denton *et al.* 2012; Doherty *et al.* 1987; Rajagopal *et al.* 2000). For example, Denton *et al.* (2002) only found one reproductive period in late summer whereas Doherty *et al.* (1987), Rajagopal *et al.* (2000), observed two reproductive periods. Natural factors, such as temperature, salinity, food availability and contamination may perturb or alter gametogenesis, spawning periods and other steps of the reproductive cycle of *C. fluminea* (Cataldo and Boltovskoy 1999; Cataldo *et al.* 2001; Lehmann *et al.* 2007; Mouthon 2001; Morgan *et al.* 2003; Rajagopal *et al.* 2000). Reaching certain temperature levels is crucial to initiates several stages of *C. fluminea* reproduction (Rajagopal *et al.* 2000; Mouthon and Parghentanian 2004). For example, spawning usually initiates at temperatures around 18ºC, however, it ceases when temperature reaches around 28ºC and a weaker spawning typically occurs after a decrease of temperatures (Byrne *et al.* 2000; Denton *et al.* 2012; Rajagopal *et al.* 2000; Mouthon and Parghentanian 2004). Incubation of embryos and release of juveniles are also modulated by temperature fluctuations (Baba *et al.* 1999; Byrne *et al.* 2000; McMahon 1982). Although temperature is rather important to trigger several stages of reproduction, food availability is also important for gonad development, fecundity, embryo development and successful brooding, and increases both the number of embryos and the individual size of developing embryos (Beekey and Karlson 2003; Doherty *et al.* 1987; Mouthon 2001). Salinity range may also have important effects on larval survival and development (Baba *et al.* 1999). Environmental contamination is another parameter that might seriously affect the reproduction of *C. fluminea.* For

example, gonadal atrophy was observed after exposure to a polychlorinated biphenyl compound (Lehmann *et al.* 2007).

The populations of *C. fluminea* inhabiting the tidal freshwater areas of the estuaries of Minho (M-est) and Lima (L-est) Rivers (NW Iberian Peninsula), two neighbour estuaries, have been showing different invasive behaviours (Sousa *et al.*, 2006a; Sousa *et al.*, 2008b,d). Because so far, no significant genetics difference between the two populations were found, their comparative study may provide valuable insides on the influence of environmental factors on biological aspects determinant for the invasive behaviour of *C. fluminea* populations and thus to control it and/or mitigate the adverse effects of the invasions by this NIS. The first record of *C. fluminea* in the M-est was in 1989 (Araújo *et al.*, 1993). Since then, the population has been increasing and colonized practically all the TFA, reaching an extraordinary abundance and biomass (Sousa *et al.*, 2008b). Contrariwise, in the L-est, the species was recorded for the first time in 2002, and only with a sparse and spotted distribution with low abundance and biomass (Sousa *et al.*, 2006b). This population is believed to be in a lag time phase, possibly due to environmental conditions limiting its dispersion (e.g. salinity, chemical contamination) (Sousa *et al.* 2006a). These factors may limit the invasive behaviour of the population acting negatively on its reproductive success, for example on gametogenesis, fertilization, recruitment and/or juvenile development. The difficulty in finding individuals with less than 13 mm of shell length (Sousa *et al.* 2006a) supports this hypothesis. Thus, the main goal of the present study is to document and compare the gonads development cycle of M-est and L-est *C. fluminea* populations in relation to abiotic variation, including environmental contamination by metals.

2. Material and Methods

2.1. Chemicals

All the chemicals used were of analytical grade and purchased from Sigma-Aldrich (Germany) or Merck (Germany).

2.2. *C. fluminea* collection and tissue preparation

C. fluminea specimens were collected monthly over 14 months (from October 2011 to November 2012) in four sampling sites (10 individuals per sampling site and month): 3 sites in the M-est (M1, M2, M3) and one site in L-est (L) with a hand rake with a net at low tide. Water and sediments from the different sampling months and sites were collected at the same time as *C. fluminea* individuals. Water temperature, dissolved oxygen, conductivity and pH were measured *in situ* and water samples were collected, in triplicate. *C. fluminea* individuals were transported to the laboratory as soon as possible in a container thermally isolated with aeration. In the laboratory, they were measured (caliper 0 – 150 mm) weighted (balance), and sacrificed under cold-induced anaesthesia. The visceral mass of each animal that contains the gonad tissue was separated and fixed in 4% formalin buffered with 0.1 M phosphate buffer for 24 h. After this period, fixed visceral mass samples were placed in 70% of ethanol aqueous solution until further analysis. Fixed visceral mass samples were dehydrated by passing the tissue through a series of increasing alcohol concentrations (76%, 90% and 100%), cleared in methylbenzoate (≥ 99% purity) and rinsed in benzene (≥ 99% purity) in an autostainer (ST5010 Autostainer XL, Germany) and embedded in paraffin. Longitudinal sections with 5 µm thick (2 per visceral mass sample) were cut, always in, approximately, the middle of the sample, using a rotary microtome (Leitz 1512,Ernest Leitz Wetzlar GmbH, Austria) and stained with hematoxyline and eosine (ST5010 Autostainer XL, Germany). Slides were observed by optical microscopy (Leitz Laborlux S light microscope, Germany) to determine the sex and the gonadal phase. Because, according previous works with this species, oogenesis continues throughout almost the entire year with different size classes of oocytes always present in the follicles (Kraemer and Galloway 1986), only three gonadal phases were considered, namely phases A, B and C (Figure 1). Individuals were considered to be in phase A, when in both

gonadal sections all the follicles contained filled oocytes only (in pre-vitellogenesis and in vitellogenesis with cytoplasm, nucleus and nucleolus well-defined); Specimens were considered to be in phase B when both gonadal sections contain filled follicles with both oocytes (in pre-vitellogenesis and in vitellogenesis with cytoplasm, nucleus and nucleolus well-defined) and sperm cells (spermatogonia along the follicular walls and sperm cells approaching the follicle centre), and/or some follicles contained oocytes (in the stages previously indicates) only, whereas other contained only sperm cells (as previously described); in both cases, these individuals were considered hermaphrodites. Finally, individuals were considered to be in phase C when in both gonadal sections contains follicles almost empty (C) suggesting a post-spawning and/or resting phase.

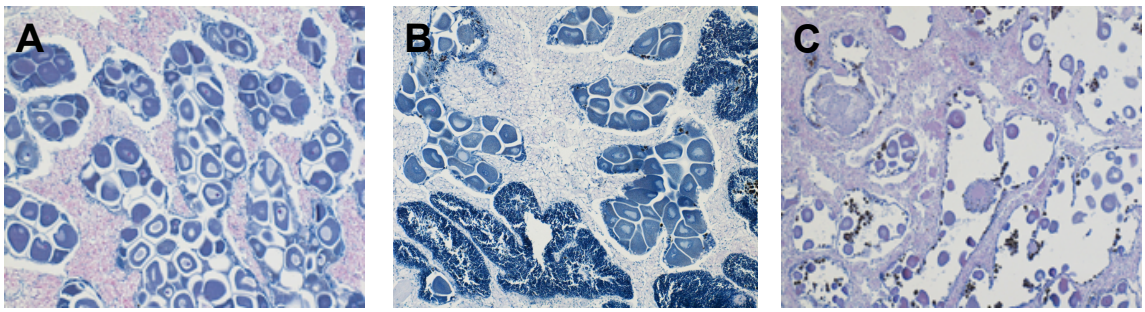


Figure IV. 1. *Corbicula fluminea* gonad sections stained with eosine-hematoxyline (with a 5x amplification). A – Gonads containing follicles with only oocytes; B – Gonads containing follicles with oocytes and sperm cells; and C – Gonads containing empty follicles.

2.3. Water and sediment analysis

In the water and sediment samples collected simultaneously to *C. fluminea*, the following parameters were determined as described in *Chapter III*: water hardness, turbidity and the concentration of ammonium, nitrates, nitrites, phosphates, iron, phenol and silica and sediments organic matter, chlorophylls *a*, *b* and *c* and granulometry.

2.4. Data analysis

Data from each water abiotic and sediment physico-chemical parameter was checked for normality of distribution and homogeneity of variances before the Analysis of Variance (ANOVA) (Zar, 1999). When these assumptions could not be full field even after data transformation, the non-parametric Kruskal–Wallis comparison test was used to compare different months and sites. When significant differences were found, the Dunn's multicomparison test was used to discriminate statistically significant treatments. The Statistics 18.0 package was used and the significance level was 0.05.

Data from gonadal development cycle phases and temporal significant water abiotic and sediment parameters simultaneously to the gonads development cycle, as well as metal concentrations both in *C. fluminea* tissues and sediments (discussed in *Chapter III*) were integrated through a Redundancy Analysis (RDA), using a model-based type of Monte Carlo permutation test (ter Braak and Prentice, 1986; ter Braak and Smilauer, 2002). In order to include metal concentrations, that at alongside with natural factors, also influence reproduction (Lehmann *et al.* 2007; Sousa *et al.* 2008a) the multivariate analysis was performed with the results obtained in October 2011, January 2012 and April 2012 (that is seasonally in autumn, winter and spring) that were the months in which samples were collected together with those collected for metal analysis. Variables included in the analysis were: gonadal development phases; IBR values (since stress condition may influence reproductive processes and vice versa); water temperature, conductivity, pH, hardness and ammonium, nitrates, nitrites, phosphates and iron concentrations; sediment organic matter, medium sand and silt and clay fraction contents and total chlorophylls; *C. fluminea* tissue concentrations of copper, manganese, nickel and mercury; and sediment concentrations of iron, chromium, copper, nickel and cadmium. Environmental parameters were chosen based on seasonal significant differences obtain in data analysis performed in *Chapter III*.

3. Results and Discussion

3.1. Natural environmental parameters variation

The results of abiotic water and physic-chemical sediments parameters indicate important spatial and temporal variability in environmental conditions (Tables IV. 1 and 2, respectively). Significant temporal variations were found for water temperature, dissolved oxygen, pH, nitrates and iron (Table IV. 3). The results showed that the highest water temperatures were observed in July, August and September and the lowest ones in January and February. Water dissolved oxygen concentrations showed, in general, an opposite trend with lower values in June and higher in April. The combination of higher temperatures and lower water dissolved oxygen concentrations should be highlighted since the combination of these two factors is particularly stressful to *C. fluminea* and bivalves in general (Cherry *et al.*, 2005; Cooper *et al.*, 2005; Johnson and McMahon, 1998; Vohmann *et al.*, 2009; Weitere *et al.*, 2009; Werner and Rothhaupt, 2008).

Spatial significant differences were found for water conductivity, hardness, nitrites, phosphates and silica and for sediment granulometry and total chlorophylls concentration (Table IV. 2). Spatial differences generally reveals an increasing gradient (upstream to downstream): water conductivity, hardness and phosphate concentration and sediment silt and clay fraction and total chlorophyll concentration were, in general, higher in downstream sampling sites (both M3 and L), suggesting that these sites are under a higher tidal influence than M1 and M2, in good agreement with findings of previous studies (Ilarri *et al.*, 2012; Sousa *et al.*, 2008a,d). The sediment grain size, in general, decreased from upstream to downstream in the M-est, whereas L-est sediments had higher percentage of coarser sands and silt and clay fraction, and lower percentage of intermediate grain sizes than the M-est sites. Finer sediments may be resuspended and dragged to locations further downstream where they are deposited, increasing the percentage of fines in these locations (Ortiz *et al.* 2013).

Table IV. 1. Abiotic water variables values determined for each sampling site and month in the tidal freshwater area of M-est and L-est, from October 2011 to November 2012. For each parameter, the values are the mean with the corresponding standard error within brackets. Temperature (T, ºC), dissolved oxygen (DO, mg/l), conductivity (Cond, µS/cm), pH (pH units), nitrites (NO_2^-, mg l/1), nitrates (NO_3^-, mg/l), ammonium (NH_4^+, mg/l), phosphates (PO_4^{3-}, mg/l), silica (SiO_2, mg/l), phenol (C_6H_5OH, mg/l), iron (Fe, mg/l), hardness ($CaCO_3$, mg/l) and turbidity (Turb, FTU).

Month	Site	T	DO	Cond	pH	Turb	$CaCO_3$	NH_4^+	NO_3^-	NO_2^-	PO_4	C_6H_6O	Si	Fe
Oct 11	M1	19.03	9.21	85.67	9.90	6.67	31.67	0.13	0.40	0.00	0.04	0.06	7.24	0.02
		(0.46)	(0.13)	(2.52)	(0.20)	(5.93)	(1.67)	(0.11)	(0.08)	(0.00)	(0.01)	(0.00)	(0.18)	(0.01)
	M2	20.50	8.35	122.67	7.47	6.67	54.44	0.08	0.26	0.01	0.02	0.09	1.65	0.03
		(0.40)	(0.08)	(0.58)	(0.03)	(4.67)	(4.19)	(0.09)	(0.03)	(0.00)	(0.01)	(0.03)	(0.10)	(0.02)
	M3	20.87	9.67	987.00	7.93	1.33	155.00	0.14	0.19	0.01	0.11	0.06	2.09	0.02
		(0.15)	(0.12)	(12.49)	(0.08)	(1.15)	(15.00)	(0.13)	(0.03)	(0.00)	(0.06)	(0.01)	(0.06)	(0.01)
	L	19.13	7.58	824.33	8.08	5.33	90.56	0.22	0.21	0.00	0.07	0.09	2.13	0.03
		(0.21)	(0.33)	(49.52)	(0.26)	(4.16)	(9.48)	(0.10)	(0.02)	(0.00)	(0.02)	(0.01)	(0.14)	(0.00)
Nov 11	M1	14.20	9.50	93.67	6.82	3.56	22.22	0.01	0.31	0.00	0.04	0.11	5.95	0.02
		(0.26)	(0.36)	(0.58)	(0.08)	(3.15)	(2.55)	(0.01)	(0.10)	(0.00)	(0.02)	(0.03)	(0.66)	(0.00)
	M2	14.73	8.40	110.00	7.20	10.44	53.89	0.21	0.34	0.01	0.12	0.08	3.20	0.05
		(0.15)	(0.10)	(0.00)	(0.02)	(0.38)	(11.10)	(0.08)	(0.01)	(0.00)	(0.03)	(0.03)	(0.13)	(0.03)
	M3	14.50	8.41	505.67	7.28	3.44	88.89	0.27	0.24	0.01	0.09	0.09	3.00	0.03
		(0.30)	(0.12)	(5.59)	(0.02)	(2.01)	(2.55)	(0.08)	(0.04)	(0.00)	(0.01)	(0.01)	(0.13)	(0.01)
	L	14.20	8.27	496.33	6.29	1.78	55.56	0.02	0.22	0.00	0.04	0.11	1.04	0.03
		(0.10)	(0.06)	(15.95)	(0.06)	(1.68)	(5.09)	(0.02)	(0.02)	(0.00)	(0.02)	(0.06)	(0.17)	(0.01)
Dec 11	M1	12.67	9.21	85.67	7.49	3.33	32.78	0.12	0.35	0.01	0.05	0.09	3.18	0.02
		(0.83)	(0.13)	(1.53)	(0.19)	(3.06)	(3.47)	(0.06)	(0.03)	(0.00)	(0.01)	(0.03)	(0.47)	(0.01)
	M2	12.67	8.35	107.33	7.68	2.89	33.33	0.06	0.41	0.01	0.05	0.06	2.64	0.04
		(0.21)	(0.08)	(5.69)	(0.17)	(3.42)	(4.41)	(0.01)	(0.07)	(0.00)	(0.01)	(0.01)	(0.94)	(0.02)
	M3	12.83	9.67	500.67	7.33	2.67	105.00	0.42	0.29	0.01	0.17	0.10	3.60	0.04
		(0.35)	(0.12)	(3.51)	(0.09)	(1.76)	(35.00)	(0.17)	(0.05)	(0.00)	(0.12)	(0.02)	(0.73)	(0.01)
	L	13.87	8.05	676.67	6.38	0.67	18.33	0.22	0.37	0.00	0.04	0.06	0.65	0.03
		(0.32)	(0.08)	(40.41)	(0.08)	(1.15)	(0.00)	(0.06)	(0.03)	(0.00)	(0.00)	(0.01)	(0.05)	(0.03)
Jan 12	M1	9.17	9.36	75.33	7.81	2.22	23.33	0.04	0.41	0.00	0.06	0.08	4.51	0.03
		(0.12)	(0.19)	(0.58)	(0.05)	(3.29)	(5.00)	(0.01)	(0.14)	(0.00)	(0.01)	(0.01)	(1.32)	(0.00)
	M2	9.10	9.59	105.67	7.67	0.67	42.22	0.04	0.69	0.01	0.07	0.07	3.85	0.02
		(0.10)	(0.39)	(0.58)	(0.24)	(1.15)	(2.55)	(0.01)	(0.05)	(0.00)	(0.01)	(0.02)	(0.42)	(0.01)
	M3	8.67	9.27	494.00	7.53	6.67	73.33	0.05	0.47	0.01	0.09	0.07	5.05	0.03
		(0.15)	(0.33)	(2.00)	(0.02)	(4.67)	(11.67)	(0.01)	(0.05)	(0.00)	(0.03)	(0.04)	(0.21)	(0.02)
	L	9.23	10.72	257.00	6.61	0.22	46.67	0.02	0.37	0.00	0.07	0.08	1.14	0.01
		(0.06)	(0.42)	(10.54)	(0.01)	(0.38)	(8.33)	(0.02)	(0.03)	(0.00)	(0.01)	(0.02)	(0.20)	(0.01)
Feb 12	M1	9.73	9.72	77.33	7.45	1.44	24.44	0.04	0.36	0.00	0.05	0.08	3.89	0.02
		(0.12)	(0.79)	(0.58)	(0.10)	(0.69)	(2.55)	(0.01)	(0.07)	(0.00)	(0.01)	(0.00)	(0.47)	(0.01)
	M2	8.50	8.87	101.33	7.82	1.78	40.56	0.05	0.50	0.01	0.09	0.06	3.93	0.01
		(0.20)	(0.71)	(0.58)	(0.04)	(2.04)	(10.18)	(0.03)	(0.05)	(0.00)	(0.02)	(0.05)	(0.61)	(0.01)
	M3	9.57	8.91	482.00	7.92	0.89	81.67	0.34	0.57	0.01	0.12	0.12	3.87	0.03
		(0.32)	(1.05)	(2.00)	(0.01)	(1.54)	(5.77)	(0.05)	(0.02)	(0.00)	(0.07)	(0.03)	(0.05)	(0.01)
	L	10.97	10.21	866.67	7.65	1.11	91.67	0.01	0.33	0.00	0.05	0.08	2.58	0.03
		(0.31)	(0.33)	(1.53)	(0.04)	(0.38)	(5.00)	(0.01)	(0.07)	(0.00)	(0.02)	(0.02)	(0.36)	(0.01)
Mar 12	M1	12.00	9.00	80.00	7.17	8.00	22.22	0.07	0.43	0.00	0.04	0.06	5.06	0.02
		(0.00)	(0.00)	(2.53)	(0.06)	(3.06)	(7.52)	(0.05)	(0.08)	(0.00)	(0.01)	(0.03)	(0.90)	(0.01)
	M2	12.00	8.00	100.93	7.50	7.78	36.11	0.04	0.42	0.01	0.05	0.07	2.52	0.03
		(0.00)	(0.00)	(6.59)	(0.26)	(3.42)	(5.85)	(0.01)	(0.07)	(0.00)	(0.02)	(0.01)	(0.24)	(0.01)
	M3	10.67	7.00	492.00	7.50	5.78	138.89	0.07	0.34	0.00	0.09	0.09	2.57	0.01
		(0.58)	(0.00)	(11.28)	(0.10)	(3.91)	(8.39)	(0.03)	(0.07)	(0.00)	(0.07)	(0.01)	(0.12)	(0.01)
	L	11.67	7.00	983.00	7.40	7.33	95.00	0.08	0.24	0.00	0.04	0.04	1.82	0.02
		(0.58)	(0.00)	(2.53)	(0.10)	(4.16)	(8.82)	(0.01)	(0.02)	(0.00)	(0.03)	(0.02)	(0.19)	(0.01)
Apr 12	M1	11.83	10.08	93.33	7.67	6.22	56.67	0.14	0.24	0.01	0.08	0.08	2.96	0.04
		(0.12)	(0.42)	(1.15)	(0.23)	(3.67)	(0.00)	(0.02)	(0.01)	(0.00)	(0.01)	(0.03)	(0.06)	(0.01)
	M2	13.37	10.51	102.33	7.66	1.33	50.56	0.17	0.47	0.01	0.06	0.10	2.63	0.07
		(0.06)	(0.13)	(1.15)	(0.01)	(2.31)	(2.55)	(0.09)	(0.02)	(0.00)	(0.02)	(0.02)	(0.08)	(0.05)
	M3	14.60	9.69	558.33	7.58	5.56	106.67	0.18	0.48	0.01	0.11	0.09	2.43	0.08
		(0.00)	(0.13)	(9.71)	(0.04)	(3.42)	(14.24)	(0.01)	(0.03)	(0.00)	(0.02)	(0.04)	(0.33)	(0.02)
	L	14.43	10.75	3316.67	7.10	5.78	285.00	0.22	0.29	0.00	0.72	0.05	5.05	0.07
		(0.06)	(0.42)	(90.74)	(0.04)	(2.14)	(6.67)	(0.02)	(0.04)	(0.00)	(0.03)	(0.02)	(0.29)	(0.05)

Table IV. 1. *Continued…*

Month	Site	T	DO	Cond	pH	Turb	$CaCO_3$	NH_4^+	NO_3^-	NO_2^-	PO_4	C_6H_6O	Si	Fe
May 12	M1	13.33 (0.21)	7.35 (0.33)	88.67 (7.23)	6.95 (0.01)	3.33 (2.91)	46.11 (0.96)	0.93 (0.90)	0.64 (0.03)	0.00 (0.00)	0.12 (0.02)	0.02 (0.01)	5.55 (0.12)	0.07 (0.02)
	M2	14.20 (0.26)	6.88 (0.60)	96.33 (1.53)	7.02 (0.01)	5.56 (3.08)	36.11 (6.31)	0.59 (0.04)	0.42 (0.04)	0.02 (0.02)	0.07 (0.02)	0.04 (0.03)	3.03 (0.49)	0.03 (0.01)
	M3	13.40 (0.17)	6.17 (0.30)	138.33 (2.31)	6.91 (0.01)	1.33 (2.31)	27.22 (11.34)	0.08 (0.02)	0.39 (0.03)	0.01 (0.00)	0.09 (0.03)	0.19 (0.01)	4.62 (0.18)	0.05 (0.01)
	L	13.90 (0.10)	7.58 (0.07)	446.33 (0.58)	6.55 (0.01)	4.44 (1.68)	33.89 (7.70)	0.10 (0.02)	0.36 (0.03)	0.00 (0.00)	0.05 (0.01)	0.07 (0.03)	2.32 (0.33)	0.03 (0.00)
Jun 12	M1	18.10 (0.17)	6.52 (0.04)	87.33 (1.15)	7.27 (0.03)	3.33 (2.40)	21.67 (4.41)	0.03 (0.00)	0.23 (0.02)	0.01 (0.00)	0.03 (0.01)	0.03 (0.01)	6.17 (1.22)	0.04 (0.02)
	M2	18.27 (0.06)	6.94 (0.01)	91.67 (0.58)	7.50 (0.11)	2.22 (1.68)	38.33 (0.00)	0.93 (0.33)	0.21 (0.04)	0.02 (0.00)	0.06 (0.02)	0.08 (0.02)	3.11 (0.02)	0.05 (0.01)
	M3	19.00 (0.00)	6.53 (0.01)	495.67 (4.73)	7.17 (0.01)	4.44 (2.14)	76.11 (5.85)	0.18 (0.03)	0.35 (0.03)	0.01 (0.00)	0.06 (0.01)	0.07 (0.01)	2.66 (0.13)	0.05 (0.01)
	L	14.20 (0.66)	6.33 (0.55)	404.00 (2.00)	6.89 (0.03)	2.22 (1.54)	258.89 (33.35)	0.80 (0.03)	0.25 (0.05)	0.00 (0.00)	0.08 (0.04)	0.07 (0.03)	1.81 (0.17)	0.04 (0.01)
Jul 12	M1	19.60 (0.10)	7.70 (0.17)	90.00 (3.46)	7.08 (0.07)	7.78 (2.52)	21.11 (2.55)	0.11 (0.03)	0.22 (0.01)	0.01 (0.00)	0.05 (0.00)	0.06 (0.01)	2.08 (0.33)	0.04 (0.02)
	M2	20.30 (0.10)	7.66 (0.55)	92.67 (0.58)	6.86 (0.20)	1.33 (2.31)	50.00 (15.28)	0.07 (0.01)	0.31 (0.01)	0.01 (0.00)	0.07 (0.02)	0.07 (0.01)	2.07 (0.30)	0.05 (0.04)
	M3	20.63 (0.15)	7.09 (0.10)	3120.00 (10.00)	7.98 (0.03)	2.89 (2.69)	364.44 (3.85)	0.20 (0.06)	0.34 (0.03)	0.00 (0.00)	0.14 (0.04)	0.06 (0.03)	2.93 (0.13)	0.04 (0.00)
	L	20.57 (0.06)	7.22 (0.01)	2356.67 (20.82)	7.65 (0.05)	1.11 (1.02)	245.00 (15.28)	0.30 (0.33)	0.13 (0.02)	0.00 (0.00)	0.01 (0.02)	0.05 (0.03)	5.08 (0.29)	0.00 (0.00)
Aug 12	M1	21.90 (0.10)	9.21 (0.13)	92.67 (0.58)	6.47 (0.08)	2.89 (3.01)	37.78 (1.92)	0.11 (0.01)	0.25 (0.01)	0.01 (0.00)	0.05 (0.01)	0.08 (0.05)	2.51 (0.44)	0.06 (0.03)
	M2	21.93 (0.06)	8.35 (0.08)	111.67 (0.58)	7.10 (0.77)	3.56 (1.54)	38.89 (4.19)	0.19 (0.03)	0.24 (0.01)	0.01 (0.00)	0.07 (0.02)	0.08 (0.02)	2.59 (0.22)	0.03 (0.00)
	M3	22.70 (0.10)	9.67 (0.12)	428.67 (3.21)	7.31 (0.12)	3.33 (3.33)	68.33 (7.64)	0.03 (0.01)	0.25 (0.01)	0.01 (0.00)	0.09 (0.01)	0.13 (0.05)	2.01 (0.26)	0.02 (0.01)
	L	21.13 (0.23)	7.58 (0.33)	8860.00 (121.24)	6.11 (0.05)	2.00 (1.76)	420.00 (60.09)	1.86 (0.32)	0.27 (0.03)	0.00 (0.00)	0.05 (0.02)	0.10 (0.06)	3.30 (0.07)	0.02 (0.00)
Sept 12	M1	19.40 (0.10)	9.30 (0.36)	82.00 (2.00)	7.47 (0.21)	2.00 (2.31)	29.44 (7.52)	0.00 (0.00)	0.26 (0.02)	0.00 (0.00)	0.04 (0.03)	0.06 (0.06)	3.09 (0.12)	0.15 (0.06)
	M2	21.50 (0.26)	9.77 (0.06)	107.33 (0.58)	7.80 (0.20)	2.44 (2.52)	30.56 (3.47)	0.03 (0.01)	0.25 (0.01)	0.00 (0.00)	0.11 (0.02)	0.14 (0.06)	2.78 (0.08)	0.03 (0.01)
	M3	22.33 (0.06)	9.39 (0.41)	672.00 (2.00)	7.27 (0.25)	3.56 (1.54)	130.00 (10.93)	0.10 (0.02)	0.44 (0.01)	0.01 (0.00)	0.12 (0.00)	0.09 (0.03)	2.88 (0.12)	0.12 (0.02)
	L	20.53 (0.06)	9.32 (0.46)	6393.33 (5.77)	7.59 (0.40)	0.67 (0.67)	37.78 (5.36)	0.15 (0.16)	0.19 (0.02)	0.00 (0.00)	0.06 (0.02)	0.08 (0.02)	1.73 (0.40)	0.07 (0.06)
Oct 12	M1	16.73 (0.06)	9.30 (0.35)	84.67 (2.08)	7.47 (0.25)	6.00 (3.33)	30.56 (9.77)	0.07 (0.02)	0.27 (0.01)	0.01 (0.00)	0.06 (0.01)	0.04 (0.02)	6.88 (0.19)	0.07 (0.03)
	M2	19.67 (0.15)	7.63 (0.03)	103.33 (0.58)	7.50 (0.10)	1.33 (1.33)	180.00 (10.00)	0.08 (0.02)	0.32 (0.01)	0.01 (0.00)	0.08 (0.00)	0.07 (0.02)	3.51 (0.09)	0.06 (0.04)
	M3	20.73 (0.15)	8.33 (0.04)	1285.00 (5.29)	7.30 (0.10)	3.33 (2.91)	35.00 (2.89)	1.41 (0.05)	0.24 (0.00)	0.01 (0.00)	0.09 (0.02)	0.09 (0.03)	3.45 (0.25)	0.04 (0.00)
	L	17.00 (0.10)	7.77 (0.07)	5243.33 (5.77)	7.27 (0.06)	1.33 (0.67)	301.67 (43.11)	0.03 (0.03)	0.23 (0.02)	0.00 (0.00)	0.07 (0.02)	0.22 (0.29)	3.36 (0.49)	0.07 (0.03)
Nov 12	M1	16.03 (0.25)	7.95 (0.02)	100.00 (1.00)	6.83 (0.06)	1.78 (3.08)	81.11 (6.74)	0.01 (0.01)	0.22 (0.01)	0.00 (0.00)	0.05 (0.01)	0.07 (0.05)	1.39 (0.28)	0.01 (0.01)
	M2	16.30 (0.10)	7.73 (0.01)	98.00 (1.00)	7.33 (0.06)	5.11 (3.42)	35.00 (4.41)	0.11 (0.02)	0.41 (0.02)	0.01 (0.00)	0.11 (0.03)	0.06 (0.02)	3.76 (0.43)	0.05 (0.01)
	M3	16.47 (0.15)	8.44 (0.16)	574.67 (0.58)	7.23 (0.06)	2.67 (1.76)	80.00 (5.00)	0.03 (0.01)	0.28 (0.02)	0.01 (0.00)	0.09 (0.00)	0.06 (0.02)	4.39 (0.08)	0.04 (0.00)
	L	17.17 (0.15)	7.84 (0.07)	1058.33 (3.06)	6.03 (0.01)	0.00 (0.00)	36.11 (2.55)	0.08 (0.01)	0.36 (0.05)	0.01 (0.00)	0.08 (0.00)	0.03 (0.01)	2.78 (0.50)	0.05 (0.03)

Table IV. 2. Sediments chlorophyll *a*, *b* and *c* values and organic matter percentage for each sampling site and month in the tidal freshwater area of M-est and L-est, from October 2011 to November 2012. For each parameter, the values are the mean with the corresponding standard error within brackets. Chlorophyll *a*, *b* and *c* and the sum of them (Chl *a*, Chl *b,* Chl *c*, and Chl_{total}, respectively, units are µg/l), very coarse sand (VCS, %), coarse sand (CS, %), medium sand (MS, %), fine sand (FS, %), very fine sand (VFS %) and silt + clay (S+C, %) for granulometry and organic matter (%).

Month	Site	OM	Chl_a	Chl_b	Chl_c	Chl_{Total}	VCS	CS	MS	FS	VFS	S+C
Oct 11	M1	1.60 (0.15)	2.16 (0.62)	1.39 (0.25)	2.41 (0.33)	5.96 (0.70)	2.25 (0.26)	13.97 (4.51)	77.83 (8.57)	4.25 (2.00)	0.97 (0.46)	0.35 (0.09)
	M2	4.58 (1.42)	1.31 (0.31)	0.98 (0.21)	2.15 (0.16)	4.44 (0.66)	0.72 (0.31)	0.27 (0.03)	21.74 (3.42)	63.18 (2.47)	10.84 (1.25)	1.74 (0.48)
	M3	0.73 (0.09)	1.18 (1.08)	0.58 (0.51)	1.50 (1.31)	3.26 (2.88)	0.65 (0.45)	2.95 (0.57)	78.66 (4.71)	16.25 (5.35)	1.00 (0.19)	0.26 (0.07)
	L	1.39 (0.05)	3.08 (1.01)	0.96 (0.06)	2.58 (0.47)	6.62 (0.57)	20.87 (2.32)	32.05 (1.20)	39.02 (3.04)	3.63 (0.19)	2.28 (0.18)	1.75 (0.19)
Nov 11	M1	1.13 (0.36)	1.65 (0.33)	1.05 (0.15)	2.11 (0.03)	4.81 (2.19)	2.66 (3.10)	14.64 (3.26)	79.41 (12.19)	2.73 (1.38)	1.18 (0.69)	0.40 (0.07)
	M2	3.08 (1.97)	1.03 (0.11)	0.82 (0.09)	2.00 (0.08)	3.85 (0.28)	3.68 (3.11)	0.43 (0.18)	31.83 (4.53)	57.27 (2.06)	5.93 (1.35)	0.59 (0.31)
	M3	0.60 (0.03)	1.07 (0.19)	0.77 (0.04)	1.98 (0.05)	3.82 (0.29)	0.99 (0.33)	3.62 (0.98)	82.08 (0.58)	12.74 (0.53)	0.31 (0.02)	0.16 (0.01)
	L	1.67 (0.43)	2.59 (0.62)	0.92 (0.08)	2.21 (0.09)	5.72 (0.77)	16.31 (2.25)	30.89 (3.73)	42.92 (5.31)	4.09 (1.45)	3.01 (1.79)	2.56 (1.25)
Dec 11	M1	1.09 (0.89)	1.08 (0.16)	0.84 (0.05)	2.07 (0.07)	3.99 (0.28)	10.14 (6.96)	15.57 (4.66)	69.43 (13.15)	2.09 (1.55)	0.63 (0.60)	0.31 (0.29)
	M2	1.60 (0.28)	0.78 (0.07)	0.71 (0.01)	1.93 (0.00)	3.42 (0.07)	10.79 (7.05)	0.35 (0.14)	30.96 (8.63)	51.03 (6.48)	5.81 (1.00)	0.68 (0.15)
	M3	4.09 (0.60)	1.41 (0.23)	0.93 (0.08)	2.15 (0.14)	4.49 (0.42)	3.51 (2.59)	6.61 (1.78)	53.94 (22.29)	11.49 (4.63)	15.22 (8.14)	8.76 (5.22)
	L	1.13 (0.43)	2.24 (0.49)	0.87 (0.03)	2.19 (0.03)	5.3 (0.55)	18.57 (3.08)	29.56 (6.84)	42.49 (5.50)	4.66 (1.96)	2.73 (1.59)	1.72 (0.91)
Jan 12	M1	1.01 (0.12)	1.31 (0.39)	0.85 (0.07)	2.10 (0.07)	4.26 (0.79)	34.41 (3.00)	32.33 (4.41)	30.22 (5.09)	1.10 (0.34)	0.85 (0.17)	0.79 (0.16)
	M2	1.17 (0.02)	0.95 (0.12)	0.73 (0.02)	1.98 (0.06)	3.66 (0.68)	1.32 (1.97)	0.19 (0.03)	43.65 (2.64)	50.67 (1.15)	3.64 (0.52)	0.49 (0.17)
	M3	0.71 (0.10)	0.89 (0.08)	0.73 (0.01)	1.96 (0.01)	3.58 (0.57)	6.24 (3.22)	10.45 (3.07)	70.26 (4.53)	12.20 (2.82)	0.50 (0.17)	0.17 (0.06)
	L	1.34 (0.22)	1.81 (0.42)	0.85 (0.06)	2.09 (0.10)	4.75 (2.63)	21.96 (0.81)	33.61 (3.18)	38.62 (2.83)	2.69 (0.92)	1.27 (0.68)	1.13 (0.59)
Feb 12	M1	1.21 (0.61)	1.00 (0.88)	0.60 (0.52)	1.42 (1.23)	3.02 (0.49)	5.53 (1.32)	6.58 (2.86)	79.03 (2.70)	6.06 (3.78)	1.78 (0.98)	0.77 (0.19)
	M2	1.78 (0.06)	1.92 (0.36)	0.91 (0.06)	2.21 (0.07)	5.04 (0.15)	3.68 (3.52)	0.31 (0.13)	19.55 (2.93)	62.03 (3.95)	11.36 (1.43)	2.61 (0.44)
	M3	3.49 (0.94)	4.25 (0.14)	1.34 (0.05)	2.80 (0.06)	8.39 (0.75)	1.13 (0.28)	1.04 (0.14)	14.14 (0.85)	39.40 (0.71)	36.39 (1.82)	7.38 (0.31)
	L	1.92 (0.32)	4.74 (0.70)	1.44 (0.05)	2.62 (0.13)	8.8 (2.72)	21.13 (3.02)	30.08 (1.11)	38.98 (2.60)	3.77 (1.30)	2.77 (1.28)	2.62 (0.87)
Mar 12	M1	1.50 (0.67)	2.31 (1.63)	1.28 (0.53)	2.53 (0.56)	6.12 (0.03)	1.42 (0.89)	9.28 (1.72)	86.84 (1.25)	1.12 (0.09)	0.47 (0.03)	0.26 (0.06)
	M2	0.61 (0.27)	0.97 (0.02)	0.77 (0.03)	1.98 (0.02)	3.72 (1.35)	15.48 (6.90)	0.32 (0.19)	25.84 (3.02)	50.15 (6.09)	6.88 (1.77)	1.15 (0.47)
	M3	1.51 (0.15)	6.55 (1.24)	1.63 (0.13)	3.00 (0.33)	11.18 (2.50)	8.62 (4.60)	5.18 (1.74)	11.49 (2.29)	48.84 (3.68)	20.74 (4.06)	4.56 (0.95)
	L	1.02 (0.05)	2.74 (0.87)	1.03 (0.12)	2.18 (0.11)	5.95 (0.22)	26.32 (2.41)	31.05 (2.14)	34.06 (1.78)	3.64 (0.55)	2.39 (0.41)	2.45 (0.48)
Apr 12	M1	1.37 (1.13)	1.39 (0.19)	0.96 (0.05)	2.09 (0.02)	4.44 (5.36)	5.16 (5.21)	14.15 (2.85)	78.42 (7.27)	1.07 (0.18)	0.47 (0.07)	0.32 (0.06)
	M2	1.37 (0.14)	3.50 (0.19)	1.09 (0.50)	2.51 (0.77)	7.1 (0.94)	3.21 (4.14)	0.53 (0.12)	33.50 (3.70)	55.87 (6.11)	5.32 (0.92)	0.71 (0.18)
	M3	5.65 (1.10)	2.68 (4.11)	1.26 (0.16)	2.56 (0.17)	6.5 (0.71)	0.36 (0.01)	0.74 (0.09)	13.65 (2.29)	41.43 (0.83)	37.16 (2.37)	6.15 (0.44)
	L	4.16 (2.12)	3.12 (0.62)	1.08 (0.07)	2.35 (0.12)	6.55 (1.15)	22.17 (1.77)	29.89 (1.18)	34.93 (1.93)	4.73 (0.27)	3.96 (0.44)	4.04 (0.47)

Table IV. 2. *Continued…*

Month	Site	OM	Chl_a	Chl_b	Chl_c	Chl_{Total}	VCS	CS	MS	FS	VFS	S+C
May 12	M1	0.99 (0.05)	4.96 (0.53)	1.51 (0.12)	3.27 (0.32)	9.74 (0.16)	60.07 (3.76)	23.91 (1.97)	12.96 (2.48)	0.77 (0.05)	0.95 (0.01)	1.13 (0.18)
	M2	1.88 (0.73)	1.31 (0.71)	0.79 (0.05)	2.32 (0.15)	4.42 (0.12)	0.07 (0.03)	0.12 (0.03)	59.90 (0.55)	36.89 (0.48)	2.47 (0.24)	0.42 (0.16)
	M3	1.04 (0.20)	1.27 (0.05)	0.81 (0.03)	2.27 (0.06)	4.35 (0.3)	16.16 (4.62)	16.66 (3.11)	25.21 (1.12)	15.29 (1.63)	12.63 (3.93)	13.48 (2.96)
	L	11.03 (13.60)	2.16 (0.05)	1.03 (0.04)	2.75 (0.15)	5.94 (0.07)	19.77 (2.44)	29.86 (0.12)	38.66 (3.03)	2.49 (0.72)	1.17 (0.77)	0.67 (0.52)
Jun 12	M1	0.87 (0.10)	0.68 (0.23)	0.16 (0.02)	0.25 (0.01)	1.09 (0.06)	1.90 (0.75)	16.39 (5.35)	78.69 (4.94)	1.66 (0.86)	0.74 (0.16)	0.33 (0.05)
	M2	1.34 (0.12)	0.36 (0.07)	0.05 (0.01)	0.11 (0.03)	0.52 (1.53)	10.51 (7.47)	0.72 (0.08)	56.56 (1.96)	27.90 (4.31)	2.57 (0.85)	1.42 (0.57)
	M3	2.79 (0.39)	0.98 (0.03)	0.26 (0.26)	0.37 (0.41)	1.61 (1.00)	0.85 (0.55)	0.98 (0.26)	11.10 (1.30)	41.65 (0.38)	36.84 (2.31)	8.30 (0.93)
	L	1.87 (0.57)	1.93 (0.88)	0.44 (0.13)	0.94 (0.17)	3.31 (1.75)	21.25 (1.27)	34.45 (2.63)	36.99 (1.38)	3.09 (0.54)	1.85 (0.37)	2.08 (0.32)
Jul 12	M1	1.17 (0.16)	3.17 (0.74)	1.27 (0.29)	2.71 (0.27)	7.15 (0.27)	3.79 (2.62)	15.45 (0.72)	77.99 (1.15)	3.10 (1.24)	1.40 (1.39)	1.06 (1.48)
	M2	1.23 (0.06)	0.78 (1.20)	0.75 (0.05)	2.09 (0.14)	3.62 (2.91)	1.79 (0.09)	1.29 (0.05)	68.74 (8.14)	22.47 (0.82)	2.33 (0.46)	0.89 (0.26)
	M3	0.66 (0.06)	0.97 (0.08)	0.66 (0.59)	1.67 (1.47)	3.3 (0.66)	3.73 (2.99)	9.40 (4.82)	75.21 (9.12)	9.12 (1.60)	0.37 (0.09)	0.28 (0.09)
	L	2.49 (0.12)	1.88 (0.85)	0.92 (0.07)	2.32 (0.13)	5.12 (2.57)	22.46 (1.53)	25.21 (1.44)	25.37 (1.52)	4.10 (0.79)	2.89 (0.70)	3.36 (1.12)
Aug 12	M1	0.79 (0.08)	2.56 (0.47)	1.47 (0.49)	3.41 (1.81)	7.44 (0.13)	3.63 (1.88)	16.62 (2.49)	75.88 (3.75)	2.48 (2.30)	0.72 (0.47)	0.29 (0.22)
	M2	1.16 (0.05)	0.86 (0.36)	0.73 (0.02)	2.01 (0.04)	3.6 (1.58)	4.81 (3.78)	0.79 (0.12)	57.39 (2.78)	33.48 (0.59)	2.41 (0.08)	0.80 (0.27)
	M3	2.41 (0.19)	4.62 (0.07)	1.55 (0.19)	2.97 (0.26)	9.14 (0.48)	30.40 (5.72)	18.81 (12.18)	15.82 (6.66)	23.85 (10.82)	8.40 (5.89)	1.96 (1.29)
	L	1.28 (0.12)	2.09 (1.13)	0.90 (0.05)	2.20 (0.08)	5.19 (0.20)	24.45 (4.60)	31.72 (0.86)	36.17 (4.32)	3.49 (0.95)	1.89 (0.74)	1.92 (0.71)
Sept 12	M1	0.88 (0.25)	1.02 (0.36)	0.77 (0.04)	1.94 (0.03)	3.73 (0.03)	13.68 (1.12)	14.38 (0.80)	69.65 (1.84)	1.61 (0.13)	0.31 (0.02)	0.17 (0.02)
	M2	1.46 (0.33)	0.72 (0.13)	0.69 (0.01)	1.97 (0.04)	3.38 (0.55)	0.65 (0.61)	0.25 (0.20)	31.89 (7.21)	67.96 (12.33)	3.40 (0.36)	0.33 (0.08)
	M3	3.17 (0.12)	2.54 (0.03)	1.01 (0.07)	2.42 (0.20)	5.97 (0.30)	0.83 (0.57)	1.22 (0.39)	19.24 (2.60)	40.64 (0.65)	31.76 (1.10)	6.00 (0.48)
	L	2.16 (0.47)	2.09 (0.29)	0.89 (0.05)	2.18 (0.14)	5.16 (0.23)	20.85 (5.59)	28.37 (4.02)	35.99 (2.32)	6.40 (2.71)	4.76 (3.02)	3.55 (2.28)
Oct 12	M1	0.82 (0.10)	1.36 (0.12)	0.87 (0.03)	2.22 (0.04)	4.45 (0.16)	15.74 (0.87)	17.69 (0.45)	63.83 (2.41)	1.66 (0.84)	0.76 (0.88)	0.65 (0.87)
	M2	1.66 (0.30)	0.97 (0.17)	0.74 (0.02)	2.07 (0.08)	3.78 (1.11)	6.38 (1.63)	0.66 (0.10)	49.47 (5.46)	42.37 (3.65)	3.29 (0.47)	0.66 (0.09)
	M3	3.26 (0.10)	4.64 (0.07)	1.23 (0.13)	2.87 (0.14)	8.74 (0.15)	1.80 (0.34)	1.73 (0.42)	15.16 (2.93)	43.66 (0.75)	30.15 (3.48)	6.95 (0.21)
	L	1.64 (0.30)	1.41 (0.86)	0.78 (0.02)	2.19 (0.02)	4.38 (0.17)	21.89 (1.03)	29.48 (1.96)	40.00 (0.72)	4.70 (1.21)	2.34 (1.00)	1.20 (0.48)
Nov 12	M1	1.73 (0.52)	2.31 (0.18)	1.28 (0.15)	2.53 (0.03)	6.12 (0.34)	3.03 (1.54)	4.35 (0.39)	67.04 (3.41)	15.17 (2.19)	5.61 (1.08)	2.80 (0.17)
	M2	1.53 (0.69)	0.97 (0.33)	0.77 (0.09)	1.98 (0.08)	3.72 (0.05)	5.99 (4.93)	0.19 (0.07)	37.52 (3.66)	52.70 (4.16)	2.50 (0.79)	0.43 (0.14)
	M3	1.34 (0.65)	6.55 (0.11)	1.63 (0.04)	3.00 (0.05)	11.18 (3.01)	0.35 (0.59)	1.48 (0.92)	87.75 (1.66)	8.29 (0.38)	0.21 (0.03)	0.34 (0.05)
	L	0.90 (0.80)	2.74 (0.62)	1.03 (0.08)	2.18 (0.09)	5.95 (2.08)	20.02 (1.38)	31.36 (0.13)	39.47 (2.08)	3.74 (0.87)	2.08 (0.86)	1.77 (0.72)

Table IV. 3. Results of the non-parametric Kruskal-Wallis fo the one-way analysis of variance of abiotic water and sediment parameters performed to investigate significant differences among sampling sites and among months. Temperature (T), dissolved oxygen (DO), conductivity (Cond), pH (pH), nitrites (NO_2^-), nitrates (NO_3^-), ammonium (NH_4^+), phosphates (PO_4^{3-}), silica (SiO_2), phenol (C_6H_5OH), iron (Fe), hardness ($CaCO_3$), turbidity (Turb) total chlorophylls (Chl_{total}) and organic matter (OM).

Parameter	Sampling Month			Sampling Site		
	df	H	p	df	H	p
T	13	51.767	0.000	3	0.704	0.872
DO	13	41.927	0.000	3	1.901	0.593
Cond	13	13.719	0.394	3	34.181	0.000
pH	13	27.978	0.009	3	6.160	0.104
Turb	13	19.791	0.101	3	6.459	0.091
$CaCO_3$	13	8.366	0.819	3	20.984	0.000
NH_4^+	13	17.496	0.178	3	4.017	0.260
NO_3^-	13	26.031	0.017	3	5.836	0.120
NO_2^-	13	2.887	0.998	3	33.390	0.000
PO_4^{3-}	13	8.643	0.799	3	23.291	0.000
C_6H_6O	13	17.933	0.160	3	5.143	0.162
Si	13	8.404	0.816	3	9.553	0.023
Fe	13	28.972	0.007	3	0.416	0.937
OM	13	9.543	0.731	3	7.434	0.059
VCS	13	7.297	0.886	3	25.600	0.000
CS	13	2.810	0.999	3	46.770	0.000
MS	13	7.528	0.873	3	12.033	0.007
FS	13	3.098	0.998	3	44.656	0.000
VFS	13	6.457	0.928	3	18.566	0.000
S+C	13	7.053	0.899	3	14.887	0.002
Chl_{total}	13	18.953	0.125	3	8.911	0.030

3.2. Gonadal development cycle

In the present study it was observed individuals with follicles containing only oocytes and individuals with follicles containing both oocytes and sperm sometimes in the same follicle (Figure IV. 2). Individuals with only male reproductive tissue were never observed and, in general male reproductive tissue was less common than female tissue. A previous study also observed that male reproductive tissue was less

common than female tissue (Kennedy et al 1985) and, according to Houki *et al.* (2011), the occurrence of *C. fluminea* specimens with male reproductive tissue only were never found in populations outside of their native range. Results showed a higher percentage of individuals with male reproductive tissue among all sampling sites in spring (Figure IV. 2). Kraemaer and Galloway (1986) also observed that spermatogenesis is a seasonal phenomenon.

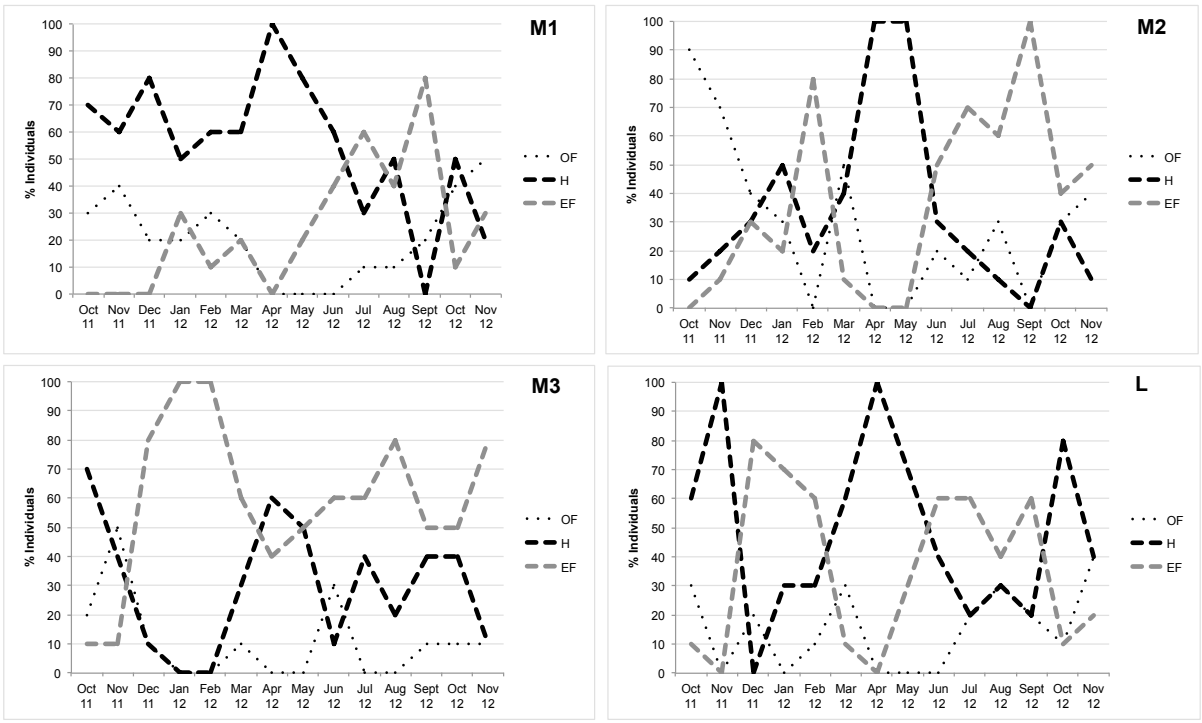


Figure IV. 2. Montly variation (October 2011 to November 2012) of the relative percentages of *Corbicula fluminea* individuals with follicles filled with oocytes only (OF), follicles filled with both oocytes and sperm cells and/or separated follicles filled with oocytes and with sperm cells in the same individual (H), and predominantly empty follicles (EF) estimated in sampling sites of the M-est (M1, M2 and M3) and the L-est (L).

Hermaphroditism is considered a further adaptation that maximises the reproductive output (Reed *et al.* 2013) and is particularly important for invasive *C. fluminea* since self-fertilization is their greatest, if not their only, reproductive mechanism outside their natural range (Pigneur *et al.* 2011). Additionally, spermatogenesis was already observed to accelerate the reproductive process

(Kraemer and Galloway 1986). Concerning sampling sites, results indicate that reproductive success may be greater at M1 since it has longer higher percentage of hermaphrodites. In fact, Sousa *et al.* (2008b) suggests that recruitment sites in M-est might be located in upstream areas since they only observed juveniles in that areas. Contrariwise, a high percentage (90%) of individuals containing only female reproductive tissue was observed in M2 in October 2011 (Figure IV. 2). Since self-fertilization is considered the main reproductive mechanism of *C. fluminea* outside their native range (Pigneur *et al.* 2011), individuals containing only female reproductive tissue cannot succeed in reproducing. M2 is located near the mouth of the Louro River, a tributary of the M-est that is one of the most contaminated rivers in Galicia (Concha-Graña *et al.*, 2006; Farkas *et al.*, 2007; Filgueiras *et al.*, 2004; Lavilla *et al.*, 2010). Since this high percentage was not observed in any other location or time of sampling, a punctual discharge of contaminants from the Louro River may be diminishing the male tissue production, which probably impair the reproduction of *C. fluminea*.

Advanced gametes were present in clams through the year as well as oogonial and spermatogonial proliferation (Figure IV. 3). A previous study also observed that *C. fluminea* gametogenesis is continuous (Byrne *et al.* 2000) and different size classes of oocytes are always present in the follicles (Kraemer and Galloway 1986).

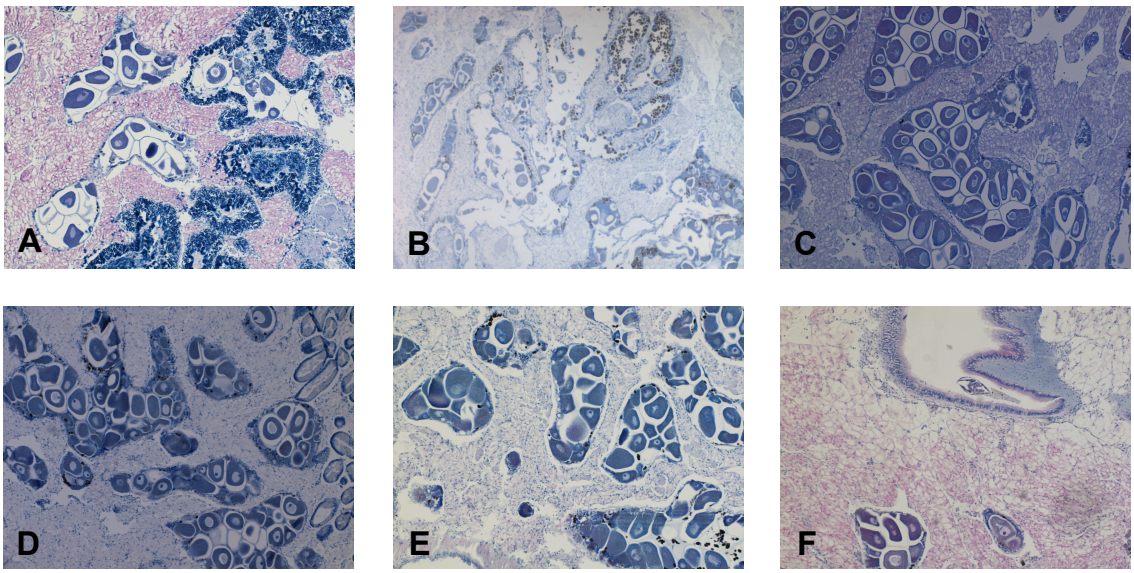


Figure IV. 3. Gonad sections from October (A), December (B), March (C), April (D), May (E) and August (F) showing a similar gametogenic state with advanced gametes

The amount of gonad tissue increased during the breeding season resulting in a reduction in the connective tissue space (Figure IV. 4). There was no evidence of complete spawning. Thus, further analyses are needed in order to understand the alteration of the amounts of the different tissues.

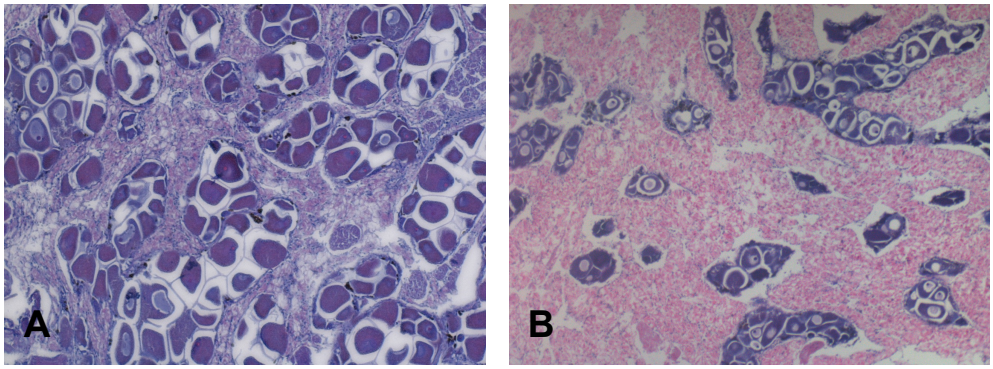


Figure IV. 4. Amount of gonad tissue during (A) and after (B) the breeding season resulting in a reduction in the connective tissue space.

Although there was no clear seasonal pattern in gametogenesis it was observed a seasonal pattern in the relative percentage of hermaphrodites and empty follicles most probably indicative of higher reproductive periods. Results showed, in general, higher relative percentage of hermaphrodite individuals in autumn and spring (Figure IV. 5) which is in accordance with most of the previous studies that observed that *C. fluminea* reproduces twice a year in temperate regions (Mouthon 2001b; Sousa *et al.* 2008a; Wittmann *et al.* 2008). Higher reproductive periods occurred in general at the same time in all sampling sites assessed and there were no substantial differences between M-est and L-est *C. fluminea* populations that might explain the differences observed between their invasive behaviours. Nevertheless, reproductive output, which also includes the number of juveniles formed and released and their survival, may be contributing to the differences observed, unfortunately, this study does not cover the production and survival of juveniles and further studies are needed in this context.

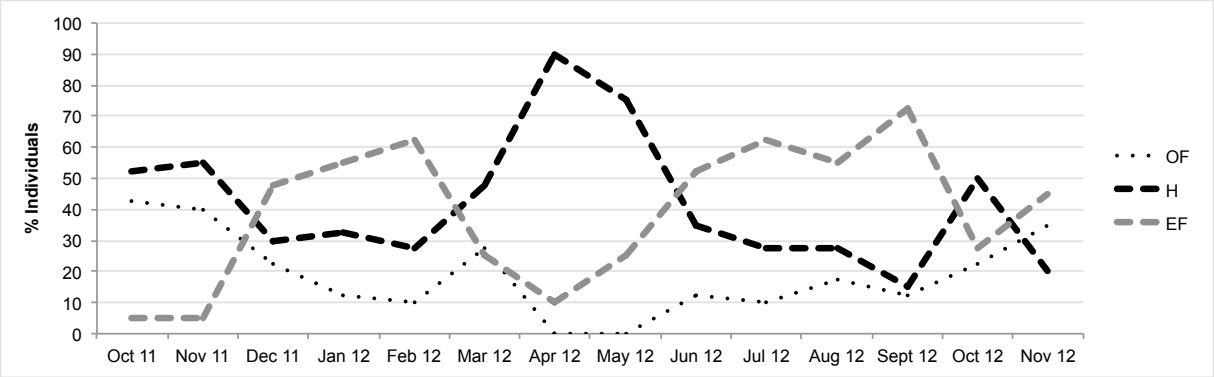


Figure IV. 5. Graphical representation relative percentage of *C. fluminea* individuals with follicles filled with only oocytes (OF), follicles filled with both oocytes and sperm and/or separated follicles filled with oocytes and with sperm observed in the same individual (H) and empty follicles (EF) calculated by the monthly (from October 2011 to November 2012) average between all sampling sites in study (M1, M2 and M3 in M-est and L in L-est) representative of general gonadal development cycle in temperate regions.

3.2. Relating gonadal development cycle and environmental parameters

The reproduction of *C fluminea* is known to vary greatly according to its location suggesting a strong influence of environmental parameters (Rosa *et al.* 2014). Different phases of gonadal development cycle were associated with different environmental parameters (Figure IV. 7). Hermaphroditism was closely associated with most of the water and sediment parameters included in the analysis: water conductivity, hardness and phosphate, nitrite and iron concentrations; and sediment organic matter, total chlorophyll content and granulometry. Hermaphroditism was closely associated with greater availability of food, in this case a higher concentration of total chlorophyll, which in itself is a food source for *C. fluminea* (Foe and Knight 1986; Lauritsen 1986); organic matter that can also be used as a food source by *C. fluminea* (Boltovskoy *et al.* 1995; Cahoon and Owen 1996; Hakenkamp and Palmer 1999); and nutrients (phosphate and nitrites) that are important for the quality of aquatic ecosystems food since among mineral nutrients, nitrogen and phosphorus are particularly important, promoting algal and plankton productivity (Elser *et al.* 2000; Sterner *et al.* 2008), leading to higher food resources for *C. fluminea*. Hermaphroditism is considered a further adaptation that enables the species to

maximize their reproductive output (Reed *et al.* 2013). The number of reproductive events and larval survival depends, besides other factors, on the food availability (Cataldo & Boltovskoy 1999, Mouthon 2001a,b). Therefore, if there is more available food there is a need to become more efficient in the reproductive output, which demonstrates the opportunist character of this species that is capable of rapidly exploiting favorable conditions (Mouthon 2001b).

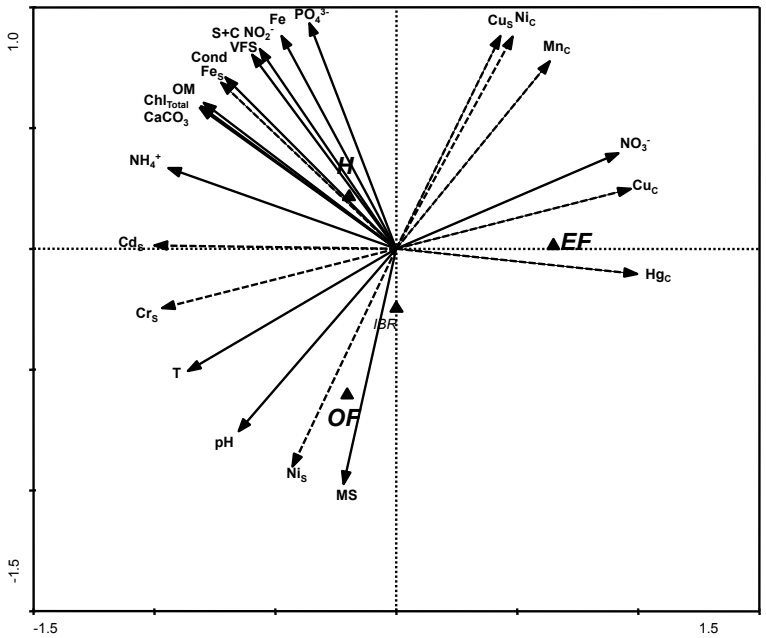


Figure IV. 6. Redundancy analysis ordination diagram displaying the scores for biological variables (represented by triangles) showing correlations with water and sediment parameters (represented by straight arrows) and metal concentrations (represented by dotted arrows): the first axis (horizontal) significantly explained 56.7% and second (vertical) 43.3% of the variability. The biological variables are: Relative percentage of follicles completely filled with only oocytes (OF), relative percentage of follicles completely filled with oocytes and sperm in the same and/or separated follicles filled with oocytes and sperm in the same individual (H), empty follicles (EF) and the integrated biomarker response index (IBR). The quantitative environmental variables are: temperature (T), dissolved oxygen (DO), pH, conductivity (Cond), NH_4^+ (Ammonium), NO_2^- (nitrites) and NO_3^- (nitrates) and the concentration of Ni, Cr, Cu and Fe in sediments and Mn, Ni, Cu and Hg in *C. fluminea* tissues.

Empty follicles were closely associated with higher concentrations of copper and mercury in *C. fluminea* tissues. Mussels can loose up to 70 % of their biomass during spawning (Duinker and Mortensen 1999) while metal content can still remain the same if not associated with gonad tissue (Philips 1976; Boyden 1977), which can explain the close association between these metal concentrations and empty follicles. Follicles filled with only oocytes were closely associated with sediment nickel concentration. Nickel was already observed to decrease the germinal seminiferous epithelium of mice (Lukac *et al.* 2014), which may explain the association between nickel concentration, and the lack of male gonad tissue. The restriction of sperm production can inhibit the success of reproduction of this species since their mechanism of reproduction is by self-fertilization (Pigneur *et al.* 2011). A previous study also observed that the presence of toxic substances might be diminishing the recruitment in populations in the Parana River Delta (Boltovskoy *et al.* 1997).

Conclusion

Although the gametogenic condition of the follicle did not appear to change over the year, the amount of gonad tissue increased during the breeding season resulting in a reduction in the connective tissue space and it was observed a seasonal pattern in the relative percentage of hermaphrodites and empty follicles most probably indicative of higher reproductive periods. Results showed, in general, higher relative percentage of hermaphrodite individuals in autumn and spring in accordance with most of the previous studies that observed that *C. fluminea* reproduces twice a year in temperate regions. Higher reproductive periods occurred in general at the same time in all sampling sites assessed and there were no substantial differences between M-est and L-est *C. fluminea* populations that might explain the differences observed between their invasive behaviours. However, reproductive output also includes the number of juveniles formed and their release and survival, which may be contributing to the invasive behaviour differences observed between M-est and L-est, unfortunately, this study does not cover the production and survival of juveniles and further studies are needed in this context.

The integrated analysis of data indicated an association between high percentages of hermaphroditism and high concentrations of nutrients, organic matter

and chlorophylls, suggesting a good synchronism between fertilization/spawning and food availability that is crucial for larvae survival and performance.

Insofar, a study of the *C. fluminea* gametogenic cycle correlated with the environmental variables it is essential to contribute to the knowledge on the mechanisms of the successful spread of this species.

Acknowledgments

This research was partially supported by the European Regional Development Fund (ERDF) through the COMPETE - Operational Competitiveness Programme and national funds through FCT – Foundation for Science and Technology, under the projects NISTRACKS (PTDC/AAC-AMB/102121; FCOMP-01-0124-FEDER-008556) and PEst-C/MAR/LA0015/2013. C. Oliveira had a PhD fellowship from FCT (SFRH/BD/68423/2010), supported by “Programa Operacional Potencial Humano do QREN Portugal 2007-2013” and national founds from the “Ministério da Ciência e Tecnologia e Ensino Superior” (MCTES – POPH-QREN-Tipologia 4.1).

Chapter V

General Discussion and Final Conclusions

C. fluminea is one of the 100 worst invasive species in Europe and is well known by its rapid and extensive spread (DAISIE 2015). It has several characteristics that make it a highly invasive species (Fureder and Pockl 2007; McMahon 2002; Sousa *et al.* 2006a; Vohmann *et al.* 2009), such as earlier sexual maturity, short life span, rapid growth and high fecundity. Despite the considerable amount of studies that were carried out on *C. fluminea*, the individual environmental factors influencing its invasive behaviour and their mode of action are not completely understood (Sousa *et al.* 2008a). Thus, the experimental work done in the scope of this thesis contributed to increase the knowledge on how environmental factors, with a special focus on pollution, may influence the invasive behaviour of *C. fluminea* populations in tidal fresh waters (TFAs) of South Europe estuaries through potential effects on summer induced stress leading to massive mortality events, health status along the year, and gonad developmental cycle. The general approach used was the comparison of the natural populations of *C. fluminea* inhabiting the TFAs of the estuaries of Minho (M-est) and Lima (L-est) Rivers (Nw coast of the Iberian Peninsula), which have been showing differences in their invasive behaviour (Sousa *et al.* 2006b, 2007a).

The environmental characterization of both systems, based on abiotic water and physico-chemical sediment parameters (*Chapters II, III* and *IV*) showed, in general, similar abiotic conditions (temperature, dissolved oxygen, conductivity, among others) but higher concentrations of nitrites and nitrates in the M-est than in the L-est. The seasonal monitoring study on the sediment metal concentrations (*Chapter III*) showed higher total metal concentrations in the L-est than in M-est, with Al, Mn and Se being the metals contributing most to the differences found.

The study on the stress levels of M-est and L-est *C. fluminea* populations, potentially leading to summer massive mortality events, assessed through the integrated biomarker response index (IBR) in relation to environmental parameters (*Chapter II*) indicated higher stress of both populations in July/August than in September/October. The main environmental factors associated with high stress levels in *C. fluminea* were increased water

temperature and conductivity, and nutrients and ammonium concentrations. Because the levels of these factors were also associated in July/August in the L-est when this population showed the highest levels of stress and no massive mortality events were described so far for the L-est population, other factors seem to be important for the occurrence of such events. Population density may be a particularly important one. In fact, in areas with high densities of individuals, demand for food and water oxygen would be higher than in areas with less specimens. Because *C. fluminea* is very sensitive to low water oxygen concentrations (Belanger *et al.* 1991; Johnson and McMahon 1998; Matthews and McMahon 1999) that in general occur in the summer in both M-est and L-est (Table III.1), a higher density of individuals competing for this factor together with the increased water temperature and conductivity (potentially indicative of increased concentrations of some environmental concentrations such as metals), and water ammonium levels (Table III.1) to which *C. fluminea* is also very sensitive (Cherry *et al.* 2005), likely increases the stress and causes higher mortality than at lower population density. This hypothesis may explain why massive mortality events occurred in the M-est population that has an extremely high density and biomass (Sousa *et al.* 2005, 2008e) and they were not detected in the L-est population that has a considerably lower density and biomass (Sousa *et al.* 2006a). Overall, the findings of *Chaper II* indicate that summer environmental conditions increase the stress levels of *C. fluminea* populations in TFAs of the NW Iberian Peninsula estuaries, decreasing their health status and leading to massive mortality events when the populations have high densities.

The study on the seasonal variation of the stress levels of *C. fluminea*, assessed through the IBR in relation to water abiotic and sediment physic-chemical parameters and the sediment metal concentrations (*Chapter III*) indicated higher stress levels in *C. fluminea* inhabiting M3 and L sampling sites (the most downstream sampling sites in study). The main environmental factors associated with higher stress levels were the coarser sands and silt/clay fraction and the concentrations of Mn, Al, Se and organic matter in sediments, and water temperature, mostly in L-est sampling site. However, the metal concentration in the soft body of individuals inhabiting L-est was lower suggesting that this population could efficiently detoxify, eliminate

and/or avoid excessive concentrations of these particular metals (Baudrimont *et al.* 2002; Marigómez *et al.* 2002). The mechanisms of metal elimination and/or avoidance may probably be inducing higher stress levels (Valko *et al.* 2005) levels that could be limiting the invasive behaviour of this population. In addition, the lack of the necessary energy for establishment and dispersion of the population, which could be redirected to cope with metal contamination, might be also contributing for the differences observed between the invasive behaviours of *C. fluminea* inhabiting in L-est and M-est. Overall, the findings of the *Chapter III* indicate higher stress levels in L-est population closely associated with Mn, Al, Se sediment concentrations which might be influencing the dispersion and establishment of this species in this estuary contributing to the differences observed between the invasive behaviours of *C. fluminea* populations inhabiting L-est and M-est.

The study on the gonadal development cycle in relation to to water abiotic and sediment physic-chemical parameters and the sediment metal concentrations (*Chapter IV*) indicate that hermaphrodite individuals was present all over the year but with higher percentages in spring an autumn and mostly associated with higher quantity of food. Because, in *C. fluminea*, the fertilization requires the simultaneous occurrence of oocytes and sperm, since self-fertilization is their greatest, if not their only, reproductive mechanism outside their natural range (Pigneur *et al.* 2011), these findings suggest an opportunist character of this species that is capable of rapidly exploiting favorable conditions (Mouthon 2001b) by synchronizing the peaks of fertilization and the availability of nutritional resources that are crucial for larval survival. Higher reproductive periods occurred in general at the same time in all sampling sites assessed and there were no substantial differences between M-est and L-est *C. fluminea* populations that might explain the differences observed between their invasive behaviours. However, the hypothesis that reproductive output could be contributing to the differences observed cannot be discarded since it also includes the number of juveniles produced and released and their survival. Unfortunately, this study does not cover the production and survival of juveniles and further studies are needed in this context. Nevertheless, the gonadal development cycle data integration with the environmental parameters showed a close association between a

higher percentage of individuals with follicles filled with only oocytes and the concentration of nickel in sediments which could be impairing the development of sperm (Lukac *et al.* 2014). The restriction of sperm production can inhibit the success of reproduction of this species since their mechanism of reproduction is by self-fertilization (Pigneur *et al.* 2011). A previous study also observed that the presence of toxic substances might be diminishing the recruitment in populations in the Parana River Delta (Boltovskoy *et al.* 1997). Overall, the findings of *Chapter IV* indicate that despite pollution seem to be influencing the success of reproduction of this species, the gonadal development cycle does not seem to be contributing to the differences observed between the invasive behaviours of M-est and L-est populations since both populations showed spawning/fertility peaks in spring and autumn in accordance with most of the previous studies that observed that *C. fluminea* reproduces twice a year in temperate regions (Sousa *et al.* 2008a).

In conclusion results showed that pollution (metal concentration and increased nutrients concentrations) induce higher stress levels in *C. fluminea* possible limiting their invasive behaviour since individuals exposed to these stressful conditions have to cope with pollution induced stress instead of investing in the population establishment and dispersal. *C. fluminea* inhabiting in both downstream sampling sites (M3 and L-est) showed, in general, higher stress conditions than those inhabiting upstream sampling sites. *C. fluminea* individuals inhabiting the most upstream sampling sites in M-est might be enough to contribute to the high abundance observed in this estuary, which probably does not occur in L-est since *C. fluminea* individuals were only previously observed in sites more downstream when compared with M-est population (Sousa *et al.* 2006a,b). In fact, juveniles were only observed in upstream sampling sites in M-est (Sousa *et al.* 2008b) corroborating this hypothesis. Therefore, to achieve the goals posed by The European Union Convention on Biodiversity Strategy (European Commission 2011) for 2020, to control or eradicate the priority species, eradication and/or control measures should be applied upstream in the TFA of estuaries, where it seems that this species shows higher reproductive output, in order to reduce the probability of a new and rapid dispersion of the species, since *C. fluminea* fecundity is extremely high, estimated at around 35 000 per hermaphroditic

individual per breeding season (McMahon 2002). Contrariwise, despite pollution seem to be diminish the male tissue production, the gonadal development cycle does not seem to be contributing to the differences observed between the invasive behaviours of M-est and L-est populations since both populations showed similar fertility/spawning peaks. However reproduction also involves the number of juveniles produced and their survival, important parameters that were not investigated in the present Thesis but should be addressed in the future in order to complete the information obtained.

Overall, the findings of the present Thesis increase the knowledge on the factors contribution to the invasive behaviour of C. fluminea in TDF areas of temperate regions indicating that pollution may be restricting the invasive behaviour of *C. fluminea* by decreasing their health status. In addition, under the scenarios of human population exponential growth and global climate changes, a better exploration of natural resources and ecosystem services, and the improvement of environmental quality are crucial. In the last decades, evidences from several studies have been drawing attention to the services that some non-indigenous invasive species (NIS), mostly known by the negative ecological and economic impacts that they generally cause in invaded ecosystems, may provide if an adequate management of their invasions is achieved (McLaughlan *et al.*, 2014). The work done within the present thesis shows that *C. fluminea* can be as a suitable indicator of environmental quality, since *C. fluminea* accumulated 7 metals (Cr, Cu, Zn, Se, As, Cd, Pb) with bioconcentration factors (BSAF) ranging from ≈ 2 (Cr) to ≈ 36 (Cu) (*Chapter III*). In ecosystems invaded by this NIS, the use of natural populations of this species in environmental monitoring programmes may help to control the invasions and mitigate its negative impacts. Moreover, because the species has a high filtration rate (McMahon, 2002; Phelps, 1994) removing a considerable amount of microalgae and other organisms and particles from the water column, and accumulates several metals (Table III. 3), natural populations contribute to clean the water and remove toxic chemicals from the abiotic component of the ecosystem. Due to these properties, the species has also potential for further exploration in relation to

bioremediation, as previously suggested by other authors (Rosa *et al.*, 2014; Simonit and Perrings, 2011). For instance, in invaded ecosystems, the use of *C. fluminea* in environmental quality monitoring and bioremediation in controlled conditions, may help to control and mitigate the adverse effects of the invasion by this species, in addition to the direct advantages of other services that natural populations may provide.

Chapter VI

References List

Araújo, R., Moreno, D., Ramos, M. (1993). The Asiatic clam *Corbicula fluminea* (Müller, 1774)(Bivalvia: Corbiculidae) in Europe. *American malacological bulletin 10*, 39–49.

Aldridge, D.W., McMahon, R.F. (1978). Growth, fecundity, and bioenergetics in a natural population of the Asiatic freshwater clam, *Corbicula manilensis* Phillippi, from north central Texas. *Journal of Molluscan Studies 44*, 49–70.

Baba, K., Tada, M., Kawajiri, T., Kuwahara, Y. (1999). Effects of temperature and salinity on spawning of the brackish water bivalve *Corbicula japonica* in Lake Abashiri, Hokkaido, Japan. *Marine Ecology Progress Series 180*, 213–221.

Barfield, M.L., Farris, J.L., Black, M.C. (2001). Biomarker and bioaccumulation responses of Asian clams exposed to aqueous cadmium. *Journal of Toxicology and Environmental Health, Part A 63*, 495–510.

Baudrimont, M., Andres, S., Durrieu, G., Boudou, A. (2002). The key role of metallothioneins in the bivalve *Corbicula fluminea* during the depuration phase, after *in situ* exposure to Cd and Zn. *Aquatic Toxicology 63*, 89-102.

Baur, B., Schmidlin, S. (2007). Effects of invasive non-native species on the native biodiversity in the river Rhine. In: Nentwig, W. (Ed.) *Biological Invasions 193*, Springer, Berlin 441 p.

Beekey, M.A., Karlson, R.H. (2003). Effect of food availability on reproduction and brood size in a freshwater brooding bivalve. *Canadian Journal of Zoology 81*, 1168–1173.

Belanger, S.E. (1991). The effect of dissolved oxygen, sediment, and sewage treatment plant discharges upon growth, survival and density of Asiatic clams. *Hydrobiologia 218*, 113–126.

Belanger, S.E., Farris, J.L., Cherry, D.S., Cairns, J. (1986). Growth of Asiatic clams (Corbicula sp.) during and after long-term zinc exposure in field-located and laboratory artificial streams. *Archives of Environmental Contamination and Toxicology 15*, 427–434.

Belanger, S.E., Farris, J.L., Cherry, D.S., Cairns, J. (1990). Validation of *Corbicula fluminea* growth reductions induced by copper in artificial streams

and river systems. *Canadian Journal of Fisheries and Aquatic Sciences 47*, 904-914.

Beliaeff, B., Burgeot, T. (2002). Integrated biomarker response: a useful tool for ecological risk assessment. *Environmental Toxicology and Chemistry 21*, 1316–1322.

Beran, L. (2006) Spreading expansion of *Corbicula fluminea* (Mollusca: Bivalvia) in the Czech Republic. *Heldia 6*, 187–192.

Bettencourt, A.M., Bricker, S.B., Ferreira, J.G., Franco, A., Marques, J.C., Melo, J.J., Nobre, A., Ramos, L., Reis, C.S., Salas, F., Silva, M.C., Simas, T., Wolff, W.J. (2004). TICOR—Typology and reference conditions for Portuguese transitional and coastal waters. *Development of guidelines for the application of the European Union Water Framework Directive*. INAG/IMAR, Lisbon, 100 p.

Bigot, A., Minguez, L., Giambérini, F., Rodius, F. (2011). Early Defense Responses in the Freshwater Bivalve *Corbicula fluminea* Exposed to Copper and Cadmium: Transcriptional and Histochemical Studies. *Environmental Toxicology 26*, 623-632.

Bird, R.P., Draper, A.H. (1984). Comparative studies on different methods of malondyhaldehyde determination. *Methods in Enzymology 105*, 299–305.

Boltovskoy, D., Correa, N., Cataldo, D. Stripeikis, J., Tudino, M. (1997). Environmental stress on *Corbicula fluminea* (Bivalvia) in the Parana River delta (Argentina) complex pollution-related disruption of population structures. *Archiv für Hydrobiologie 138*, 483–507.

Boltovskoy D., Izaguirrel I., Correa N. (1995). Feeding selectivity of *Corbicula fluminea* (Bivalvia) on natural phytoplankton. *Hydrobiologia 312*, 171–182.

Boyden, C.R. (1977). Effect of size upon metal content of shellfish. *Journal of Marine Biological Association of the United Kingdom 57*, 675-714.

Bradford, M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. *Analytical Biochemistry 72*, 248–59.

Brancotte, V., Vincent, T. (2002). L ’ Invasion Du Réseau Hydrographique Français Par Les Mollusques Corbicula Spp. Modalite De Colonisation Et

Role Preponderant Des Canaux De Navigation. *Bulletin français de la pêche et de la pisciculture 365-366*, 325–337.

Britton, J.C., Morton, B. (1982). A dissection guide, field and laboratory manual for the introduced bivalve *Corbicula fluminea*. *Malacological Review 3*, 82 p.

Brockerhoff, E.G., Kimberley, M., Liebhold, A.M., Haack, R.A., Cavey, J.F. (2014) Predicting how altering propagule pressure changes establishment rates of biological invaders across species pools. *Ecology 95*, 594–601.

Byers, J.E. (2002). Impact of non-indigenous species on natives enhanced by enthropogenic alteration of selection regimes. *Oikos 97*, 449–457.

Byrne, R.A., McMahon, R.F. (1994). Behavioural and physiological responses to emersion in freshwater bivalves. *American Zoologist 34*, 194-204.

Byrne, M., Phelps, H., Church, T. (2000). Reproduction and development of the freshwater clam *Corbicula australis* in southeast Australia. *Hydrobiologia 418*, 185–197.

Caffrey, J.M., Baars, J., Barbour, J.H., Boets, P., Boon, P., Davenport, K., Dick, J.T.A., Early, J., Edsman, L., Gallagher, C., Gross, J., Heinimaa, P., Horrill, C., Hudin, S., Hulme, P.E., Hynes, S., MacIsaac, H.J., McLoone, P., Millane, M., Moen, T.L., Moore, N., Newman, J., O'Conchuir, R., O'Farrel, M., O'Flynn, C., Oidtmann, B., Renals, T., Ricciardi, A., Roy, H., Shaw, R., van Valkenburg, J.L.C.H., Weyl, O., Williams, F., Lucy, F.E. (2014). Tackling invasive alien species in Europe : the top 20 Issues. *Management of Biological Invasions 5*, 1–20.

Cairrão, E., Couderchet, M., Soares, a M.V.M., Guilhermino, L. (2004). Glutathione-S-transferase activity of Fucus spp. as a biomarker of environmental contamination. *Aquatic Toxicology 70*, 277–86.

Cantanhêde, G., Hahn, N. S., Gubiani, É. A., Fugi, R. (2008). Invasive molluscs in the diet of *Pterodoras granulosus* (Valenciennes, 1821) (Pisces, Doradidae) in the Upper Paraná River floodplain, Brazil. *Ecology of Freshwater Fish 17*, 47-53.

Cataldo, D., Boltovskoy D. (1999). Population dynamics of *Corbicula fluminea* (Bivalvia) in the Paraná river delta (Argentina). *Hydrobiologia 380*, 153-163.

Cataldo, D., Colombo, J. C., Boltovskoy, D., Bilos, C., Landoni, P. (2001). Environmental toxicity assessment in the Paraná river delta (Argentina): simultaneous evaluation of selected pollutants and mortality rates of *Corbicula fluminea* (Bivalvia) early juveniles. *Environmental Pollution 112*, 379–89.

Cherry, D.S., Scheller, J.L., Cooper, N.L., Bidwell, J.R. (2005). Potential effects of Asian clam (*Corbicula fluminea*) die-offs on native freshwater mussels (Unionidae) I : water-column ammonia levels and ammonia toxicity. *Journal of the North American Benthological Society 24*, 369–380.

Cid, A., Picado, A., Correia, J.B., Chaves, R., Silva, H., Caldeira, J., de Matos, A.P., Diniz, M.S. (2015). Oxidative stress and histological changes following exposure to diamond nanoparticles in the freshwater Asian clam *Corbicula fluminea* (Müller, 1774). *Journal of Hazardous Materials 284*, 27-34.

Clairborne, A. (1985). Catalase activity. In: Greenwald RA (ed) *CRC handbook of methods in oxygen radical research*. CRC Press, Boca Raton 283-284.

Colautti, R.I., Macisaac, H.J. (2004). A neutral terminology to define “invasive” species. *Diversity and Distributions 10*, 135-141.

Colombo, J.C., Bilos, C., Campanaro, M., Rodriguez-Presa, M.J., Catoggio, J.A. (1995). Bioaccumulation of polychlorinated biphenyls and chlorinated pesticides by the Asiatic clam *Corbicula fluminea*: Its use as a sentinel organism in the Rio de La Plata Estuary, Argentina. *Environmental Science and Technology 29*, 914-927.

Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs.

Concha-Graña, E., Turnes-Carou, M.I., Muniategui-Lorenzo, S., López-Mahía, P., Prada-Rodríguez, D., Fernández-Fernández, E. (2006). Evaluation of HCH isomers and metabolites in soils, leachates, river water and sediments of a highly contaminated area. *Chemosphere 64*, 588–95.

Conners, D.E., Westerfield, S.M., Feyko, A., Black, M.C. (1999). Lead accumulation in soft tissues and shells of Asiatic clams (*Corbicula fluminea*). In: *Proceedings of the Georgia Water Resources Conference*, held March 30-31, at the University of Georgia. Kathryn J. Hatcher (ed) Institute of Ecology, University of Georgia, Athens, Georgia.

Cooper, N.L., Bidwell, J.R., Cherry, D.S. (2005). Potential effects of Asian clam (*Corbicula fluminea*) die-offs on native freshwater mussels (Unionidae) II: porewater ammonia. *Journal of the North American Benthological Society 24*, 381–394.

Costantini, D., Omo, G.D. (2015) Oxidative stress predicts long-term resight probability and reproductive success in *Scopoli's shearwater* (Calonectris diomedea). *Conservation Physiology 3*, 1-7.

Cotter, E., Malham, S.K., O'Keeffe, S., Lynch, S.A., Latchford, J.W., King, J.W., Beaumont, A.R., Culloty, S.C. (2010). Summer mortality of the Pacific oyster, *Crassostrea gigas*, in the Irish Sea: The influence of growth, biochemistry and gametogenesis. *Aquaculture 303*, 8–21.

Cravo, A., Pereira, C., Gomes, T., Cardoso, C., Serafim, A., Almeida, C., Rocha, T., *et al.* (2012). A multibiomarker approach in the clam *Ruditapes decussatus* to assess the impact of pollution in the Ria Formosa lagoon, South Coast of Portugal. *Marine Environmental Research 75*, 23–34.

Cribb, A.E., Leeder, J.S., Spielberg, S.P. (1989). Use of a microplate reader in an assay of glutathione reductase using 5,50-dithiobis (2-nitrobenzoic acid). *Analytical Biochemistry 183*, 195–196.

Crooks J.A. (2002). Characterizing ecosystem-level consequences of biological invasions: the role of ecosystem engineers. *Oikos 97*, 153–166.

Cummings, K.S., Graf, D.L. (2010). Mollusca: Bivalvia. in J. H. Thorp and A. P. Covich (eds). *Ecology and classification of North American freshwater invertebrates*. Academic Press, Amsterdam, The Netherlands, 309–384 p.

DAISIE (2014) Delivering alien invasive species inventories for Europe (DAISIE). http://www.europe-aliens.org (Accessed in September 2014).

DAISIE (2015) Delivering alien invasive species inventories for Europe (DAISIE). http://www.europe-aliens.org (Accessed in September 2015).

Darrigran G. (2002). Potential impact of filter-feeding invaders on temperate inland freshwater environments. *Biological Invasions 4*, 145-156.

Davis, M.A. (2009). *Invasion Biology*. UK: Oxford University Press, 244 p.

Dégremont, L., Ernande, B., Bédier, E., Boudry, P. (2007). Summer mortality of hatchery-produced Pacific oyster spat (*Crassostrea gigas*). I. Estimation of genetic parameters for survival and growth. *Aquaculture 262*, 41–53.

Delgado, M., Camacho, A.P. (2005). Histological study of the gonadal development of *Ruditapes decussatus* (L.) (Mollusca : Bivalvia) and its relationship with available food. *Scientia Marina 69*, 87–97.

Denton, M.E., Chandra, S., Wittmann, M.E., Reuter, J., Baguley, J.G. (2012). Reproduction and Population Structure of *Corbicula fluminea* in an Oligotrophic Subalpine Lake. *Journal of Shellfish Research 31(1)*, 145-152.

Doherty, F.G. (1990). The Asiatic clam, Corbicula spp., as a biological monitor in freshwater environments. *Environmental Monitoring and Assessment 15*, 143–181.

Doherty, F.G., Cherry, D.S. (1988). Tolerance of the Asiatic clam Corbicula spp. to lethal levels of toxic stressors - a review. *Environmental Pollution 51*, 269–313.

Doherty, F.G., Cherry, D.S., Cairns Jr., J. (1987). Valve closure responses of the Asiatic clam *Corbicula fluminea* exposed to cadmium and zinc. *Hydrobiologia 153*, 159–167.

Duinker, A., Mortensen, S. (1999). Kvalitet av skjellet kritisk punkt for en voksende eksportnæring. *Norsk Fiskeoppdrett 19*, 30-32.

Duman, F., Urey, E., Kar, M. (2013). Temporal Variation of Metal Concentrations of Creek Sediment Samples. *Polish Journal of Environmental Studies 22*, 1335–1339.

ECHA http://echa.europa.eu/information-on-chemicals/information-from-existing-substances-regulation?p_p_id=viewsubstances_WAR_echarevsubstanceportlet&p_p_lifecycle=0&p_p_state=normal&p_p_mode=view&p_p_col_id=column-1&p_p_col_pos=1&p_p_col_count=2&_viewsubstances_WAR_echarevsubstanceportlet_keywords=&_viewsubstances_WAR_echarevsubstanceportlet_advancedSearch=false&_viewsubstances_WAR_echarevsubstanceportlet_andOperator=true&_viewsubstances_WAR_echarevsubstanceportlet_orderByCol=staticField_-105&_viewsubstances_WAR_echarevsubstanceportlet_orderByType=asc&_viewsubstances_WAR_echarevsubstanceportlet_delta=200 assessed on 1st July 2015.

El-Moselhy, Kh., Othaman, M., El-Azem, A.I., Abd, H., El-Metwally, M.E.A. (2014). Bioaccumulation of heavy metals in some tissues of fish in the Red Sea, Egypt. *Egyptian Journal of Basic and Applied Sciences I*, 97-105.

Ellis, G., Goldberg, D.M. (1971). An improved manual and semi-automatic assay for NADP-dependent isocitrate dehydrogenase activity, with a description of some kinetic properties of human liver and serum enzyme. *Clinical Biochemistry 2*, 175–185.

Ellman, G.L., Courtney, K.D., Andres, V., Featherstone, R.M. (1961). A new rapid colorimetric determination of acetylcholinesterase activity. *Biochemical Pharmacology 7*, 88–95.

Elser, J.J., Sterner,R.W., Galford, A.E., Chrzanowski, T.H., Findlay, D.L., Mills, K.H., Paterson, M.J., Stainton, M.P., Schindler, D.W. (2000). Pelagic C:N:P stoichiometry in a eutrophied lake: responses to a whole-lake food-web manipulation. *Ecosystems 3*, 293–307.

Engel, K., Tollrian, R., Jeschke, J.M. (2011). Integrating biological invasions, climate change, and phenotypic plasticity. *Communicative & Integrative Biology 4*, 247–250.

European Comission (2014) Proposal for a regulation of the European parliament and of the council on the prevention and management of the introduction and spread of invasive alien species COM (2014) 317, Strasbourg, France, October 24, 2014, 49 pp

European Commission (2011) Our life insurance, our natural capital: an EU biodiversity strategy to 2020. Communication from the Commission of the European Parliament, the Council, the Economic and Social Committee and the Committee of the Regions COM (2011) 244, Brussels, Belgium, May 3, 2006, 17 pp

European Commission (2013) Proposal for a regulation of the European parliament and of the council on the prevention and management of the introduction and spread of invasive alien species COM (2013) 620 final, COD (2013) 0307, Brussels, Belgium, September 9, 2013, 36 pp

Evdokimov, V.V., Matrosova, I.V. (2009). Seasonal characteristics of gametogenesis of some marketable hydrobionts. *Cell and Tissue Biology 3*, 593–602.

Facon, B., Genton, B.J., Jarne, J.S.P., Estoup, A., Patrice, D. (2006). A general eco-evolutionary framework for understanding bioinvasions. *Trends in Ecology and Evolution 21*, 130-135.

Farcy, E., Burgeot, T., Haberkorn, H., Auffret, M., Lagadic, L., Allenou, J.P., Budzinski, H., Mazzella, N., Pete, R., Heydorff, M., Menard, D., Mondeguer, F., Caquet, T. (2013). An integrate environmental approach to investigate biomarker fluctuations in the blue mussel *Mytilus edulis* in the Vilaine estuary, France. *Environmental Science and Pollution Research 20*, 630–650.

Farkas, A., Erratico, C., Vigano, L. (2007). Assessment of the environmental significance of heavy metal pollution in surficial sediments of the River Po. *Chemosphere 68*, 761–768.

Farris, J.L., Van Hassel, J.H., Belanger, S.E., Cherry, D.S., Cairns, J. (1988). Application of cellulolytic activity of Asiatic clams (Corbicula sp.) to in-stream monitoring of power plant effluents. *Environmental Toxicology and Chemistry 7*, 701–713.

Ferreira, A.M., Micaelo, C., Vale, C. (2003). Are coastal resources of NW Portugal fingerprinting hydrocarbons released from the Prestige accident? *Ciencias Marinas 29*, 109–114.

Figuerola, J., Green A. (2002). Dispersal of aquatic organisms by waterbirds: a review of past research and priorities for future studies. *Freshwater Biology 47*, 483-494.

Filgueiras, A.V., Lavilla, I., Bendicho, C. (2004). Evaluation of distribution, mobility and binding behaviour of heavy metals in surficial sediments of Louro River (Galicia , Spain) using chemometric analysis : a case study. *Science of the Total Environment 330*, 115–129.

Foe, C., Knight, A. (1986). A Method for Evaluating the Sublethal Impact of Stress Employing *Corbicula fluminea.* in Prezant, R. S. (ed.), *Proceedings of the Second International Corbicula Symposium, American Malacological Bulletin*, Special Edition 2, pp. 133-142.

Frasco, M.F., Guilhermino, L. (2002). Effects of dimethoate and betanaphthoflavone on selected biomarkers of *Poecilia reticulate*. *Fish Physiology and Biochemistry 26*, 149-156.

Fried, B., Emili, S. (1987). Experimental infection of *Corbicula fluminea* (Bivalvia: Corbiculidae with Echinostoma revolutum cercariae. *Journal of Parasitology 73*, 655–656.

Fritz, L. W., Lutz, R.A. (1986). Environmental Perturbations Reflected in Internal Shell Growth Patterns of *Corbicula fluminea* (Mollusca: Bivalvia). *The Veliger 28*, 401-417.

Fureder, L., Pockl, M. (2007). Ecological traits of aquatic NIS invading Austrian fresh waters. *Biological invaders in inland waters: Profiles, distribution, and threats 2*, 233–257.

García-esquivel, Z., Bricelj, V.M., Gonzalez-gomez, M.A. (2001). Physiological basis for energy demands and early post-larval mortality in the Pacific oyster, *Crassostrea gigas*. *Journal of Experimental Marine Biology and Ecology 263*, 77–103.

Gerd, G., Weeda, E., Gabbott, P.A., Zoophysiologie, I., Bonn, U., Avz, I., Allee, E. (1978). Changes in the Level of Octopine during the Escape Responses of the Scallop *Pecten maximus* (L.). *Journal of Comparative Physiology 124*, 121–127.

Gollasch, S. (2006). Overview on introduced aquatic species in European navigational and adjacent waters. *Helgoland Marine Research 60*, 84– 89.

González-Flores, E., Tornero-Campante, M.A., Sandoval-Castro, E., Pérez-Magaña, A., Gordillo-Martínez, A. J. (2011). Biodisponibilidad y fraccionamiento de metales pesados en suelos agrícolas enmendados con biosólidos de origen municipal. *Revista Internacional de Contaminación Ambiental 27*, 290-301.

Graczyk, T.K., Fried., B. (1998). Echinostomiasis: a common but forgotten food-borne disease. *The American Journal of Tropical Medicine and Hygiene 58*, 501-504.

Gravato, C., Guimarães, L., Santos, J., Faria, M., Alves, A., Guilhermino, L. (2010). Comparative study about the effects of pollution on glass and yellow eels (*Anguilla anguilla*) from the estuaries of Minho, Lima and Douro Rivers (NW Portugal). *Ecotoxicology and Environmental Safety 73*, 524-533.

Guilhermino, L., Lopes, M.C., Carvalho, A.P., Soares, A.M. (1996). Acetylcholinesterase activity in juveniles of *Daphnia magna* Straus. *Bulletin of Environmental Contamination and Toxicology 57*, 979–985.

Guimarães, L., Medina, M.H., Guilhermino, L. (2009). Health status of Pomatoschistus microps populations in relation to pollution and natural stressors: implications for ecological risk assessment. *Biomarkers 17*, 62–77.

Gutiérrez, J.L., Jones, C.G., Strayer, D.L., Iribarne O.O. (2003). Mollusks as ecosystem engineers: the role of shell production in aquatic habitats. *Oikos 101*, 79–90.

Habig, W.H., Pabst, M.J., Jacoby, W.B. (1974). Glutathione S-transferases. The first enzimatic step in mercapturic acid formation. *The Journal of Biological Chemistry 249*, 7130-7139.

Hakenkamp C.C., Palmer M.A. (1999). Introduced bivalves in freshwater ecosystems: the impact of *Corbicula* on organic matter dynamics in a sandy stream. *Oecologia 119*, 445-451.

Hakenkamp, C.C., Ribblett, S.G., Palmer, M.A., Swan, C.M., Reid, J.W., Goodison, M.R. (2001). The impact of an introduced bivalve (*Corbicula fluminea*) on the benthos of a sandy stream. *Freshwater Biology 46*, 491–501.

Hedtke, S.M., Stanger-Hall, K., Baker, R.J., Hillis, D.M. (2008). All-male asexuality: Origin and maintenance of androgenesis in the Asian clam *Corbicula*. *Evolution 62*, 1119–1136.

Hellmann, J.J., Byers, J.E., Bierwagen, B.G., Dukes, J.S. (2008). Five potential consequences of climate change for invasive species. *Conservation Biology* 22, 534–543.

Hill, N., Johnston, E. L., King, C. K., Simpson, S.L. (2011). Physico-chemical changes in metal-spiked sediments deployed in the field: implications for the interpretation of in situ studies. *Chemosphere 83*, 400–408.

Hornbach, D.J. (1992). Life history traits of a riverine population of the Asian clam *Corbicula fluminea*. *American Midland Naturalist 127*, 248–257.

Houki, S., Mitsuya, Y., Takako, H., Akira, K. (2011). Origin and possible role of males in hermaphroditic androgenetic *Corbicula* clams. *Zoological science 28*, 526-531.

Hubenov, Z., Trichkova, T., Kenderov, L., Kozuharov, D. (2013). Distribution of *Corbicula fluminea* (Mollusca: Corbiculidae) over an eleven-year period of its invasion in Bulgaria. *Acta Zoologica Bulgarica 65*, 315–326.

Huvet, A., Normand, J., Fleury, E., Quillien, V., Fabioux, C., Boudry, P. (2010). Reproductive effort of Pacific oysters: A trait associated with susceptibility to summer mortality. *Aquaculture 304*, 95–99.

IARC (1993). Beryllium, cadmium, mercury, and exposures in the glass manufacturing industry. IARC Monogr Eval Carcinog Risks Hum, 58, 1–415.

IARC (2009). Arsenic, metals, fibres, and dusts. IARC Monogr Eval Carcinog Risks Hum, 100C, 1–469.

Ilarri, M.I., Antunes, C., Guilhermino, L., Sousa, R. (2010). Massive mortality of the Asian clam *Corbicula fluminea* in a highly invaded area. *Biological Invasions 13*, 277–280.

Ilarri M.I., Freitas F., Costa-Dias S., Antunes C., Guilhermino L., Sousa R. (2012). Associated macrozoobenthos with the invasive Asian clam *Corbicula fluminea*. *Journal of Sea Research 72*, 113–120.

Ilarri, M., Sousa, R. (2011). *Corbicula fluminea* Müller (Asian Clam). In: *A handbook of global freshwater invasive species* (R.A. Francis, ed.), pp. 173–183. Publishing for a Sustainable Future, London and New York.

INAG (2000). Planos das bacias Hidrograficas dos rios Luso-Espanhois-Sintese. Caracterização recursos Hídricos, Divisão e Diagnóstico. Instituto da Água, Direcção de serviço de Recursos Subterrâneos, 398 p.

Inza, B., Ribeyre, F., Maury-Brachet, R., Boudou, A. (1997). Tissue distribution of inorganic mercury, methylmercury and cadmium in the Asiatic clam (*Corbicula fluminea*) in relation to the contamination of the water column and sediment. *Chemosphere 35*, 2817-2836.

Jeffrey, S.W., Humphrey, G.F. (1975). New spectrophotometric equations for determining chlorophylls a, b, c1 and c2 in higher plants, algae and natural phytoplankton. *Biochemie und Physiologie der Pflanzen 167*, 191–194.

Johnson, P.D., McMahon, R.F. (1998). Effects of temperature and chronic hypoxia on survivorship of the zebra mussel (*Dreissena polymorpha*) and Asian clam (*Corbicula fluminea*). *Canadian Journal of Fisheries and Aquatic Sciences 55*, 1564–1572.

Kalinina, G.G., Matrosova, I.V., Evdokimova, A.V., Evdokimov, V.V. (2006). Seasonal characteristics of gametogenesis in the *Corbicula japonica*. *Tsitologiia 48*, 149–52.

Kamburska, L., Lauceri, R., Beltrami, M., Boggero, A., Cardeccia, A., Guarneri, I., Manca, M., Riccardi, N. (2013). Establishment of *Corbicula fluminea* (O.F. Müller, 1774) in Lake Maggiore: a spatial approach to trace the invasion dynamics. *BioInvasions Records 2*, 105–117.

Karatayev, A., Padilla, D., Minchin, D. (2007). Changes in global economies and trade: the potential spread of exotic freshwater bivalves. *Biological Invasions 9*, 161–180.

Karatayev, A.Y., Burlakova, L.E., Padilla, D.K. (2005). Contrasting distribution and impacts of two freshwater exotic suspension feeders, *Dreissena polymorpha* and *Corbicula fluminea*. In Dame, R. & S. Olenin (eds), *The comparative role of suspension feeders in ecosystems.* NATO Science Series: IV–Earth and Environmental Sciences. Springer, Dordrecht, Netherlands, 239–262 pp.

Karatayev, A.Y., Burlakova, L.E., Padilla, D.K., Johnson, L.E. (2003). Patterns of spread of the zebra mussel (*Dreissena polymorpha* (Pallas): the continuing invasion of Belarussian Lakes. *Biological Invasions 5*, 213–221.

Kennedy, V.S., Huekelem, L.V. (1985). Gametogenesis and larval production in a population of the introduced Asiatic Clam, *Corbicula* sp. (Bivalvia:Corbiculidea), in Maryland. *The Biological Bulletin 168*, 50-60.

Kolar, C.S., Lodge, D.M. (2001). Progress in invasion biology: predicting invaders. *Trends in Ecology & Evolution 16*, 199–204.

Komaru, A., Kawagishi, T., Konishi, K. (1998). Cytological evidence of spontaneous androgenesis in the freshwater clam *Corbicula leana* Prime. *Development Genes and Evolution 208*, 46–50.

Kraemer, L.R., Galloway, M.L. (1986). Larval development of *Corbicula fluminea* (Müller) (Bivalvia, Corbiculacea) - An appraisal of heterochrony. *American Malacological Bulletin 4*, 61-79.

Larson, D.L., Phillips-Mao, L., Quiram, G., Sharpe, L., Stark, R., Sugita, S., Weiler, A. (2011). A framework for sustainable invasive species management: Environmental, social, and economic objectives. *Journal of Environmental Management 92*, 14–22.

Lauritsen, D.D. (1986). Filter-feeding in *Corbicula fluminea* and its effect on seston removal. *Journal of the North American Benthological Society 5*, 165–172.

Lavilla, I., Garrido, J., Bendicho, C. (2010). A biogeochemical approach to understanding the accumulation patterns of trace elements in three species of dragonfly larvae : evaluation as biomonitors. *Journal of Environmental Monitoring 12*, 724–730.

Lee, T., Siripattrawan, S., Ituarte, C.F., O Foighil, D. (2005). Invasion of the clonal clams: *Corbicula* lineages in the New World. *American Malacological Bulletin 20*, 113-122.

Lee, C.E. (2002). Evolutionary genetics of invasive species. *Trends in Ecology and Evolution 17*, 386-391.

Lee, S., Kim, S-J., Ju, S-J., Pak, S-J., Son, S-K.,Yang, J., Han S. (2015). Mercury accumulation in hydrothermal vent mollusks from the southern Tonga Arc, southwestern Pacific Ocean. *Chemosphere 127*, 246–253.

Legeay, A., Achard-Joris, M., Baudrimont, M., Massabuau, J-C., Bourdineaud, J-P. (2005). Impact of cadmium contamination and oxygenation levels on biochemical responses in the Asiatic clam *Corbicula fluminea*. *Aquatic Toxicology 74*, 242–253.

Lehmann, D.W., Levine, J.F., McHugh, Law, J. (2007) Polychlorinated biphenyl exposure causes gonadal atrophy and oxidative stress in *Corbicula fluminea* clams. *Toxicologic Pathology 35*, 356–65.

Lima, I., Moreira, S.M., Osten, J.R.-V., Soares, A.M.V.M., Guilhermino, L. (2007). Biochemical responses of the marine mussel *Mytilus galloprovincialis* to petrochemical environmental contamination along the North-western coast of Portugal. *Chemosphere 66*, 1230–1242.

Livingstone, D.R., Stickle, W.B., Kapper, M.A., Wang, S., Zurburg, W. (1990). Further studies on the phylogenetic distribution of pyruvate oxidoreductase activities. *Comparative Biochemistry and Physiology Part B* 97, 661–666.

Lockwood, J.L., Hoopes, M.F., Marchetti ,M.P. (2007) Invasion Ecology. Blackwell Publishing Ltd, 304 p.

Lodge, D.M., Williams, S., MacIsaac, H.J., Hayes, K.R., Leung, B., Reichard, S., Mack, R.N., Moyle, P.B., Smith, M., Andow, D.A., Carlton, J.T., McMichael, A. (2006) Biological invasions: Recommendations for U.S. policy and management. *Ecological Applications 16*, 2035–2054.

Lukac, N.,, Massanyi ,P., Krockova, J., Toman, R., Danko, J., Stawarz, R., Formicki, G. (2014). Effect of Nickel on Male Reproduction. *Universal Journal of Agricultural Research 2*, 250-252.

Lushchak, V.I. (2011) Environmentally induced oxidative stress in aquatic animals. *Aquatic Toxicology 101*, 13–30.

Maanan, M. (2008). Heavy metal concentrations in marine molluscs from the Moroccan coastal region. *Environmental Pollution 153*, 176–183.

Mackie, G.L., Claudi, R. (2010). Monitoring and control of macrofouling mollusks in fresh water systems. Boca Raton, FL: CRC Press. 508 p.

Marigómez, I., 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.

Matthews, M.A., McMahon, R.F. (1999). Effects of temperature and temperature acclimation on survival of zebra mussels (*Dreissena polymorpha*) and asian clams (*Corbicula fluminea*) under extreme hypoxia. *Journal of Molluscan Studies 65*, 317- 325.

McDowell, B., Byers, J., Rosemond, A., McDowell, W. (2013). Effects of Mass Mortality of an Abundant Invasive Species on Ecosystem Function. Proceedings of the 2013 Georgia Water Resources Conference, held April 10–11, 2013, at the University of Georgia.

McLaughlan, C., Gallardo ,B., Aldridge, D.C. (2014). How complete is our knowledge of the ecosystem services impacts of Europe's top 10 invasive species? *Acta Oecologica 54*, 119–130.

McMahon, R.F. (2000). Invasive characteristics of the freshwater bivalve *Corbicula fluminea*. In: Nonindigenous Freshwater Organisms: Vectors, Biology and Impacts (ed Claudi R), pp. 315–343. CRC Press LLC, Boca Raton.

McMahon, R. (1991). Mollusca: Bivalvia. in Thorp, J. H. and A. P. Covich. (eds.) *Ecology and Classification of North American Freshwater Invertebrates*. Academic Press, Inc., New York. 911p.

McMahon, R.F. (1999). Invasive characteristics of the freshwater bivalve *Corbicula fluminea*. In R. Claudi and J. H. Leach (eds.) *Nonindigenous*

freshwater organisms: Vectors, biology, and impacts. CRC Press, Boca Raton, 315 – 343 pp.

McMahon, R.F., Williams, C.J. (1986). A Reassessment of Growth Rate, Life Span, Life Cycles and Population Dynamics in a Natural Population and Field Caged Individuals of *Corbicula fluminea* (Müller) (Bivalvia: Corbiculacea). In Prezant, R. S. (ed.) *Proceedings of the Second International Corbicula Symposium, American Malacological Bulletin*, Special Edition No. 2, pp. 151-166.

McMahon, R.F. (1982). The occurrence and spread of the introduced Asiatic freshwater clam *Corbicula fluminea* (Müller) in North America: 1924 - 1982. *The Nautilus 96*, 134 - 141.

McMahon, R.F. (2002). Evolutionary and physiological adaptations of aquatic invasive animals : r selection versus resistance. *Canadian Journal of Fisheries and Aquatic Sciences 59*, 1235–1244.

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.

Minchin, D., Lucy, F., Sullivan, M. (2002). Zebra mussel: impacts and spread. In Leppäkoski, E., Gollasch, S., Olenin, S. (eds). *Invasive aquatic species of Europe: distribution, impacts and management.* Dordrecht: Kluwer Academic Publishers, 135-148 p.

Minchin, D. (2009). *Corbicula fluminea* (Müller), Asian clam (Corbiculidae, Molluska). In: *Handbook of alien species in Europe: DAISIE*. Volume 3 of Invading Nature - Springer Series in Invasion Ecology (ed.) Illustrated 428 pp.

Minchin, D. (2014). The distribution of the Asian clam *Corbicula fluminea* and its potential to spread in Ireland. *Management of Biological Invasions 5*, 165–177

Monteiro, M., Quintaneiro, C., Nogueira, J., Morgado, F., Soares, A.M., Guilhermino, L. (2007). Impact of chemical exposure on the fish Pomatoschistus microps Krøyer (1838) in estuaries of the Portuguese Northwest coast. *Chemosphere 66*, 514–22.

Moreira, S.M., Moreira-Santos, M., Guilhermino, L., Ribeiro, R. (2006). An in situ post exposure feeding assay with *Carcinus maenas* for estuarine sediment-overlying water toxicity evaluations. *Environmental Pollution* 139, 318–29.

Morgan, D.E., Keser, M., Swenarton, J.T., Foertch, J.F. (2003). Population dynamics of the Asiatic clam, *Corbicula fluminea* (Muller) in the Lower Connecticut River: Establishing a foothold in New England. *Journal of shellfish research 22*, 193–203.

Morton, B. (1986). Corbicula in Asia - an updated synthesis. *American Malacological Bulletin, Special Edition 2*, 113-124.

Morton, B. (1977). Freshwater fouling bivalves; Fort Worth, Texas, USA: Texas Christian University. 1–14 pp.

Mouthon, J. (2001a). Life cycle and population dynamics of the Asian clam *Corbicula fluminea* (Bivalvia: Corbiculidae) in the Rhone River at Creys-Malville (France). *Archiv Fur Hydrobiologie 151*, 571-589.

Mouthon, J. (2001b). Life cycle and population dynamics of the Asian clam Corbicula fluminea (Bivalvia: Corbiculidae) in the Saone River at Lyon (France). *Hydrobiologia 452*, 109-119.

Mouthon, J. (1981). Sur la présence en France et au Portugal de *Corbicula* (Bivalvia, Corbiculidae) originaire dé Asie. *Basteria 45*, 109–116.

Mouthon, J., Daufresne, M. (2006). Effects of the 2003 heat wave and climatic warming on mollusc communities of the Saone: a large lowland river and of its two main tributaries (France). *Global Change Biology 12*, 441–449.

Mouthon, J., Parghentanian, T. (2004). Comparison of the life cycle and population dynamics of two Corbicula species, *C. fluminea* and *C. fluminalis* (Bivalvia: Corbiculidae) in two French canals. *Archiv für Hydrobiologie 161*, 267–287.

MSFD, 2008. Directive 2008/56/EC of the European Parliament and the Council of 17 June 2008 Establishing a Framework for Community Action in the Field of Marine Environmental Policy (Marine Strategy Framework Directive).

Myrand, B., Guderley, H., Himmelman, J. (2000). Reproduction and summer mortality of blue mussels *Mytilus edulis* in the Magdalen Islands,

southern Gulf of St. Lawrence. *Marine Ecology Progress Series 197*, 193–207.

Nally, R.M. (2000). Regression and model-building in conservation biology, biogeography and ecology: the distinction between– and reconciliation of–'predictive' and "explanatory" models. *Biodiversity Conservation 9*, 655–671.

Nentwig, W. (2007) Pathways in animal invasions. *Biological Invasions* 11–28 pp.

Netpae, T., Phalaraksh, C., Wongkham, W. (2012). Antioxidant Enzyme Activities and DNA Damage as Biomarker of Copper Effect on *Corbicula fluminea*. *Electronic Journal of Biology 8*, 19-23.

Nicolodi, J., Toldo, Jr. E., Farina, L. (2011). Wave dynamics and resuspension in Lake Guaíba (Brazil) with implications on points of water abstraction for human supply. *Journal of Coastal Research 64*, 1550–1554.

Occhipinti-Ambrogi, A., Galil, B.S. (2004). A uniform terminology on bioinvasions: A chimera or an operative tool? *Marine Pollution Bulletin 49*, 688–694.

Ohkawa, H. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry 95*, 351-358.

Ojaveer, H., Galil, B.S., Gollasch, S., *et al.* (2014). Identifying the top issues of marine invasive alien species in Europe. *Management of Biological Invasions 5*, 81–84.

Oliveira, C., Vilares, P., Guilhermino, L. (2015). Integrated biomarker responses of the invasive species *Corbicula fluminea* in relation to environmental abiotic conditions: A potential indicator of the likelihood of clam's summer mortality syndrome. *Comparative Biochemistry and Physiology Part A 182*, 27-37.

Ortiz, C.A., Ashton, A., Nepf, H. (2013). Mean and turbulent velocity fields near rigid and flexible plants and the implications for deposition. *Journal of Geophysical Research: Earth Surface118*, 2585–2599.

Ortmann, C., Grieshaber, M.K. (2003). Energy metabolism and valve closure behaviour in the Asian clam *Corbicula fluminea*. *Journal of Experimental Biology 206*, 4167–4178.

Parchaso, F., Thompson, J.K. (2002). Influence of hydrologic processes on reproduction of the introduced bivalve *Potamocorbula amurensis* in northern San Francisco Bay, California. *Pacific Science 56*, 329–345.

Park, G.M., Chung, E.Y. (2004). Histological studies on hermaphroditism, gametogenesis and cyclic changes in the structures of marsupial gills of the introduced Asiatic calm, *Corbicula fluminea*, and the Korean clam, *Corbicula leana*. *Journal of Shellfish Research, 23*, 179 - 184.

PBH (2001). Plano de Bacia Hidrográfica do Rio Minho, Relatório Final, Ministério do Ambiente e do Ordenamento do Território, Instituto da Água, IP.

Pérez-Quintero, J.C. (2008) Revision of the distribution of *Corbicula fluminea* (Müller 1744) in the Iberian Peninsula. *Aquatic Invasions 3*, 355–358.

Perrings, C., Dehnen-Schmutz, K., Touza, J., Williamson, M. (2005). How to manage biological invasions under globalization. *Trends in Ecology and Evolution 20*, 212–215.

Pfenninger, M., Reinhardt, F., Streit B. (2002). Evidence for cryptic hybridization between different evolutionary lineages of the invasive clam genus Corbicula (Veneroida, Bivalvia). *Journal of Evolutionary Biology 15*, 818-829.

Phelps, H. L. (1994). Potential for *Corbicula* in aquaculture. *Journal of Shellfish Research 13*, 319.

Phillips, D.J.H. (1976). The common mussel *Mytilus edulis* as an indicator of pollution by zinc, cadmium, lead and copper. I. Effects of environmental variables on uptake of metals. *Marine Biology 38*, 59-69.

Pigneur, L.M., Marescaux, J., Roland, K., Etoundil, E., Descy, J.P., Doninck, K.V. (2011). Phylogeny and androgenesis in theinvasive Corbicula clams (Bivalvia, Corbiculidae) in Western Europe. *BMC Evolutionary Biology 11*, 147.

Pimentel, D., Pimentel, M., Wilson, A. (2007). Plant, animal, and microbe invasive species in the United States and world. In: Nentwig W (ed), *Biological invasions*. Springer, Berlin, 315–330 pp.

Planelló, R., Servia, M. J., Gómez-Sande, P., Herrero, O., Cobo, F., Morcillo, G. (2013). Transcriptional Responses, Metabolic Activity and

Mouthpart Deformities in Natural Populations of *Chironomus riparius* Larvae Exposed to Environmental Pollutants. *Environmental Toxicology 30*, 383-95.

Power, M., Tilman, D., Estes, J., Menge, B. (1996). Challenges in the quest for keystones. *Bioscience 46*, 609–620.

Prezant, R.S., Chalermwat, K. (1984). Flotation of the Bivalve *Corbicula fluminea* as a Means of Dispersal. *Science 225*, 1491–1493.

Pueyo, M., Rauret, G., Lück, D., Yli-Halla, M., Muntau, H., Quevauviller, Ph., López-Sánchez, J.F. (2001). Certification of the extractable contents of Cd, Cr, Cu, Ni, Pb and Zn in a freshwater sediment following a collaboratively tested and optimised three-step sequential extraction procedure. *Journal of Environmental Monitoring 3*, 243-250.

Quintaneiro, C., Monteiro, M., Pastorinho, R., *et al.* (2006). Environmental pollution and natural populations: a biomarkers case study from the Iberian Atlantic coast. *Marine Pollution Bulletin 52*, 1406–13.

Rahel, F.J., Olden, J.D. (2008). Assessing the effects of climate change on aquatic invasive species. *Conservation Biology 22*, 521-33.

Rajagopal, S., van der Velde, G., bij de Vaate, A. (2000). Reproductive biology of the Asiatic clams *Corbicula fluminalis* and *Cobicula fluminea* in the river Rhine*. Arch für Hydrobiol 149*, 403–420.

Rao, C.R.M., Sahuquillo, A., López-Sánchez, J.F. (2008). A review of the different methods applied in environmental geochemistry for single and sequential extraction of trace elements in soils and related materials. *Water Air and Soil Pollution 189*, 291-333.

Rauret, G., López-Sánchez, J. F., Sahuquillo, A., Rubio, R., Davison, C. M., Ure, A. M., Quevauviller, Ph. (1999). Improvement of the BCR three step sequential extraction procedure prior to the certification of new sediment and soil reference materials. *Journal of Environmental Monitoring 1*, 57-61.

Reed, A.J., Thatje, S., Linse, K. (2013). An unusual hermaphrodite reproductive trait in the Antarctic brooding bivalve *Lissarca miliaris* (Philobryidae) from the Scotia Sea, Southern Ocean. *Polar Biology* 36, 1–11.

Reis, P.A., Guilhermino, L., Antunes, C., Sousa, R. (2014). Assessment of the ecological quality of the Minho estuary (Northwest Iberian Peninsula) based on metal concentrations in sediments and in *Corbicula fluminea*. *Limnetica, 33,* 161-174.

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

Ren, J., Luo, J., Ma, H., Wang, X., Ma, L.Q. (2013). Bioavailability and oxidative stress of cadmium to Corbicula fluminea. *Environmental Science: Processes & Impacts 15*, 860-869.

Renard, E., Bachmann, V., Cariou, M.L., Moreteau, J.C. (2000). Morphological and molecular differentiation of invasive freshwater species of the genus *Corbicula* (Bivalvia, Corbiculidea) suggest the presence of three taxa in French rivers. *Molecular Ecology 9*, 2009-2016.

Ribeiro, D.C., Costa, S., Guilhermino, L., In press. A framework to assess the vulnerability of estuarine systems for use in ecological risk assessment. *Ocean & Coastal Management*.

Rifi, M., Le Pennec, G., Ben, S.M., Ben, S.J. (2011). Reproductive strategy of the invasive cockle *Fulvia fragilis* in the Bay of Tunis (Tunisia). *Journal of the Marine Biological Association of the United Kingdom 91*, 1465–1475.

Rodrigues, A.P., Oliva-Teles, T., Mesquita, S.R., Delerue–Matosc, C., Guimarães, L. (2014). Integrated biomarker responses of an estuarine invertebrate to high abiotic stress and decreased metal contamination. *Marine Environmental Research 101*, 101–114.

Roman, J., Darling, J.A. (2007). Paradox lost: genetic diversity and the success of aquatic invasions. *Trends in Ecology & Evolution 22*, 454–464.

Rosa, I.C., Pereira, J.L., Costa, R., *et al.* (2012). Effects of Upper-Limit Water Temperatures on the Dispersal of the Asian Clam *Corbicula fluminea*. *PLoS One 7:e46635*.

Rosa, I.C., Pereira, J.L., Costa, R., Gomes, J., Pereira, M.L., Gonçalves, F. (2014). Dispersal of *Corbicula fluminea*: factors influencing the invasive clam's drifting behavior. *Annales de Limnologie - International Journal of Limnology 50*, 37–47.

Rosa, I.C., Pereira, J.L., Gomes, J., Saraiva, P.M., Gonçalves, F., Costa, R. (2011). The Asian clam *Corbicula fluminea* in the European freshwater-dependent industry: A latent threat or a friendly enemy? *Ecological Economics 70*, 1805–1813.

Rubal, M., Guilhermino, L., Medina, M. H. (2009). Individual, population and community level effects of subtle anthropogenic contamination in estuarine meiobenthos. *Environmental Pollution 157,* 2751–2758.

Rybalkina, S.M., Maiorova, M.A., Anisimov, A.P., Kravchenko, D.N. (2013). The gametogenesis and sexual cycle of the bivalve *Corbicula japonica* Prime (1864) in the mouth of the Kievka River (Sea of Japan). *Russian Journal of Marine Biology 39*, 253–264.

Sahuquillo, A., López-Sánchez, J.F., Rauret, G., Ure, A.M., Muntau, H., Quevauviller, P. (2002). Sequential extraction procedures for sediment analyses. In: Methodologies for soil and sediment fractionation studies, Edited by Quevauviller P., pages 10-27; The Royal Society of Chemistry.

Sakai, A., Allendorf, F., Holt, J. (2001). The Population Biology of Invasive Species. *Annual Review of Ecology, Evolution, and Systematics 32*, 305–320.

Salman, J. (2011). The Clam *Pseudodontpsis euphraticus* (Bourguignat, 1852) as a Bioaccumulation Indicator Organism of Heavy Metals in Euphrates River-Iraq. *Journal of Babylon University/Pure and Applied Sciences 11*, 116-125.

Samain, J.F., Dégremont, L., Soletchnik, P.,Haure, J., Bédier, E., Ropert, M., Moal, J., Huvet, A., Bacca, H., VanWormhoudt, A., Delaporte, M., *et al.* (2007). Genetically based resistance to summermortality in the Pacificoyster (*Crassostrea gigas*) and its relationship with physiological, immunological characteristics and infection process. *Aquaculture 268*, 227–243.

Samain, J.F., McCombie, H. (2008). Summer Mortality of Pacific Oyster Crassostrea gigas. Ed. Ifremer/Quae, 379 pp.

Santos, S., Vilar, V. J. P., Alves, P., Boaventura, R. A., Botelho, C. (2013). Water quality in Minho/Miño River (Portugal/Spain). *Environmental Monitoring and Assessment 185*, 3269–3281.

Schmidlin, S., Schmera, D., Ursenbacher, S., Baur, B. (2012). Separate introductions but lack of genetic variability in the invasive clam *Corbicula* spp. in Swiss lakes. *Aquatic Invasions 7*, 73–80.

Sebesvari, Z., Ettwig, K. F., Emons, H. (2005). Biomonitoring of tin and arsenic in different compartments of a limnic ecosystem with emphasis on Corbicula fluminea and Dikerogammarus villosus. *Journal of Environmental Monitoring 7*, 203–207.

Shoults-Wilson, W.A., Peterson, J.T., Unrine, J.M., Rickard, J., Black, M.C. (2009). The Asian clam *Corbicula fluminea* as a biomonitor of trace element contamination: accounting for different sources of variation using an hierarchical linear model. *Environmental Toxicology and Chemistry 28,* 2224–2232.

Simard, M.A., Paquet, A., Jutras, C., Robitaille, Y., Blier, P.U., Courtois, R., Martel, A.L. (2012). North American range extension of the invasive Asian clam in a St. Lawrence River power station thermal plume. *Aquatic Invasions 7*, 81–89.

Simberloff, D., Martin, J., Genovesi, P., Maris, V., Wardle, D.A., Aronson, J., Courchamp, F., Galil, B., García-Berthou, E., Pascal, M. (2012). Impacts of biological invasions: what's what and the way forward. *Trends in Ecology & Evolution 28*, 58– 66.

Simonit, S., Perrings, C. (2011). Sustainability and the value of the 'regulating' services: Wetlands and water quality in Lake Victoria. *Ecological Economics 70*, 1189–1199.

Soletchnik, P., Lambert, C., Costil, K. (2005). Summer mortality of *Crassostrea gigas* (Thunberg) in relation to environmental rearing conditions. *Journal of shellfish research 24*, 197–207.

Sousa, R., Antunes, C., Guilhermino, L. (2008a). Ecology of the invasive Asian clam *Corbicula fluminea* (Müller, 1774) in aquatic ecosystems : an overview. *Annales de Limnologie - International Journal of Limnology 44*, 85–94.

Sousa, R., Antunes, C., Guilhermino, L. (2006a). Factors influencing the occurrence and distribution *of Corbicula fluminea* (Müller, 1774) in the River Lima estuary. *Annales de Limnologie - International Journal of Limnology 42*, 165–171.

Sousa, R., Antunes, C., Guilhermino, L. (2007a). Species composition and monthly variation of the Molluscan fauna in the freshwater subtidal area of the River Minho estuary. *Estuarine, Coastal and Shelf Science 75*, 90–100.

Sousa, R., Dias, S., Antunes, C. (2006b). Spatial Subtidal Macrobenthic Distribution in Relation to Abiotic Conditions in the Lima Estuary, NW of Portugal. *Hydrobiologia 559*, 135–148.

Sousa, R., Dias, S., Guilhermino, L., Antunes, C. (2008b). Minho River tidal freshwater wetlands: threats to faunal biodiversity. *Aquatic Biology 3*, 237–250.

Sousa, R., Freire, R., Rufino, M. (2007b). Genetic and shell morphological variability of the invasive bivalve Corbicula fluminea (Müller, 1774) in two Portuguese estuaries. *Estuarine Coastal and Shelfish Science 74*, 166–174.

Sousa, R., Guilhermino, L., Antunes, C. (2005). Molluscan fauna in the freshwater tidal area of the River Minho estuary, NW of Iberian Peninsula. *Annales de Limnologie - International Journal of Limnology 41*, 141–147.

Sousa, R., Ilarri, M., Souza, A.T., Antunes, C., Guilhermino, L. (2011). Rapid decline of the greater European peaclam at the periphery of its distribution. *Annales de Limnologie - International Journal of Limnology 47*, 211–219.

Sousa, R., Nogueira, A., Antunes , C., Guilhermino, L. (2008c). Growth and production of *Pisidium amnicum* in the freshwater tidal area of the River Minho estuary. *Estuarine Coastal and Shelfish Science 79*, 467–474.

Sousa, R., Nogueira, A., Gaspar, M. *et al.* (2008d). Growth and extremely high production of the non-indigenous invasive species *Corbicula fluminea* (Müller, 1774): Possible implications for ecosystem functioning. *Estuarine Coastal and Shelfish Science 80,* 289–295.

Sousa, R., Rufino, M., Gaspar, M., Antunes, C., Guilhermino, L. (2008e) Abiotic impacts on spatial and temporal distribution of *Corbicula fluminea* (Müller, 1774) in the River Minho Estuary, Portugal. *Aquatic Conservation: Marine and Freshwater Ecosystems 110*, 98–110.

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.

Spann, N., Aldridge, D. C., Griffin, J. L., Jones, O. (2011). Size-dependent effects of low level cadmium and zinc exposure on the metabolome of the Asian clam, *Corbicula fluminea*. *Aquatic Toxicology 105*, 589–99.

Sterner, R.W., Andersen, T., Elser, J.J. *et al.* (2008). Scale-dependent carbon:nitrogen:phosphorus seston stoichiometry in marine and freshwaters. *Limnology and Oceanography 53*, 1169-1180.

Strayer, D.L., Caraco, N.F., Cole, J.J. Findlay, S., Pace, M.L. (1999). Transformation of freshwater ecosystems by bivalves: a case study of zebra mussels in the Hudson River. *Bioscience 49*, 19–27.

Szefer, P., Ali, A.A., Ba-Haroon, A.A., Rajehc, A.A., Geldon, J., Nabrzyskid, M. (1999). Distribution and relationships of selected trace metals in molluscs and associated sediments from the Gulf of Aden, Yemen. *Environmental Pollution 106*, 299–314.

Takabe, Y., Tsuno, H., Nishimura, F., Guan, Y., Mizuno, T., Matsumura, C., Nakano, T. (2011). Applicability of *Corbicula* as a bioindicator for monitoring organochlorine pesticides in fresh and brackish waters. *Environmental Monitoring and Assessment 179*, 47–63.

ter Braak, C.J.F. (1986). Canonical correspondence analysis: a new eigenvector technique for multivariate direct gradient analysis. *Ecology 67*, 1167–1179.

ter Braak, C.J.F., Smilauer, P. (2002). CANOCO Reference manual and CanoDraw for Windows User's guide: software for Canonical Community Ordination (version 4.5). Microcomputer Power, Ithaca.

Tomaru, Y., Kawabata, Z., Nakano, S. (2001). Mass mortality of the Japanese pearl oyster *Pinctada fucata* martensii in relation to water temperature, chlorophyll a and phytoplankton composition. *Diseases of Aquatic Organisms 44*, 61–68.

Torres, M.A., Testa, C.P., Gaspari, C., Masutti, M.B., Panitz, C.M.N., Curi-Pedrosa, R., Almeida, E.A., Di Mascio, P., Filho, D.W. (2002). Oxidative stress in the mussel *Mytella guyanensis* from polluted mangroves on Santa Catarina Island, Brazil. *Marine Pollution Bulletin 44*, 923-932.

Tran, D., Boudou, A., Massabuau, J.C. (2001). How oxygenation level influences cadmium accumulation pattern in the asiatic clam Corbicula fluminea: a laboratory and field study. *Environmental Toxicology and Chemistry 20*, 2073-80.

Tremblay, R., Myrand, B., Guderley, H. (1998). Temporal variation of lysosomal capacities in relation to susceptibility of mussels, *Mytilus edulis* , to summer mortality. *Marine Biology 132*, 641–649.

Tsangaris, C., Hatzianestis, I., Catsiki, V.-A., Kormas, K.A., Strogyloudi, E., Neofitou, C., Andral, B., Galgani, F. (2011). Active biomonitoring in Greek

coastal waters: application of the integrated biomarker response index in relation to contaminant levels in caged mussels. *Science of the Total Environment 412-413*, 359–365.

UNEP http://www.unep.org/chemicalsandwaste/ assessed on 1st July 2015.

Urrutia, M.B., Ibarrola, I., Iglesias, J.I.P., Navarro, E. (1999). Energetics of growth and reproduction in a high-tidal population of the clam *Ruditapes decussatus* from Urdaibai Estuary (Basque Country, N. Spain). *Journal of Sea Research 42*, 35–48.

USEPA http://water.epa.gov/scitech/methods/cwa/pollutants.cfm assessed on 1st July 2015.

Valko, M., Morris, H., Cronin, M.T. (2005). Metals toxicity and oxidative stress. *Current Medical Chemistry 12*, 1161-1208.

Vasanthi, L.A., Revathi, P., Arulvasu, C., Munuswamy, N. (2012). Biomarkers of metal toxicity and histology of *Perna viridis* from Ennore estuary Chennai, south east coast of India. *Ecotoxicology and Environmental Safety 84*, 92–99.

Vaughn, C.C., Hakenkamp, C.C. (2001). The functional role of burrowing bivalves in freshwater ecosystems. *Freshwater Biology 46,* 1431-1446.

Vidal, M.L., Bassèeres, A., Narbonne, J.F. (2001). Potential biomarkers of trichloroethylene and toluene exposure in *Corbicula fluminea*. *Environmental Toxicology and Pharmacology 9*, 87–97.

Voelz, N.J., McArthur, J.V., Rader, R.B. (1998). Upstream mobility of the Asiatic clam *Corbicula fluminea*: identifying potential dispersal agents. *Journal of Freshwater Ecology 13*, 39-45.

Vohmann, A., Borcherding, J., Kureck, A., bij de Vaate, A., Arndt, H., Weitere, M. (2009). Strong body mass decrease of the invasive clam *Corbicula fluminea* during summer. *Biological Invasions 12*, 53–64.

Way, C.M., Hornbach, D.J., Millerway, C.A., Payne, B.S., Miller, A.C. (1990). Dynamics of filter feeding in *Corbicula fluminea* (Bivalvia, Corbiculidae). *Canadian Journal of Zoology ‐ Revue Canadienne De Zoologie 68*, 115-120

Weitere, M., Vohmann, A., Schulz, N. Linn, C., Dietrich, D., Arndt, H. (2009). Linking environmental warming to the fitness of the *invasive Corbicula fluminea*. *Global Change Biology 15,* 2838–2851.

Werner, S., Rothhaupt, K.-O. (2008). Mass mortality of the invasive bivalve *Corbicula fluminea* induced by a severe low-water event and associated low water temperatures. *Hydrobiologia 613*, 143–150.

Wilson, J.R.U., Dormontt, E.E., Prentis, P.J., *et al.* (2009). Something in the way you move: dispersal pathways affect invasion success. *Trends in Ecology & Evolution 24*, 136–144.

Wittmann, M., Reuter, J., Schladow, G., Hackley, S., Allen, B., Chandra, S., *et al.* (2008). Asian clam (*Corbicula fluminea*) of Lake Tahoe : Preliminary scientific findings in support of a management plan, (December). (http://terc.ucdavis.edu/research/AsianClam2009.pdf). Tahoe Environmental Research Center, Reno, NV. Assessed in September 2014.

Xiao, B-C., Li, E-C., Du, Z-Y., Jiang, R-I., Chen, L-Q., Yu, N. (2014). Effects of temperature and salinity on metabolic rate of the Asiatic clam *Corbicula fluminea* (Müller, 1774). *SpringerPlus 3*, 455.

Yeager, M.M., Cherry, D.S. (1994). Feeding and burrowing behaviors of juvenile rainbow mussels, *Villosa iris* (Bivalvia:Unionidae). *Journal of the North American Benthological Society 13,* 217–222.

Zar, J.H. (1999). Biostatistical Analysis, 4th edn. Prentice Hall: New Jersey.

Zavaleta E.S., Hobbs R.J., Money H.A. (2001). Viewing invasive species removal in a whole ecosystem context. *Trends in Ecology & Evolution 16*, 454-459.

Zaza, S., Balogh, K., Palmery, M., Pastorelli, A. A., Stacchini, P. (2015). Human exposure in Italy to lead, cadmium and mercury through fish and seafood product consumption from Eastern Central Atlantic Fishing Area. *Journal of Food Composition and Analysis 40*, 148–153.

Annex I

ELSEVIER LICENSE
TERMS AND CONDITIONS

Dec 17, 2015

This is a License Agreement between Cristiana Oliveira ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited The Boulevard,Langford Lane Kidlington,Oxford,OX5 1GB,UK
Registered Company Number	1982084
Customer name	Cristiana Oliveira
Customer address	Rua João Allen nº 155 3.4A Porto, 4200-007
License number	3771520248457
License date	Dec 17, 2015
Licensed content publisher	Elsevier
Licensed content publication	Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology
Licensed content title	Integrated biomarker responses of the invasive species Corbicula fluminea in relation to environmental abiotic conditions: A potential indicator of the likelihood of clam's summer mortality syndrome
Licensed content author	Cristiana Oliveira,Pedro Vilares,Lúcia Guilhermino
Licensed content date	April 2015
Licensed content volume number	182
Licensed content issue number	n/a
Number of pages	11
Start Page	27
End Page	37
Type of Use	reuse in a thesis/dissertation
Portion	full article
Format	both print and electronic
Are you the author of this Elsevier article?	Yes
Will you be translating?	No
Title of your	May pollution restrict the invasive behaviour of the non-indigenous

thesis/dissertation	species Corbicula fluminea?
Expected completion date	Jan 2016
Estimated size (number of pages)	200
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 EUR
VAT/Local Sales Tax	0.00 EUR / 0.00 GBP
Total	0.00 EUR
Terms and Conditions	

INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.
3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:
"Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."
4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.
5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com)
6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.
7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.
8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be

deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.
9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.
10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.
11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.
12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).
13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.
14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

LIMITED LICENSE

The following terms and conditions apply only to specific license types:
15. **Translation**: This permission is granted for non-exclusive world **English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article.
16. **Posting licensed content on any Website**: The following terms and conditions apply as follows: Licensing material from an Elsevier journal: All content posted to the web site must maintain the copyright information line on the bottom of each image; A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx or the Elsevier homepage for books at http://www.elsevier.com; Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.
Licensing material from an Elsevier book: A hyper-text link must be included to the Elsevier homepage at http://www.elsevier.com . All content posted to the web site must maintain the

copyright information line on the bottom of each image.

Posting licensed content on Electronic reserve: In addition to the above the following clauses are applicable: The web site must be password-protected and made available only to bona fide students registered on a relevant course. This permission is granted for 1 year only. You may obtain a new license for future website posting.
17. **For journal authors:** the following clauses are applicable in addition to the above:
Preprints:
A preprint is an author's own write-up of research results and analysis, it has not been peer-reviewed, nor has it had any other value added to it by a publisher (such as formatting, copyright, technical enhancement etc.).
Authors can share their preprints anywhere at any time. Preprints should not be added to or enhanced in any way in order to appear more like, or to substitute for, the final versions of articles however authors can update their preprints on arXiv or RePEc with their Accepted Author Manuscript (see below).
If accepted for publication, we encourage authors to link from the preprint to their formal publication via its DOI. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help users to find, access, cite and use the best available version. Please note that Cell Press, The Lancet and some society-owned have different preprint policies. Information on these policies is available on the journal homepage.
Accepted Author Manuscripts: An accepted author manuscript is the manuscript of an article that has been accepted for publication and which typically includes author-incorporated changes suggested during submission, peer review and editor-author communications.
Authors can share their accepted author manuscript:

- immediately
  - via their non-commercial person homepage or blog
  - by updating a preprint in arXiv or RePEc with the accepted manuscript
  - via their research institute or institutional repository for internal institutional uses or as part of an invitation-only research collaboration work-group
  - directly by providing copies to their students or to research collaborators for their personal use
  - for private scholarly sharing as part of an invitation-only work group on commercial sites with which Elsevier has an agreement
- after the embargo period
  - via non-commercial hosting platforms such as their institutional repository
  - via commercial sites with which Elsevier has an agreement

In all cases accepted manuscripts should:

- link to the formal publication via its DOI
- bear a CC-BY-NC-ND license - this is easy to do
- if aggregated with other manuscripts, for example in a repository or other site, be shared in alignment with our hosting policy not be added to or enhanced in any way to appear more like, or to substitute for, the published journal article.

Published journal article (JPA): A published journal article (PJA) is the definitive final record of published research that appears or will appear in the journal and embodies all value-adding publishing activities including peer review co-ordination, copy-editing,

formatting, (if relevant) pagination and online enrichment.
Policies for sharing publishing journal articles differ for subscription and gold open access articles:
Subscription Articles: If you are an author, please share a link to your article rather than the full-text. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help your users to find, access, cite, and use the best available version.
Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.
If you are affiliated with a library that subscribes to ScienceDirect you have additional private sharing rights for others' research accessed under that agreement. This includes use for classroom teaching and internal training at the institution (including use in course packs and courseware programs), and inclusion of the article for grant funding purposes.
Gold Open Access Articles: May be shared according to the author-selected end-user license and should contain a CrossMark logo, the end user license, and a DOI link to the formal publication on ScienceDirect.
Please refer to Elsevier's posting policy for further information.
18. **For book authors** the following clauses are applicable in addition to the above:
Authors are permitted to place a brief summary of their work online only. You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version. **Posting to a repository:** Authors are permitted to post a summary of their chapter only in their institution's repository.
19. **Thesis/Dissertation**: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for Proquest/UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission. Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

Elsevier Open Access Terms and Conditions
You can publish open access with Elsevier in hundreds of open access journals or in nearly 2000 established subscription journals that support open access publishing. Permitted third party re-use of these open access articles is defined by the author's choice of Creative Commons user license. See our open access license policy for more information.
Terms & Conditions applicable to all Open Access articles published with Elsevier:
Any reuse of the article must not represent the author as endorsing the adaptation of the article nor should the article be modified in such a way as to damage the author's honour or reputation. If any changes have been made, such changes must be clearly indicated.
The author(s) must be appropriately credited and we ask that you include the end user license and a DOI link to the formal publication on ScienceDirect.
If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source it is the responsibility of the user to ensure their reuse complies with the terms and conditions determined by the rights holder.
Additional Terms & Conditions applicable to each Creative Commons user license:
CC BY: The CC-BY license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article and to make commercial use of the Article (including reuse and/or resale of the Article by commercial entities), provided the user gives appropriate credit (with a link to the formal publication through the relevant

DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by/4.0.
CC BY NC SA: The CC BY-NC-SA license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article, provided this is not done for commercial purposes, and that the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. Further, any new works must be made available on the same conditions. The full details of the license are available at http://creativecommons.org/licenses/by-nc-sa/4.0.
CC BY NC ND: The CC BY-NC-ND license allows users to copy and distribute the Article, provided this is not done for commercial purposes and further does not permit distribution of the Article if it is changed or edited in any way, and provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, and that the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by-nc-nd/4.0.
Any commercial reuse of Open Access articles published with a CC BY NC SA or CC BY NC ND license requires permission from Elsevier and will be subject to a fee.
Commercial reuse includes:

- Associating advertising with the full text of the Article
- Charging fees for document delivery or access
- Article aggregation
- Systematic distribution via e-mail lists or share buttons

Posting or linking by commercial companies for use by customers of those companies.

20. **Other Conditions**:

v1.8

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.