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THE ASIAN CLAM: DISPERSAL, IMPACTS AND POTENTIAL BENEFITS

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THE ASIAN CLAM: DISPERSAL, IMPACTS AND POTENTIAL BENEFITS

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LEGAL DETAILS

In compliance with what is stated in Decree-Law nº 216/92 of October 13th, it is hereby declared that the author of this thesis participated in the creation and execution of the experimental work leading to the results here stated, as well as in their interpretation and writing of the respective manuscripts.

This thesis includes two scientific papers published in international journals, one submitted article and two articles in preparation originated from part of the results obtained in the experimental work referenced as:

- **Rosa IC,** Pereira JL, Costa R, Gonçalves F & Prezant R (2012) Effects of upper-limit water temperatures on the dispersal of the Asian clam *Corbicula fluminea*. PLoS ONE 7(10): e46635. doi:10.1371/journal.pone.0046635

- **Rosa IC,** Pereira JL., Gomes J, Saraiva PM, 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. doi: 10.1016/j.ecolecon.2011.05.006

- **Rosa IC,** Costa R, Gonçalves F & Pereira JL. Bioremediation of a metal rich efluente by the invasive bivalve *Corbicula fluminea*. Water Research (*submitted*)

- **Rosa IC,** Gomes J, Pereira ML, Pereira JL, Costa R & Gonçalves F. Dispersal of *Corbicula fluminea*: Factors influencing the invasive clam's drifting behavior. Freshwater Biology (submitted).

- **Rosa IC**, Pereira JL, Gonçalves F & Costa R. Sensivity of the invasive bivalve *Corbicula fluminea* to candidate control chemicals: The role of dissolved oxygen conditions (*in preparation*)

Mais uma vez, às minhas avós…

Se pelo menos pudéssemos viver duas vezes:
a primeira vez, para cometer todos os inevitáveis erros; a segunda para lucrar com eles
David Lawrence

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SUMMARY

Invasive species are currently one of the world's major environmental threats due to their damaging impacts, both at the ecological and economic levels.

The freshwater clam *Corbicula fluminea*, commonly known as the Asian clam, is amongst the most severe invasive species – it is currently present around the world and, in addition to its effects in the invaded ecosystems, the establishment of populations has damaging consequences to the freshwater-dependent industries because of its biofouling activity. The knowledge on the management and control of this pest greatly increased over the past decades, concomitantly with the species' increasing spread and impacts. However, several questions remain unaddressed, and effective and environmentally acceptable mitigation of the nuissance is still a challenge. Particularly in Portugal, a gateway through which *C. fluminea* entered Europe 30 years ago, the systematic monitoring of the pest spread has been lacking.

This thesis provides information on the problematic of the Asian clam invasions, addressing the invasive process under a holistic perspective that covers the species' dispersal, impacts and control. The studies included here are relevant at the local level, with the pest's current distribution and industrial impacts in Portugal being outlined for future reference. At a more general level, knowledge to ground the improvement of pest management is also given. In this latter context, dispersal mechanisms, the improvement of current control methods and the possibility of taking profit from the invader were investigated.

The species' dispersal was addressed by examining the influence of environmental change (increased temperature was used as a proxy) in the clams' flotation behavior, and assessing the role played by particular genetic aspects and seasonality in the production of the mucous threads that promote clams' drifting. The results suggest that the clams' flotation behavior is stimulated by increasing water temperature. Also, data do not confirm the generally assumed seasonality of the mucous drogue line production, with local adaptation phenomena being pointed out as a reasonable cause for the controversial records. The information provided on the pest's flotation behavior is essential to assess and effectively deal with its dispersal, eventually developing strategies to prevent it. The impacts of the pest in Portuguese freshwater-dependent industries together with its current distribution in the country were then assessed. Although the Asian clam has been present in Portugal since the 1980's, its dispersal and impacts in Portugal remain relatively mild, possibly because the invasion is still standing in the lag phase before the rapidly increasing population growth stage or due to frequent phenomena of massive die-

offs occurring in populations established in waterbodies across the country. The documentation of the species' impacts in Portugal may assist official entities on the implementation of integrated management policies in countries at risk of invasion or where the pest has recently arrived. As the relevance of continuing the search for more efficient control solutions for the Asian clam was recognized, the potential of combining biocide application with depressed oxygen conditions was investigated. Preliminary results suggest that hypoxia increases the efficiency of some biocides, which establishes the grounds for future research using intermediate, more suitable, levels of dissolved oxygen combined with selected biocides. Finally, and provided the shortcomings that still exist in available control solutions for the Asian clam and the recognition that the pest may be of hard, if not impossible, eradication from fouled systems, the potential for its use as bioremediation tool was explored using a metal-bearing effluent as an experimental model. The species was able to remove a significant fraction of the effluent metals, which translated into a confirmed decrease of its environmental toxicity. The potential of *C. fluminea* as a bioremediator for industrial effluents opens an avenue for offsetting the damaging effects of established infestations.

RESUMO

As espécies invasoras são atualmente uma das maiores ameaças ambientais devido aos seus impactos negativos tanto a nível ecológico como económico.

A amêijoa de água doce *Corbicula fluminea*, também conhecida por amêijoa asiática, está entre as espécies invasoras mais preocupantes – está atualmente presente em praticamente todo o mundo e, a juntar aos seus impactos negativos perpetrados em ecossistemas invadidos, o estabelecimento de populações tem consequências perniciosas nas indústrias dependentes de água doce devido à sua atividade de *biofouling*. O conhecimento sobre a gestão e controlo desta espécie tem aumentado bastante durante as últimas décadas, a par com o aumento da sua dispersão e impactos. No entanto, são várias as questões que se mantêm sem resposta e a mitigação do dano de uma forma efetiva e aceitável do ponto de vista ambiental permanece ainda como um desafio. Particularmente em Portugal, a porta de entrada de *C. fluminea* na Europa há 30 anos, existem lacunas na monitorização sistemática da dispersão desta invasora.

A presente tese fornece informação no contexto da problemática das invasões pela amêijoa asiática, recorrendo a uma abordagem holística do processo invasor de modo a cobrir aspetos como a dispersão, os impactos e o controlo da espécie. Os estudos aqui incluídos apresentam relevância a nível local, na medida em que se centram na distribuição atual e impactos nas indústrias em Portugal. A um nível mais geral, foi também gerado novo conhecimento científico que pode ser aplicado no sentido de melhorar a gestão da invasora. Neste contexto, os mecanismos de dispersão, o melhoramento de métodos de controlo e a possibilidade de tirar proveito do invasor foram investigados.

A dispersão da espécie foi estudada examinando a influência das alterações ambientais (o aumento da temperatura foi usado como modelo) no movimento de "flutuação" acima do substrato da amêijoa em estádios iniciais de vida, bem como o papel desempenhado por aspetos genéticos e pela sazonalidade na produção do muco que promove esse movimento. Os resultados sugerem que a "flutuação" é estimulada pelo aumento da temperatura. Por outro lado, os dados não permitem confirmar a ideia generalizada de que a produção do muco está relacionada com fenómenos sazonais, sendo que fenómenos de adaptação local são apontados como uma causa provável para a discrepância sinalizada. A informação sobre o movimento de "flutuação" é essencial para se caracterizar em maior detalhe a dispersão da espécie, permitindo eventualmente desenvolver estratégias de prevenção nesse sentido. Em seguida, os impactos de *C. fluminea* nas indústrias portuguesas dependentes de água doce e a sua atual distribuição

no país foram estudados. Apesar da amêijoa asiática estar presente em Portugal desde os anos 80, verificou-se que a sua dispersão é ainda limitada e que os impactos económicos da espécie permanecem relativamente moderados. Tal cenário poderá ser explicado pelo facto de a invasão ainda estar na fase estacionária (fase *lag*) que precede o rápido crescimento das populações nos locais invadidos ou pela ocorrência de fenómenos de mortalidade massivas características de *C. fluminea* e que ocorrem em populações já estabelecidas no país. A documentação dos impactos desta espécie em Portugal pode auxiliar entidades oficiais na implementação de políticas de gestão integrada em países que se encontrem em risco de invasão ou onde a peste chegou recentemente. Reconhecendo a relevância de continuar a procurar soluções de controlo mais eficientes para a amêijoa asiática, foi investigado o potencial da aplicação de biocidas em combinação com condições de baixo oxigénio dissolvido. Os resultados preliminares sugerem que a hipoxia aumenta a eficácia de alguns biocidas, o que proporciona caminhos futuros para a investigação, nomeadamente abordando níveis de oxigénio intermédios em combinação com biocidas. Finalmente, e tendo em conta as lacunas que ainda existem nas soluções de controlo disponíveis, assim como as dificuldades associadas à erradicação da peste de sistemas afetados, foi explorado o potencial de *C. fluminea* para ser usado como ferramenta de bioremediação usando um efluente rico em metais como modelo experimental. A amêijoa removeu uma fração significativa de metais do efluente, o que se traduziu na diminuição da sua toxicidade ambiental. O potencial de *C. fluminea* como bioremediadora de efluentes industriais abre portas para de alguma forma compensar os efeitos nocivos das infestações já estabelecidas.

INTRODUCTORY NOTE

Biological invasions are a growing worldwide threat to the ecological and economic well-being of the planet. Invasion starts with the introduction of a non-indigenous species into a new habitat, which is generally promoted by the human action. The invasive process then proceeds through a serious of sequential stages until a stable population is established. During this process, invaded ecosystems and human-made structures tend to be significantly damaged, the reason why adequate management strategies have to be applied.

The freshwater clam *Corbicula fluminea*, commonly known as the Asian clam, is amongst the most serious invasive species due to its ecological impacts and biofouling activity. The species is presently spread from its Asiatic native range across all over the world. While the body of knowledge on *C. fluminea* biology, impacts and management greatly enlarged over the past 40 years, many questions remain unsolved and there is still room for improving pest monitoring and control programmes. In particular, in Portugal, a gateway through which the species entered Europe 30 years ago, integrated management of potential threatens posed by the *C. fluminea* invasion have been somewhat neglected. In this context, this thesis had a twofold aim. First, and considering the insipiency of local (i.e. national-wide) pest management strategies targeted at the Asian clam, this thesis intends to update the information on the distribution and economic impacts of the Asian clam in Portugal. Second, this thesis intends to generate new knowledge that support the design of improved management practices, with the focus given: (i) to particular aspects of the species biology that can explain dispersal patterns; (ii) to the species tolerance to chemical and physical treatment; (iii) to the bioremediation abilities as beneficial service that the species can provide. The Asian clam problematics has thus been addressed from a holistic perspective, covering a range of pest management-related topics, including the species dispersal, potential impacts due to the invasion of in new areas, control of established populations and the possibility of taking profit from the presence of the invader when accepting it becomes unavoidable (Figure I.1). Consistently with such a perspective, the thesis is organized in seven chapters being the central five distributed by these four main topics as represented in Figure I.1.

Chapter 1 defines and describes fundamental concepts within the context of biological invasions. Such background information frames the problematics of invasive species and the rationale behind the holistic perspective adopted in this thesis. Chapter 1 also presents the Asian clam as one of the world's most important invasive species, reviewing the species' major biological features, its dispersal, its impacts and the methods

currently in use to control the bivalve. This chapter thus provides general information supporting the subsequent chapters. Chapters 2 to 6 constitute the main body of the thesis in the sense that they present actual research results. These chapters were formatted as journal articles – some have already been published, some were submitted to peer-reviewed journals and one is awaiting submission until additional data can be integrated – and therefore some conceptualization notes, rationale description, methods detailing and literature-based discussion parts can be found repeated across them.

Dispersal is a determinant step in any biological invasion process. It has been proven that a mucous-assisted flotation behavior constitutes an important mode of dispersal in *C. fluminea*. Understanding the factors influencing the production of such mucous and related clams' drifting mechanism may thus provide relevant information for pest management. Chapters 2 and 3 address this topic, with Chapter 2 studying the influence of temperature in the clams' flotation behavior, and Chapter 3 investigating whether genetic or seasonal factors affect the mucous production process.

One of the main concerns related to invasive species is the impacts they are responsible for. In the case of biofoulers such as *C. fluminea*, not only ecological, but also economic losses due to their presence in human-made structures and the industrial environment have to be considered. Since the introduction of *C. fluminea* in Portugal, a wide range of ecological impacts have been reported in freshwater ecosystems. However, the damage caused by the species at the industrial level has not been systematically documented. Chapter 4 provides such documentation along with information on the bivalve's current distribution in Portugal.

As the effective and environmentally friendly mitigation of Asian clam populations remains a challenge, chapter 5 intends to contribute to the search of improved control solutions by examining the potential of combining biocide application with hypoxic conditions.

Frequently, the eradication of established Asian clam populations is unachievable. The possibility of taking profit from the pest could thus offset its damaging impacts. In chapter 6, this idea is explored by assessing the use of *C. fluminea* as a bioremediation tool applied to metal-bearing effluent treatment.

Finally, in chapter 7, concluding remarks on the different studies and on their integration, as well as future directions for the research following this thesis are presented.

Background information	Species dispersal	Impacts	Control	Taking profit from the pest	Final considerations
Chapter 1 Biological invasions: The general theory and the particular case of *Corbicula fluminea*	**Chapter 2** Effects of upper-limit water temperatures on the dispersal of the Asian clam *Corbicula fluminea*	**Chapter 4** The Asian clam *Corbicula fluminea* in the European freshwater-dependent industry: A latent threat or a friendly enemy?	**Chapter 5** Sensivity of the invasive bivalve *Corbicula fluminea* to candidate control chemicals: The role of dissolved oxygen conditions	**Chapter 6** Bioremediation of a metal-rich effluent by the invasive bivalve *Corbicula fluminea*	
	Chapter 3 Dispersal of *Corbicula fluminea*: Factors influencing the invasive clam's drifting behavior				

Figure I.1: Roadmap for this dissertation.

CHAPTER 1

Biological invasions: The general theory and the particular case of *Corbicula fluminea*

1.1. Biological invasions as a global threat

Flora and fauna have evolved through the years with the presence of physical barriers that prevented the migration of the species beyond their native range. This has contributed to the great biodiversity of the planet and to the isolation of endemic species. However, as human activities grew, these physical barriers tended to disappear or its circumvention became facilitated causing the spread of several species outside their native area. The species whose presence in a given area is due to intentional or accidental introduction as a result of human activity were conventionally designated non-indigenous species (NIS) (some authors refer to these species also as alien, exotic, introduced, allochtonous or non-native) (Richardson et al. 2000). In several cases, alien species do not successfully adapt following arrival to a new habitat and hence they do not establish. However, in other situations the organisms survive, grow and reproduce, the population becomes widespread and locally dominant causing serious damages to the ecosystem and frequently to human activities. Local ecology is frequently disrupted, human health can be threatened and serious economic effects can constitute a final outcome. In these scenarios the ecosystem is actually facing an invasion by a non-indigenous species (Colautti and MacIsaac 2004, Davis and Thompson 2001, European Comission 2008) and the species can be classified as an actual invasive species.

The invasive process has been described as a series of sequential stages. This structured approach to define the invasion process facilitates the identification and development of adjusted management strategies. Davis (2009) synthesizes the process in three fundamental stages: (i) dispersal; (ii) establishment; and (iii) persistence and spread. A successful invasion only occurs when the species crosses these three stages. Dispersal is usually a complex, multifactor process which determines the arrival of individuals (*i.e.* propagules) to a given area outside of its native range (Colautti and MacIsaac 2004, Davis 2009). This stage is strongly dependent on factors such as the dispersal vector, the duration of the process, the conditions encountered along the way, and the nature and quality of the propagules (*e.g.* life stage and overall physiological condition) (Davis 2009). After reaching the new habitat, the alien species needs to establish, i.e. persist long enough to reproduce. To successfully overcome this stage, the population needs to accomplish four tasks: (i) to find a niche where abiotic conditions stand within its tolerance range; (ii) to be able to exploit the resources available to get the necessary energy for maintenance, growth, and reproduction; (iii) to find the gametes of a mate (in case of out-crossing species); (iv) and to avoid pre-reproductive mortality (Davis 2009). Finally, to be a successful

invader, the introduced NIS must persist for subsequent generations and spread well beyond the original point of entry. In this last stage, the range of phenotypic and genotypic plasticity of the population play an important role (Davis 2009).

Biological invasions are a serious worldwide problem. For example, approximately 50,000 alien invasive species are estimated to exist in the US (Pimentel et al. 2005) and more than 11,000 in Europe (DAISIE, 2013) both in aquatic and terrestrial ecosystems. Several characteristics relating ultimately to the severity of ecological effects and economical impacts noticed following invasion define a restricted group of very hazardous NIS. DAISIE (2013), for example, lists the 100 worst biological invaders, most of them plants, but a significant part includes animals and within these several aquatic mollusks such as *Dreissena polymorpha and Crassostrea gigas*.

NIS are responsible for severe ecological and economic impacts (Sousa et al. 2012). In this context, the attention of researchers and policy makers in biological invasions has been growing consistently, being the topic actually considered as a major pervasive aspects of global environmental change (Rahel and Olden 2008). Regarding the policy response to invasive species threats, the present European legislation agrees in the application of a three-way approach (European Comission 2008): (i) prevention, (ii) early detection and eradication, and (iii) control and long-term containment. Prevention is generally far more cost-effective and environmentally sustainable than reactive strategies and requires an action before the beginning of the invasion process (Perrings 2005, Simberloff et al. 2013). It involves measures that reduce the likelihood of invasions such as the launching and implementation of directives defined by specialized legislation and regulation, risk analysis, and border control (Bax 2003, Davis 2009, European Comission 2008). In cases where an alien species has already been introduced, early detection and rapid eradication are the most cost-effective measures to prevent future consequences (Zavaleta et al. 2001). The success of these measures requires an efficient communication system either by creating an early warning mechanism to inform other areas which are also at risk of invasion or by establishing an adequate platform for exchanging information on potential eradication strategies (European Comission 2008). Early detection efforts may also include the regular monitoring of a specific site to detect dispersal trends (Davis 2009). In turn, a successful eradication will generally benefit biological diversity. However, insufficient or incorrect planning can cause unwanted and/or unexpected impacts on native species that ultimately reflect in unbalance at the ecosystem level (Zavaleta et al. 2001). To avoid this, it is crucial to create coordinated eradication programs overseen by experts (European Comission 2008). When prevention, early

detection and eradication fail or whenever eradication is not feasible, control and/or containment measures should be implemented. Generally, the proposed control methods may include physical, mechanical, chemical and biological methods or integrated approaches using combinations of different methods. Control methods can be classified as proactive or reactive depending on whether it is designed to prevent the settlement of the invader or to target populations already established, respectively (Mackie and Claudi 2010, Sousa et al. 2012). In cases where control actions immediately result in high mortality or removal, it is important to monitor the ecosystem for an extended period of time, complemented by preventive measures against reintroductions (Sousa et al. 2012).

There are currently legislation documents and European policies that indeed propose adequate solutions for the invasive species problematic (e.g. Bax 2003). However, these solutions are inefficient or not implemented at all. The lack of communication and coordination between the different stakeholders (e.g. scientists, policy makers, government, and industry) is an additional identified problem that prevents the success of the implementation of comprehensive and integrated management systems for biological invasions; in fact, the same “language” is not often spoken and effective discussion between actors with different backgrounds is hampered (Colautti and MacIsaac 2004, Davis and Thompson 2001). One should also bear in mind that measures taken at a national level might temporary work. However if the problem persists in this boundaries the probability of a reinvasion increases (Bax 2003). The society may also play a key role in managing biological invasions. Public awareness and educational actions on the problematic (e.g. alerting to the hazardous potential of invasive-like exotic species trading, domestic raising and disposal; publicizing the main items that can allow recognizing a biological invasion and the main accessible warning systems) may add significantly to the societal contribute on the management of invasive species, especially regarding prevention and early detection steps.

Complete eradication of an established invasive population is extremely difficult and a clean, side-effects free solution is hardly available. In fact, in face of an invasive species that is for long established in a given site, stakeholders tend to favor maintaining the invader (Simberloff et al. 2013) since, as the establishment period enlarges, the management costs tend to increase whilst the success of any eradication measure tends to decrease.

Amongst aquatic ecosystems, lakes, estuaries and rivers are particularly prone to biological invasions due to the large number and variety of dispersal vectors and

anthropogenic disturbance agents to which they are susceptible (Cohen and Carlton 1998, Ricciardi and MacIsaac 2000). Bivalve species have most of the characteristics allowing successful establishment in non-native aquatic habitats (McMahon 2002), which configures these organisms as potential invaders. The Asian clam, *Corbicula fluminea* is an example of a bivalve that underwent global range expansion outside its native range with high success as an invasive species. Although some common biological features are common to all invasive species, particular traits may explain the extent of the invasion success. In this way, and given the species selected as the research focus in this thesis, background information on the biology of the Asian clam *C. fluminea* is provided in the following sections of the present chapter.

1.2. Biology, impacts and control of the Asian clam *C. fluminea*

1.2.1. Taxonomy

It is generally accepted that the taxonomic status of *Corbicula* genus is unsolved. The uncertainties are mostly due to considerable phenotypic variation in shell morphology, colour, and ornamentation, strengthening different reproductive strategies and ecological differences that characterize the genus (Glaubrecht et al. 2003, Park and Kim 2003, Pigneur et al. 2011, Sousa et al. 2007). As a consequence, taxonomic studies focusing on Asian, European, and American populations of the Asian clam are controversial. Considering the populations inhabiting exclusively freshwaters, there are presently three morphotypes identified (Table 1.1), however no agreement exists as to the correspondence between genetic haplotypes and designated species. An additional confounding factor is the very common occurrence of androgenesis in this genus (Byrne M. et al. 2000, Ishibashi et al. 2003, Komaru and Kawagishi 1998), thus distinct nuclear lineages can be ultimately grouped within the same mitochondrial cluster. To avoid this situation when exploring phylogenetic relationships, nuclear and mitochondrial data should be combined and interpreted along with morphological observations to avoid biased conclusions (Hedtke et al. 2008, Park and Kim 2003, Pigneur et al. 2011).

Table 1.1. Freshwater *Corbicula* morphotypes described in the literature.

European Haplotypes			**American Haplotypes**			**Asian Haplotypes**		
Haplotype	Nomenclature	Reference	Haplotype	Nomenclature	Reference	Haplotype	Nomenclature	Reference
I	*C. fluminea*	Renard et al. 2000	Form A	*C. cf. leana*	Hedtke et al. 2008, Siripattrawan et al. 2000	FW5	*C. leana*	Park and Kim 2003
H2	*C. sp.* (Form R)	Pfenninger et al. 2002	Form A	*C. sp.* (Form A)	Lee et al. 2005			
1	*C. sp.* (Form R)	Pigneur et al. 2011						
Morph-1	*C. fluminea*	Bodis et al. 2011						
IV	*C. fluminea*-like	Renard et al. 2000	Form C	*C. sp.* (Form C)	Lee et al. 2005	FW17	*C. sp*	Park and Kim 2003
H4	*C. sp.* (Form S)	Pfenninger et al. 2002)						
2	*C. sp.* (Form S)	Pigneur et al. 2011						
V	*C. fluminalis*	Renard et al. 2000	Form B	*C. cf. fluminea*	Hedtke et al. 2008, Siripattrawan et al. 2000	FW1	*C. fluminea*	Park and Kim 2003
3	*C. sp.* (Rlc)	Pigneur et al. 2011	Form B	*C. sp.* (Form B)	Lee et al. 2005			
Morph-2	*C. sp.*(morph-2)							

1.2.2. Reproduction, life cycle and population structure

C. fluminea is a hermaphroditic and androgenetic species (Kraemer and Lott 1977, Park and Chung 2004, Pigneur et al. 2011, Rajagopal et al. 2000) and, although self-fertilization has been observed, cross-fertilization seems to be the most common in this species (Kraemer and Galloway 1986, Park and Chung 2004). Contrasting with other hermaphroditic bivalve mollusks, the Asian clam is proto-oogamous and oogenesis is initiated before spermatogenesis (Britton and Morton 1982).

In general, sexual maturity is reached within the first year following spawning, generally at a shell length of 10 mm (Ituarte 1985, Mouthon 2001b). In mature clams, incubation and spawning occurs in the inner demibranchs of the gills (King et al. 1986). After fertilization and early cell divisions, a trocophore is formed which evolves into a straight-hinged stage that then develops into a pediveliger stage (100-112 h after spawning) (King et al. 1986). Then, juveniles (length mean ± SD: 221 ± 10 µm) are released out of the gills into the water, already shelled and with adductor muscles, foot, statocysts, gills, and digestive system fully formed (King et al. 1986, McMahon 2002). Juveniles are well adapted to the benthic compartment and the species hence lack a planktonic stage (King et al. 1986, Phelps 1994). Juveniles are released in large quantities (up to 700 juveniles $clam^{-1}$ day^{-1} (Aldridge and McMahon 1978)) but high mortality rates during the first three weeks are common (Britton 1982, King et al. 1986). They present a very high growth rate (shell length as large as 29 mm reached within the first year of growth (Aldridge and McMahon 1978)), which is partly due to their higher filtration and assimilation rate compared to older organisms (McMahon 2002).

C. fluminea presents a wide range of intraspecific variation in life-history traits and in the population structure patterns as assessed in different sites (Table 1.2). When considering populations mostly composed by adult clams, maximum shell length ranges between 19 and 51.6 mm. Seasonal variation can be observed in population densities, with different studies indicating maximum densities ranging between 60 and 16.198 clams m^{-2}. Life span is in general 2-3 years however, some studies reported populations with longevity of 5 years. During their life span, clams seem to reproduce once or twice a year. In the cases where only one recruitment event was reported, it occurred from early summer to early autumn depending on the population. In cases where two recruitment events were observed, they occurred in spring/early summer, and again in late summer/autumn, which means that juveniles born in the spring can contribute to the immediately following autumn reproductive event. Differences in the annual number of

Table 1.2. Summary of shell length, density, life span and recruitment events found for several *C. fluminea* populations in different studies. The study site location, sampling date and water temperature range are also shown. This summary result from a thorough literature search and only studies reporting data on at least 4 of the columns were considered.

Study	Study site	Sampling date	Water temperature range (ºC)	SL range (mm)	Clam density (clams m^{-2})	Recruitment events		Life span (years)
						Number	Time of the year	
Aldridge and McMahon 1978	Lake Arlington, USA	Sep 1974 – Jan 1976	12.2-32.2	0.2-40	17.7-94.6	2	Apr-Jul & Aug-Dec	2
Ituarte 1985	Punta Atalaya, Argentina	Nov 1982-Apr 1985	~11-27	~3 - ~30	36-1132	1	September	3
McMahon and Williams 1986	Trinity River, USA	Sep 1980 – Dec 1982	4.8-29	2.6- ~45	305-16198	2	Mar-Jul & Aug-Nov	3
Hornbach 1992	Mechums River, USA	Oct 1982-Oct 1983	0- ~26	0.4-19	173-1495	1	July	2-3
French and Schloesser 1996	St. Clair River, USA	1988-1990	0.5-12.5	1.8– 5.3	18-187	-	-	-
Cataldo and Boltovskoy 1998	Paraná River Delta, Argentina	Oct 1995-Oct 1996	10-29	0.2-33	379-2609	1	Oct-Nov	3
Rajagopal et al. 2000	Lek River, Netherlands	Aug 1991-Jan 1993	~2-~24	-	-	2	May/Jun & Sep	-
Mouthon 2001a	Saone River, France	Sep 1986-Dec 1999	0.5-27.2	1.5- ~29	~120-934	2	Jun/Jul & Aug/Sep	5
Mouthon 2001a	Rhône River, France	Sep 1996-Dec 1999	3-23.5	~0.5 - ~29.3	~300-5266	1	Jul-Sep	5
Morgan et al. 2003	Connecticut River, USA	Aug 1993-Nov 1994	-1.7-30.6	-	52-114	1	Jun-Sep	-
Mouthon and Parghentanian 2004	Loire River, France	Dec 2001-May 2003	[Ice]-25	0.5- ~34	88-< 4000	2	Jan-Feb & May-Oct	2.5-3
Sousa et al. 2006	Lima River, Portugal	Aug 2004 & Aug 2005	20.1-22.9	13.0-51.6	60	-	-	-
Schmidlin and Baur 2007	Alrhine River, Switzerland	Mar 2003-Oct 2003	7-24	1-24	83-339	1	Jun-Jul	-
Sousa et al. 2008c	Minho River, Portugal	Jan 2005- Aug 2006	6.7-23.1	1.85-41.83	92-2152	-	-	2-3
Franco et al. 2012	Mondego Estuary, Portugal	Dec 2007-Dec 2008	10.1-25.4	0.92-37	419-6632	Continuous	-	-
Present study	Casal de São Tomé, Portugal	Oct 2011-Dec 2012	11.8-20.5	6-32	1353-6352	2	Jun-Sep & Nov	-

reproductive events between consecutive years at the same site were also reported (McMahon and Williams 1986). These reproductive events are accompanied by a decline in clam physiological condition consistent with the decrease in biomass caused by the release of developed juveniles (French and Schloesser 1996)

The variation noticed in life-history traits when different populations are compared may relate to genetic dissimilarities, but most probably environmental conditions are the main factor determining such variation. In fact, factors such as temperature, available food resources, and contamination, were already proven to affect clam growth and physiological condition, population structure and/or spawning (Cataldo and Boltovskoy 1998, Cataldo et al. 2001, Morgan et al. 2003, Mouthon 2001b, Sousa et al. 2008c). Temperature, for example, seems to be an essential trigger for the spermatogenesis (Kraemer and Galloway 1986), spawning (Rajagopal et al. 2000), and clam growth (Cataldo and Boltovskoy 1998, Morgan et al. 2003). Some authors also concluded that food availability may be as important as thermal regimes for some life-cycle events such as recruitment (Cataldo and Boltovskoy 1998, Mouthon and Parghentanian 2004, Rajagopal et al. 2000).

1.2.3. Habitat and feeding preferences

Some controversy exists regarding *C. fluminea* classification as a freshwater- or brackish water species. Some authors include the species in brackish group despite its obvious preference for freshwater (Evans et al. 1977), but the classification as a freshwater species with high tolerance to estuarine habitats has been the most commonly used (Britton 1982). This high tolerance has been explained as a result of a recent brackish water ancestral, since *Corbiculidae* is a family grouping mainly estuarine bivalves (Britton 1982).

The Asian clam occurs both in lenthic and lotic habitats (Britton 1982). This species is rarely found in intermittent streams or temporary ponds, possibly due to its low tolerance to aerial exposure (Byrne et al. 1991), and it clearly prefers high-order tributaries (i.e. above second order streams) (Karatayev et al. 2005b, Schmidlin and Baur 2007). Water current strongly determines *C. fluminea* presence in a given site, not only because it affects food availability but also because it strongly defines the type of sediment, which is particularly important for infaunal bivalves such as this species (Karatayev et al. 2003, Schmidlin and Baur 2007). The preferred substrate of the Asian clam has been argued to be composed of sand and gravel, both typically found in low current water bodies (bij de Vaate and Greijdanus-Klaas 1990). *C. fluminea* preferentially colonizes waterbodies without considerable seasonal hydrological oscillations, ample

oxygen supply, and sediments rich in organic material, being commonly found in the limnetic portions of the ecosystems (Britton 1982, Karatayev et al. 2003, Schmidlin and Baur 2007, Sousa et al. 2008b).

As already referred, organic matter in streambed is a factor that can determine the presence of *C. fluminea* in a given site. This clam is primarily a suspension-feeder filtering phytoplankton and detritus from the water column at very high rates (Cherry et al. 1980, Silverman et al. 1995). Besides that, the Asian clam is a pedal-feeder using the organic matter available in the sediment has a food resource (Hakenkamp and Palmer 1999). This feeding alternative can play an important role especially in oligotrophic habitats (present study, Mouthon 2001a).

1.2.4. Current distribution and vectors of dispersal

C. fluminea is native from southeast China, Korea and southeast Russia but underwent a massive global range expansion over the last century (Araujo et al. 1993). Recently it has already been reported in almost all North America (reviewed in Mackie and Claudi 2010), South America (Beasley et al. 2003, Ituarte 1985), and all over Europe (e.g. Beran 2006, bij de Vaate and Greijdanus-Klaas 1990, Elliott 2008, Paunović 2007, Pérez-Bote and Fernández 2008, Rosa et al. 2011).

The introduction of these invasive bivalves in non-native areas has been attributed to several causes not always related with human activities. Some suggested that the vectors of dispersal are transportation via ballast water, as bait used by fishermen, and on bird's feet, and entangled in macrophytes (Britton 1982, Counts 1986).

1.2.5. Invasion success

Several parameters may determine the distribution and occurrence of *C. fluminea,* in several stages of its life cycle. The Asian clam is considered a susceptible species to environmental stress when compared with sphaeriids and unionaceans (for more details see McMahon (1991)). Its range of tolerance to different environmental stressors was synthesized by Mackie and Claudi (2010) and some new information within this context can be found in the present thesis. According to Mackie and Claudi (2010), clams survive to a wide water temperatures ranging between 2 and 36 ºC and the organisms are apparently very tolerant to low dissolved oxygen levels in water (lower tolerance limit of 0.5 mg L^{-1}). On the other hand, *C. fluminea* does not tolerate waters with pH below 5 and hardness below 3 mg $CaCO_3$ L^{-1} (Mackie and Claudi 2010). The same authors concluded that the upper tolerance limits for conductivity and total dissolved solids are of 12,600 µS cm^{-1} and 8400 mg L^{-1}, respectively. The upper tolerance limit of *C. fluminea*

for salinity is 8, however, in situations where acclimation is allowed or exposure occurs for short periods, the clam can tolerate salinities up to 20-22 (Evans et al. 1977, Ilarri and Sousa 2011). During the studies carried out within the context of the present thesis the Asian clam was found in oligotrophic waters with almost no chlorophyll *a* (mean ± standard deviation: 0.73 ± 1.01 µg/ L^{-1}) whereas 25 µg L^{-1} chlorophyll *a*, typically found in mesotrophic systems, seems to be the upper tolerance limit of the species (Mackie and Claudi 2010).

The reduced tolerance to particular environmental stressors can lead to high mortality rates as it has been reported in many studies (Cooper et al. 2005, Ilarri et al. 2011, Schmidlin and Baur 2007, Werner and Rothhaupt 2008). Other factors such as overpopulation, alteration of flow regimes, contamination, epidemics, and parasites can also act alone or in synergy promoting massive die-offs (Britton and Morton 1982, French and Schloesser 1996, Morgan et al. 2003, Vohmann et al. 2009). However, *C. fluminea* has a remarkable ability to rapidly recover from population crashes (often within one year (Britton 1982)), which reflects its ecological profile as a typical r-strategist . This evidences that broad physiological tolerance to a large range of environmental stressors is not mandatory for the success of invasive species, as argued by McMahon 2002. Short life span, early maturity, high fecundity, biannual juvenile release patterns, high growth rates, hermaphroditism and androgenesis, small juvenile size and extensive capacity for dispersal are rather key features of the species biology that confer important advantage in the invasion process (McMahon 2002, Pigneur et al. 2011, Sousa et al. 2006, Vohmann et al. 2009).

The traits that favor the invasion success of *C. fluminea* are also the basis for the species ability to impair the invaded ecosystems and to promote the malfunction of infested underwater industrial structures, as detailed in the following section.

1.2.6. Impacts

Table 1.3 summarizes the ecological and industrial impacts of *C. fluminea* that have been reported in the literature. The classification of the ecological impacts is not straightforward due to the complexity of biotic-biotic and biotic-abiotic interactions that establish ecosystems. In fact, an impact viewed as negative for a given species or group of species can be beneficial for another species or for the overall ecosystem health. For example, if the Asian clam extraordinary filtering abilities can be noxious to competitor native species, benefits to the ecosystem at a higher level can arise provided that the eutrophication process is more easily delayed or avoided.

Table 1.3. Summary of the ecologic and industrial impacts of *C. fluminea*. The column +/- indicates whether the ecological impacts are positive (+) or negative (-). When all the industrial impacts are considered as being negative that column was not considered.

	+/-	Impacts	Reference
Ecological	+	Shelter and substrate for other species	Strayer and Malcom 2007, Werner and Rothhaupt 2007
	+	Source of organic matter and inorganic nutrients in the substrate for pelagic and benthic species	Cantanhêde et al. 2008, Sousa et al. 2008a, Vaughn and Hakenkamp 2001
	+	Decrease in eutrophication levels and turbidity	Phelps 1994
	-	Dominance in the benthic biomass by substitution and/or reduction of the available habitat for other species	Schmidlin and Baur 2007, Strayer 1999, Vaughn and Hakenkamp 2001, Williams et al. 2001
	-	Competition for food with other filtering and benthic species	Cohen. et al. 1984, Hakenkamp and Palmer 1999, Lauritsen 1986, Strayer 1999, Williams et al. 2001
	-	Ingestion of sperm, glochidia, and newly metamorphosed juveniles of native bivalves	Strayer 1999
	-	Excessive production of ammonia and oxygen depletion following massive die-off episodes	Ilarri et al. 2011, Strayer 1999
	-	Changes in nutrient cycles	Hakenkamp and Palmer 1999, Vaughn and Hakenkamp 2001
	-	Vectors of parasites and other pathogenic agents	Chung et al. 2001
	Industry	**Impacts**	**Reference**
Industrial	Electric power stations	Clogging of condensers and water distribution systems in areas with a reduced perimeter and low flow	Isom 1986, Johnson et al. 1986, MacPhee 1986, McMahon 1977, Page et al. 1986, Potter and Liden 1986, Rosa et al. 2011, Smithson 1986
		Loss of performance and efficiency in the condensers due to high pressures and cleaning activities	Potter and Liden 1986
		Security failure due to deficient cooling and clogging of the fire protection systems	Isom 1986, Johnson et al. 1986
		Productivity losses during cleaning operations and clams removal	Isom 1986, Johnson et al. 1986, MacPhee 1986, McMahon 1977, Page et al. 1986, Potter and Liden 1986, Rosa et al. 2011, Smithson 1986
	Drinking water treatment plants	Decrease in efficiency due to the interruption of the water flow and increase in machinery abrasion	Ingram 1959, Rosa et al. 2011, Sinclair 1964
		Changes in water odor and flavor	Smithson 1986
	Cement industries	Losses in the cement quality due to the use of sand loaded with clam shells in the production	Sinclair 1964
	Irrigation systems	Clams accumulation in low flow areas, leading to the decrease in water flow that then favors sediments deposition, which contributes additionally to the impact	Ingram 1959, Prokopovich 1969, Prokopovich and Herbert 1965, Rosa et al. 2011
		Clog of irrigation structures, which requires increased energy consumption to re-establish adequate flow levels	Rosa et al. 2011
		Waste of water during clams removal operations and increased need to repair/replace of irrigation structures	McMahon 1983, Prokopovich and Herbert 1965, Rosa et al. 2011

Industrial impacts relates to the *C. fluminea* biofouling activity, which is characterized by the growth and establishment of dense populations in different underwater structures, pipeline and equipment of water-dependent industries such as power stations, drinking water treatment plants, cement industries and irrigation systems (Table 1.3). The damages caused by Asian clam infestations, as well as the implementation of control measures for the pest, costs several billion dollars each year worldwide; this makes the Asian clam a species with serious economic impacts (Pimentel et al. 2005). In Europe, the problem does not seem to be as serious as in the United States (Rosa et al. 2011). However, based on the North American experience, a sudden increase of industrial losses due to this invasive species may occur (Rosa et al. 2011), hence the need to stimulate the development of adequate management practices for the pest, including improved control methods.

1.2.7. Control

Given the severity of *C. fluminea* impacts particularly in an industrial context, the attention devoted to the development of methods to control this pest has been increasing. Physical and mechanical, chemical and biological methods have been proposed to control the Asian clam and other biofouling bivalves.

Physical measures already suggested to alleviate fouling by the Asian clam entail the use of electric currents, electromagnetic fields, gamma radiation, heated water, and ultrasonic vibrations (Doherty and Cherry 1988, Mattice 1983, Mussalli et al. 1986). The most promising method seems to be the application of heated water; however, upper temperature tolerance limits of *C. fluminea* are very high (Mackie and Claudi 2010), thus such a control technique requires water temperatures as high that its application may require modification of the system's design or eventually newly developed systems (Mattice 1983).

Mechanical methods are generally directed towards the removing of the clams from the vulnerable structures of the industries and include the use of strainers and traps, the back flushing of clogged lines and manual removal of shells (Doherty and Cherry 1988, Isom 1986, MacPhee 1986, Mattice 1983, Rosa et al. 2011). However, some disadvantages can be ascribed to mechanical control methods, e.g., there are limitations in the use of strainers with mesh sizes small enough to prevent juveniles from entering the service water systems, and manual removal may only be possible with the plant temporary shutdown (Mattice 1983, Page et al. 1986, Rosa et al. 2011). Some authors have also suggested the combination of mechanical and chemical solutions for industrial macrofouling control (Isom 1986, Mussalli et al. 1986, Smithson 1986), but

most of the data available in the literature result from studies focused only in chemical control techniques.

Chemical methods used to control *C. fluminea* impacts consist in the addition of biocides to the industry's waterways or reservoirs (Doherty and Cherry 1988). Following the synthesis by Mattice (1983), table 1.4 summarizes chemical control methods that have been proposed as effective against the biofouling activity of the Asian clam. It is important to notice that the comparability of the available toxicity information for most of the biocides is limited since the test methodology was designed according to the specific objectives of each study. This is especially important when organisms that respond differently to the chemical challenges as different life stages (e.g. chlorine, bromine, TBTF, Polyquat; see table 1.4 for more details) or environmental temperatures (e.g. chlorine, bromine; see table 1.4 for more details) are tested. The main difficulty of chemical control is that the compound must be stable enough to be effective i.e. it must not react or decay becoming non-toxic before or as it is applied (Mattice 1983). On the other hand, the chemical *per se* or its byproducts should be non-toxic or persistent potentially affecting non-target organisms. As an example, chlorination has been historically the preferred chemical method to control a wide range of fouling organisms (e.g. bacteria, algae, fungi and invertebrates including *C. fluminea* (Jenner et al. 1998, Rajagopal et al. 2002)); Notwithstanding, chlorine byproducts were proven to be highly toxic to other organisms (Jenner et al. 1997, Rajamohan et al. 2007). In this context, the assessment of the environmental toxicity of candidate control chemicals is of key relevance, although little attention has been given so far to this recommendation (Waller et al. 1993).

Biological control is another strategy that has been suggested to apply to *C. fluminea* biofouling, namely considering the predatory potential of vertebrate and invertebrate species over the clam (Cantanhêde et al. 2008, Robinson 1988). The confirmed predators of the Asian clam are mainly fish (14 species including Barbus bocagei, *Platichthys flesus*, *Cobitis paludica*, *Ictalarus furcatus*, *Lepomis microlophus*, *Aplodinotus grunninens*) but also muskrats (*Ondatra zibenthica*), ducks, raccoons, crayfish and flatworms (Brancotte and Vincent 2002, Britton 1982, Karatayev et al. 2005a, Sousa et al. 2008c). However, none of the predator species was proven to prefer *C. fluminea* over other food items, which limits the potential of control methods based on predation. Parasites can also be considered in the control of the pest but only two parasite species are known to be associated with the Asian clam (the oligochaete *Chaetogaster limnaei* and a mite), and there are no evidences of a negative effect of these parasites in clams' abundance (Karatayev et al. 2005a).

Table 1.4. Summary of chemical solutions proposed for the control of *C. fluminea*. NI - no information available. J - Juveniles and A – Adults.

Chemical	Test conditions				Main conclusions	References
	[ ] (mg L^{-1})	T (ºC)	Exposure time	Life stage		
Chlorine	5, 7.5 and 10	35-46	30 min	J/A	▪ Mortalities related to water temperature but not to chlorine exposure	Mattice et al. 1982
	0.23-0.26	14-25	28-32 days	J/A	▪ Effective (> 90% mortality) at ~0.25 mg/L after 28 days at 20-25 ºC ▪ Effective at lower temperatures only when organisms were weakened by exposure to winter conditions ▪ Ineffective in short-term exposure ▪ Pretreatment of clams to lower concentrations of chlorine for 14 days does not enhance efficiency	Doherty et al. 1986
	0.29	7 & 23	28 days	J/A	▪ Effective (LT100) in juveniles after 17 days at 23 ºC ▪ Lower toxicity in adult clams (66.7% mortality after 28 days) ▪ Lower toxicity at 7 ºC (39 and 3.3% mortality after 28 days for juvenile and adult clams, respectively)	Belanger et al. 1991
Slimcide 364	25, 50, 100 and 200	-	3-24 h	J	▪ Effective (90% mortality at 200 mg/L after 3h)	Davis and Doherty 1985
Betz ®Slimicide C-41	5, 10, 20, 25, 50 and 100	-	1-6 days	J/A	▪ Effective (LT100) in early juveniles after 3 h at 25 mg/L ▪ Effective (LT100) in late juveniles after 24h at 50 and 100 mg/L ▪ Effective in adult clams (LT100) after 24h at 100 mg/L, and less effective in smaller concentrations even after 72h	Davis and Lyons 1986
Bromine	1.69-1.83 moles/L	14-25	28-32 days	J/A	▪ At low temperatures, more efficient in adult clams (LT50 = 5.6 days) than in juvenile clams (LT50 = 8.8 days) ▪ At higher temperatures the opposite pattern occurs (LT50= 22.3 and 22.4 days *vs* LT50=19.9 and 20.3 days)	Doherty et al. 1986
TBTF-Tributyl tin fluoride	0.0001, 0.001, 0.0032, 0.010, 0.032	20	8	J/A	▪ Effective (LC99) in juveniles at 17.4 µg/L after 4 days ▪ Effective (LC99) in adults at 60.5 µg/L after 8 days	Mussalli et al. 1986
Polyquat-Poly[oxyethylene(dimethyliminio)ethylene (dimethyliminio)ethylene dichloride]	2, 4, and 8	24	61 days	J/A	▪ Effective (LT100) in juveniles after 11 days at 2 ppm and 7 days at 8 ppm ▪ Effective (LT100) in adults after 5 days at 2-8 ppm ▪ Continuous application in service water systems killed previously settled juveniles and prevented further settlement by juveniles	McMahon and Lutey 1988
	1, 2, 4, 8 and 10	20	24-33	NI	▪ Semicontinuous application (30 min on/90 min off) proven to be more efficient than continuous application at 1, 2 4, and 10 ppm	McMahon and Chase 1997

					▪ Semicontinous application reduced chemical concentration required when compared with continuous application	
ADBAC (n-alkyl dimethylbenzyl ammonium chloride)	2	25	NI	NI	▪ Effective (LT100) after 12 h	Lyons et al. 1988 in Post et al. 2007
TCMTB 2-(thiocyanomethylthio)benzothiazole	1, 2 and 4	24	7 days	J/A	▪ Effective (LT100) after 96, 35 and 24 h at increasing concentrations in the case of juvenile clams ▪ Effective (LT100) after 160, 160 and 120 h at increasing concentrations in the case of adult clams	Hollis and Lutey 1989
Monochloramine	0.25 and 0.50	30	28 days	J/A	▪ Effective (LT100) after 8 and 4 days at 0.25 and 0.5 mg/L, respectively, against juvenile clams ▪ Effective (LT100) after 11-16 and 7-10 days a 0.25 and 0.5 mg/L, respectively, against adult clams (depending on the original site of clam collection)	Belanger et al. 1991
Monochloramine plus excess of ammonia	0.25+0.54, 0.50+0.34 and 0.75+0.37	30	28 days	J/A	▪ Toxicity was enhanced by the presence of ammonia	Belanger et al. 1991
Ammonia	0.14, 0.23, 0.60 and 1.60 (un-ionized ammonia)	30	28 days	J/A	▪ Effective (LT100) after 4-12 and 4-8 days at 0.60 and 1.60 mg/L, respectively, against juvenile clams (depending on the original site of clam collection) ▪ Effective (LT100) after 5-12 and 5 days at 0.60 and 0.1.60 mg/L, respectively, against adult clams (depending on the original site of clam collection)	Belanger et al. 1991
Bromicine	0.25, 0.50 and 0.75	30	28 days	J/A	▪ Effective (LT100) after 4 and 10 days at 0.50 and 0.75 mg/L, respectively, against juvenile clams ▪ Effective (LT100) after 16-22 and 17 days at 0.50 and 0.75 mg/L, respectively, against adult clams (depending on the original site of clam collection)	Belanger et al. 1991
Copper	0.05, 0.10, 0.20, and 0.40	20.1-28.0	30 days	A	▪ Control may be economically practical at a concentration of 0.05-0.10 mg/L ▪ Laboratory tests overestimated clam sensitivity	Belanger et al. 1991
DGH/QUAT-dodecylguanidinehydrochloride (DGH) e n-alkyl dimethylbenzyl ammonium chloride (QUAT)	3.75, 7.5, 15.0 and 20.0	15	24	Late J	▪ Effective (LT100) after 10 days at 15.0 mg/L ▪ Responsible for an increase in tissue water levels and a decrease in tissue glycogen	Bidwell et al. 1995

1.3. References

Aldridge DW, McMahon RF. 1978. Growth, fecundity, and bioenergetics in a natural population of asiatic freshwater clam, *Corbicula manilensis* Philippi, from North Central Texas. Journal of Molluscan Studies 44: 49-70.

Araujo R, Moreno D, Ramos MA. 1993. The Asiatic clam *Corbicula fluminea* (Müller, 1774) (*Bivalvia: Corbiculidae*) in Europe. American Malacological Bulletin 10: 39-49.

Bax N. 2003. Marine invasive alien species: a threat to global biodiversity. Marine Policy 27: 313-323.

Beasley CR, Tagliaro CH, Figueiredo WB. 2003. The occurrence of the Asian clam *Corbicula fluminea* in the lower Amazon Basin. Acta Amazonica 33: 317-324.

Belanger SE, Cherry DS, Farris JL, Sappington KG, Cairns J. 1991. Sensitivity of the Asiatic clam to various biocidal control agents. Journal American Water Works Association 83: 79-87.

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

Bidwell JR, Farris JL, Cherry DS. 1995. Comparative response of the zebra mussel, *Dreissena polymorpha*, and the asian clam, *Corbicula fluminea*, to DGH/QUAT, a nonoxidizing molluscicide. Aquatic Toxicology 33: 183-200.

bij de Vaate A, Greijdanus-Klaas M. 1990. The asiatic clam, *Corbicula fluminea* (Müller,1774) (*Pelecypoda, Corbiculidae*), a new immigrant in the Netherlands. Bulletin Zoologish Museum 12: 173-178.

Bodis E, Nosek J, Oertel N, Toth B, Feher Z. 2011. A comparative study of two *Corbicula* morphs (*Bivalvia, Corbiculidae*) inhabiting river Danube. International Review of Hydrobiology 96: 257-273.

Brancotte V, Vincent T. 2002. The invasion of french hydrographic system by the molluscs *Corbicula* spp details of settlement and main role of ship canals. Bulletin Francais De La Peche Et De La Pisciculture: 325-337.

Britton JC. 1982. Biogeography and ecology of the Asiatic clam, *Corbicula*, in Texas. Pages 21-30 in Davis JR, ed. Proceedings of the Symposium on Recent Benthological Investigations in Texas and Adjacent States. Austin, Texas: Aquatic Sciences Section, Texas Academy of Science.

Britton JC, Morton B. 1982. A dissection guide, field and laboratory manual for the introduced bivalve *Corbicula fluminea*. Malacological Review Supplement 3: 1-82.

Byrne RA, Shipman BN, Smatresk NJ, Dietz TH, McMahon RF. 1991. Acid-base balance during emergence in the freshwater bivalve *Corbicula fluminea*. Physiological Zoology 64: 748-766.

Byrne M, Phelps H, Church T, Adair V, Selvakumaraswamy P, Potts J. 2000. Reproduction and development of the freshwater clam *Corbicula australis* in Southeast Australia. Hydrobiologia 418: 185-197.

Cantanhêde G, Hahn NS, Gubiani ÉA, 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. 1998. Population dynamics of *Corbicula fluminea* (*Bivalvia*) in the Paraná River Delta (Argentina). Hydrobiologia 380: 153-163.

Cataldo D, Boltovskoy D, Stripekis S, Pose M. 2001. Condition index and growth rates of field caged Corbicula fluminea (*Bivalvia*) as biomarkers of pollution gradients in the Paraná river delta (Argentina). Aquatic Ecosystem Health and Management 4: 187-201.

Cherry DS, Rodgers Jr. JH, Graney RL, Cairns Jr. J. 1980. Dynamics and control of the Asiatic clam in the New River, Virginia. Virginia: Virginia Water Research Center, Virginia Polytechnic Institute and State University.

Chung P, Jung Y, Park Y, Hwang M, Soh C. 2001. *Corbicula fluminea* (*Bivalvia: Corbiculidae*): A possible second molluscan intermediate host of *Echinostoma cinetorchis (Trematoda: Echinostomatidae*) in Korea. Korean Journal of Parasitology 39: 329-332.

Cohen AN, Carlton JT. 1998. Accelerating invasion rate in a highly invaded estuary. Science 279: 555-558.

Cohen RRH, Dresler PV, Phillips EJP, Cory RL. 1984. The effect of the Asiatic clam, *Corbicula fluminea*, on phytoplankton of the Potomac River, Maryland. Limnology and Oceanography 29: 170-180.

Colautti R, MacIsaac H. 2004. A neutral terminology to define 'invasive' species. Diversity and Distributions 10: 135-141.

Cooper NL, Bidwell JR, Cherry DS. 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.

Counts CL. 1986. The zoogeography and history of the invasion of the United States by *Corbicula fluminea* (*Bivalvia: Corbiculidae*). Proceedings of the Second International Corbicula Symposium, American Malacological Bulletin - Special edition no.2.

DAISIE – European Invasive Alien Species Gateway. www.europe-aliens.org. Acessed 2013.

Davis DP, Doherty FG. 1985. Method for controlling mollusks. USA: Betz Laboratories, Inc.

Davis DP, Lyons LA. 1986. Method for controlling mollusks. USA: Betz Laboratories, Inc.

Davis MA, Thompson K. 2001. Invasion terminology: should ecologists define their terms differently than others? No, not if we want to be of any help. ESA Bulletin 82: 206.

Davis MA. 2009. Invasion Biology. UK: Oxford University Press.

Doherty FG, Farris JL, Cherry DS, Cairns Jr. J. 1986. Control of the freshwater fouling bivalve *Corbicula fluminea* by halogenation. Archives of Environmental Contamination and Toxicology 15: 535-542.

Doherty FG, Cherry DS. 1988. Tolerance of the asiatic clam *Corbicula* spp. to lethal levels of toxic stressors - A review. Environmental Pollution 51: 269-313.

Elliott P. 2008. The Asian clam (*Corbicula fluminea)* in the River Thames, London, England. Aquatic Invasions 3: 54-60.

European Comission. 2008. Comunicação da Comissão ao Conselho, ao Parlamento Europeu, ao Comité Económico e Social Europeu e ao Comité das Regiões - Por uma estratégia da UE em matéria de espécies invasivas. Brussels.

Evans J, L.P., Murphy CE, Britton JC, Newland LW. 1977. Salinity relationships in *Corbicula fluminea* (Müller 1774). Pages 193-214. First International *Corbicula* Symposium. Fort Worth, Texas, USA: Texas Christian University.

Franco JN, Ceia FR, Patricio J, Modesto V, Thompson J, Marques JC, Neto JM. 2012. Population dynamics of *Corbicula fluminea* (Müller, 1774) in mesohaline and oligohaline habitats: Invasion success in a Southern Europe estuary. Estuarine Coastal and Shelf Science 112: 31-39.

French JRP, Schloesser DW. 1996. Distribution and winter survival health of Asian clams, *Corbicula fluminea*, in the St Clair River, Michigan. Journal of Freshwater Ecology 11: 183-192.

Glaubrecht M, von Rintelen T, Korniushin AV. 2003. Toward asystematic revision of brooding freshwater corbiculidae in Southeast Asia (*Bivalvia, Veneroida*): On shell morphology, anatomy and molecular phylogenetics of endemic taxa from islands in Indonesia. Malacologia 45: 1-40.

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

Hedtke SM, Stanger-Hall K, Baker RJ, Hillis DM. 2008. All-male asexuality: Origin and maintenance of androgenesis in the Asian clam *Corbicula*. Evolution 62: 1119-1136.

Hollis CG, Lutey RW. 1989. Corbicula control methods, biofouling problems, life cycles associated to industries. USA: Buckman Laboratories International, Inc.

Hornbach DJ. 1992. Life history traits of a riverine population of the asian clam *Corbicula fluminea*. American Midland Naturalist 127: 248-257.

Ilarri MI, Sousa R. 2011. *Corbicula fluminea* Müller (Asian clam). Pages 173-184 in Francis RA, ed. A Handbook of Global Freshwater Invasive Species. New York.

Ilarri MI, Antunes C, Guilhermino L, Sousa R. 2011. Massive mortality of the Asian clam *Corbicula fluminea* in a highly invaded area. Biological Invasions 13: 277-280.

Ingram WM. 1959. Asiatic clams as potential pests in California water supplies. Journal American Water Works Association: 363-370.

Ishibashi R, Ookubo K, Aoki M, Utaki M, Komaru A, Kawamura K. 2003. Androgenetic reproduction in a freshwater diploid clam *Corbicula fluminea (Bivalvia : Corbiculidae).* Zoological Science 20: 727-732.

Isom B. 1986. Historical review of Asiatic clam (*Corbicula)* invasion and biofouling of waters and industries in the Americas in Britton J, ed. Proceedings of the Second International *Corbicula* Symposium: American Malacological Bulletin - Special edition no.2.

Ituarte CF. 1985. Growth dynamics in a natural population of *Corbicula fluminea (Bivalvia Sphaeriacea)* at Punta Atalaya, Rio de La Plata, Argentina. Studies on Neotropical Fauna and Environment 20: 217-225.

Jenner HA, Taylor CJL, vanDonk M, Khalanski M. 1997. Chlorination by-products in chlorinated cooling water of some European coastal power stations. Marine Environmental Research 43: 279-293.

Jenner HA, Whitehouse JW, Taylor CJL, Khalanski M. 1998. Cooling water management in European power stations Biology and control of fouling. Hydroécologie Appliquée 10: 1-225.

Johnson KI, Henager CH, Page TL, Hayes PF. 1986. Engineering factors influencing *Corbicula* fouling in nuclear service water systems. American Malacological Bulletin special edition nr.2: 47-52.

Karatayev AY, Burlakova LE, Kesterson T, Padilla DK. 2003. Dominance of the Asiatic clam, *Corbicula fluminea* (Müller), in the benthic community of a reservoir. Journal of Shellfish Research 22: 487-493.

Karatayev AY, Burlakova LE, Padilla DK. 2005a. Contrasting distribution and impacts of two freshwater exotic suspension feeders, *Dreissena polymorpha* and *Corbicula fluminea*. Pages 239-262 in Dame RF, Olenin S, eds. The Comparative Roles of Suspension-Feeders in Ecosystems, vol. IV. Earth and Environmental Sciences. Dordrecht, The Netherlands: Springer.

Karatayev AY, Howells RG, Burlakova LE, Sewell BD. 2005b. History of spread and current distribution of *Corbicula fluminea* (Müller) in Texas. Journal of Shellfish Research 24: 553-559.

King CA, Langdon CJ, Counts CL. 1986. Spawning and early development of *Corbicula fluminea* (*Bivalvia, Corbiculidae*) in laboratory culture. American Malacological Bulletin 4: 81-88.

Komaru A, Kawagishi T. 1998. Cytological evidence of spontaneous androgenesis in the freshwater clam *Corbicula leana* Prime. Development Genes and Evolution 208: 46-50.

Kraemer LR, Lott S. 1977. Microscopic anatomy of the visceral mass of *Corbicula (Bivalvia: Sphaeriacea*). Bulletin of the American Malacological Union, Inc.: 48-55.

Kraemer LR, Galloway ML. 1986. Larval development of *Corbicula fluminea* (Müller) (*Bivalvia, Corbiculacea*) - An appraisal of its heterochrony. American Malacological Bulletin 4: 61-79.

Lauritsen D. 1986. Assimilation of radiolabeled algae by *Corbicula*. Proceedings of the Second International *Corbicula* Symposium: American Malacological Bulletin - Special edition no.2.

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

Mackie GL, Claudi R. 2010. Monitoring and control of macrofouling mollusks in fresh water systems. Boca Raton, FL: CRC Press.

MacPhee D. 1986. A mechanical strainer design for *Corbicula* fouling prevention in the service water system at Arkansas Nuclear One, Unit 2 in Britton JC, ed. Proceedings of the Second International *Corbicula* Symposium: American Malacological Bulletin - Special edition no.2.

Mattice JS, McLean RB, Burch MB. 1982. Evaluation of short-term exposure to heated water and chlorine for control of the Asiatic clam (*Corbicula fluminea*). Tennessee, USA: Oak Ridge National Laboratory.

Mattice JS. 1983. Freshwater macrofouling and control with emphasis on *Corbicula*. Pages 1-30 in Diaz-Tous IA, Miller MJ, Mussalli IG, eds. Symposium on Condenser Macrofouling Control Technologies: The State of the Art. California, USA: Electric Power Research Institute.

McMahon RF. 1977. Shell size frequency distributions of *Corbicula manilensis* Philippi from a clam fouled steam condenser.

McMahon RF. 1983. Ecology of an invasive pest bivalve, *Corbicula* in Russel-Hunter WD, ed. The Mollusca, vol. 6. London: Academic Press, Inc.

McMahon RF, Williams CJ. 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*). Proceedings of the Second International Corbicula Symposium: American Malacological Bulletin - Special edition no.2.

McMahon RF, Lutey RW. 1988. Field and laboratories studies of the efficacy of poly[oxyethylene(dimethyliminio)ethylene(dimethyliminio)ethylene dichloride] as a biocide against the Asian clam, *Corbicula fluminea*. Charlotte: Electric Power Research Institute.

McMahon RF. 1991. Mollusca: Bivalvia in Thorp JH, Covich AP, eds. North American Freshwater Invertebrates. San Diego, California: Academic Press, Inc.

McMahon RF, Chase RA. 1997. Investigation of the efficacy of semicontinuous application of a nonoxidizing molluscicide for control of macrofouling by zebra mussels (*Dreissena polymorpha*) and Asian clams (*Corbicula fluminea*). Pages 507-522 in Ditri FM, ed. Zebra Mussels and Aquatic Nuisance Species.

McMahon RF. 2002. Evolutionary and physiological adaptations of aquatic invasive animals: r selection versus resistance. Canadian Journal of Fisheries and Aquatic Sciences 59: 1235-1244.

Morgan DE, Keser M, Swenarton JT, Foertch JF. 2003. Population dynamics of the Asiatic clam, *Corbicula fluminea* (Müller) in the Lower Connecticut River: Establishing a foothold in New England. Journal of Shellfish Research 22: 193-203.

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, 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 Fur Hydrobiologie 161: 267-287.

Mussalli YG, Duaz-Tous IA, Sickel JB. 1986. Asiatic clam control by mechanical straining and organotin toxicants in Britton JC, ed. Proceedings of the Second International *Corbicula* Symposium: American Malacological Bulletin - Special edition no.2.

Page T, Neitzel D, Simmons M. 1986. Biofouling of power plant service systems by *Corbicula* in Britton JC, ed. Proceedings of the Second International *Corbicula* Symposium: American Malacological Bulletin - Special edition no.2.

Park GM, Chung EY. 2004. Histological studies on hermaphroditism, gametogenesis and cyclic changes in the structures of marsupial gills of the introduced Asiatic clam, *Corbicula fluminea*, and the Korean clam, *Corbicula leana*. Journal of Shellfish Research 23: 179-184.

Park JK, Kim W. 2003. Two *Corbicula (Corbiculidae : Bivalvia*) mitochondrial lineages are widely distributed in Asian freshwater environment. Molecular Phylogenetics and Evolution 29: 529-539.

Paunović M. 2007. Distribution of Asian clams Corbicula fluminea (Müller, 1774) and *C. fluminalis* (Müller, 1774) in Serbia. Aquatic Invasions 2: 99-106.

Pérez-Bote JL, Fernández J. 2008. First record of the Asia clam *Corbicula fluminea* (Müller, 1774) in the Guadiana River Basin (southwestern Iberian Peninsula). Aquatic Invasions 3: 87-90.

Perrings C. 2005. Mitigation and adaptation strategies for the control of biological invasions. Ecological Economics 52: 315-325.

Pfenninger MP, 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 HL. 1994. The asiatic clam (*Corbicula fluminea*) invasion and system-level ecological change in the Potomac River estuary near Washington, DC. Estuaries 17: 614-621.

Pigneur L-M, Marescaux J, Roland K, Etoundi E, Descy J-P, Van Doninck K. 2011. Phylogeny and androgenesis in the invasive *Corbicula* clams *(Bivalvia, Corbiculidae*) in Western Europe. Bmc Evolutionary Biology 11.

Pimentel D, Zuniga R, Morrison D. 2005. Update on the environmental and economic costs associated with alien-invasive species in the United States. Ecological Economics 52: 273-288.

Post RM, Petrille JC, Lyons LA. 2007. A review of freshwater macrobiological control methods for the power industry.

Potter J, Liden L. 1986. Corbicula control at the Potomac River Steam Electric Station Alexandria, Virginia in Britton JC, ed. Proceedings of the Second International *Corbicula* Symposium. American Malacological Bulletin - Special edition no.2.

Prokopovich NP, Herbert DJ. 1965. Sedimentation in the Delta-Mendota Canal. Journal AWWA: 375-382.

Prokopovich NP. 1969. Deposition of clastic sediments by clams. Journal of Sendimentary Petrology 39: 8901-8901.

Rahel FJ, Olden JD. 2008. Assessing the effects of climate change on aquatic invasive species. Conservation Biology 22: 521-533.

Rajagopal S, van der Velde G, de Vaate AB. 2000. Reproductive biology of the Asiatic clams *Corbicula fluminalis* and *Corbicula fluminea* in the river Rhine. Archiv Fur Hydrobiologie 149: 403-420.

Rajagopal S, van der Velde G, Jenner HA. 2002. Effects of low-level chlorination on zebra mussel, *Dreissena polymorpha*. Water Research 36: 3029-3034.

Rajamohan R, Vinnitha E, Venugopalan VP, Narasimhan SV. 2007. Chlorination by-products and their discharge from the cooling water system of a coastal electric plant. Current Science 93: 1608-1612.

Renard E, Bachmann V, Cariou ML, Moreteau JC. 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.

Ricciardi A, MacIsaac HJ. 2000. Recent mass invasion of the North American Great Lakes by Ponto-Caspian species. Trends in Ecology & Evolution 15: 62-65.

Richardson DM, Pysek P, Rejmánek M, Barbour MG, Panetta FD, CJ W. 2000. Naturalization and invasion of alien plants - concepts and definitions. Diversity and Distributions 6.

Robinson JV. 1988. Ecological resistance to the invasion of a freshwater clam, *Corbicula fluminea*: Fish predation effects. Oecologia 77: 445-452.

Rosa IC, Pereira JL, Gomes J, Saraiva PM, Goncalves 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.

Schmidlin S, Baur B. 2007. Distribution and substrate preference of the invasive clam *Corbicula fluminea* in the river Rhine in the region of Basel (Switzerland, Germany, France). Aquatic Sciences 69: 153-161.

Silverman H, Achberger EC, Lynn JW, Dietz TH. 1995. Filtration and utilization of laboratory-cultured bacteria by *Dreissena polymorpha*, *Corbicula fluminea*, and *Carunculina texasensis*. Biological Bulletin 189: 308-319.

Simberloff D, et al. 2013. Impacts of biological invasions: What's what and the way forward. Trends in Ecology and Evolution 28: 58-66.

Sinclair RM. 1964. Clam pests in Tennessee water supplies. Journal American Water Works Association: 592-599.

Siripattrawan S, Park J-K, Foighil D. 2000. Two lineages of the introduced Asian freshwater clam *Corbicula* occur in North America. J. Molluscan Stud. 66: 423-429.

Smithson J. 1986. Development of a *Corbicula* control treatment at the Baldwin Power Station in Britton JC, ed. Proceedings of the Second International *Corbicula* Symposium: American Malacological Bulletin - Special edition no.2.

Sousa R, Antunes C, Guilhermino L. 2006. 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, Freire R, Rufino M, Mendez J, Gaspar M, Antunes C, Guilhermino L. 2007. Genetic and shell morphological variability of the invasive bivalve *Corbicula fluminea* (Müller, 1774) in two Portuguese estuaries. Estuarine, Coastal and Shelf Science 74: 166-174.

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, Rufino M, Gaspar M, Antunes C, Guilhermino L. 2008b. Abiotic impacts on spatial and temporal distribution of *Corbicula fluminea* (Müller, 1774) in the River Minho estuary, Portugal. Aquatic Conservation: Marine and Freshwater Ecosystems 18: 98-110.

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

Sousa R, Novais A, Costa R, Strayer D. 2012. Invasive bivalves in fresh waters: Impacts from individuals to ecosystems and possible control strategies. Hydrobiologia.

Strayer DL. 1999. Effects of alien species on freshwater mollusks in North America. Journal of the North American Benthological Society 18: 74-98.

Strayer DL, Malcom HM. 2007. Shell decay rates of native and alien freshwater bivalves and implications for habitat engineering. Freshwater Biology 52: 1611-1617.

Vaughn CC, Hakenkamp CC. 2001. The functional role of burrowing bivalves in freshwater ecosystems. Freshwater Biology 46: 1431-1446.

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.

Waller DL, Rach JJ, Cope WG, Marking LL, Fisher SW, Dabrowska H. 1993. Toxicity of candidate molluscicides to zebra mussels (*Dreissena polymorpha*) and selected nontarget organisms. Journal of Great Lakes Research 19: 695-702.

Werner S, Rothhaupt K-O. 2007. Effects of the invasive bivalve *Corbicula fluminea* on settling juveniles and other benthic taxa. Journal of the North American Benthological Society 26: 673-680.

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.

Williams EH, Bunkley-Williams L, Lilyestrom CG, Ortiz-Corps EAR. 2001. A review of recent introductions of aquatic invertebrates in Puerto Rico and implications for the management of nonindigenous species. Caribbean Journal of Science 37: 246-251.

Zavaleta ES, Hobbs RJ, Mooney HA. 2001. Viewing invasive species removal in a whole-ecosystem context. Trends in Ecology & Evolution 16: 454-459.

CHAPTER 2

Effects of upper-limit water temperatures on the dispersal of the Asian clam *Corbicula fluminea*

Effects of Upper-limit Water Temperatures on the Dispersal of the Asian Clam *Corbicula fluminea*

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2.1. Abstract

Temperature is a determinant environmental variable in metabolic rates of organisms ultimately influencing important physiological and behavioral features. Stressful conditions such as increasing temperature, particularly within high ranges occurring in the summer, have been suggested to induce flotation behavior in *Corbicula fluminea* which may be important in dispersal of this invasive species. However, there has been no experimental evidence supporting this hypothesis. It was already proven that *C. fluminea* drift is supported by a mucilaginous drogue line produced by mucocytes present in the ctenidia. Detailed microscopic examination of changes in these cells and quantification of clam flotation following one, two and three weeks of exposure to 22, 25 and 30ºC was carried out so that the effects of increasing water temperatures in dispersal patterns could be discussed. Results show that changes in temperature triggered an acceleration of the mucocytes production and stimulated flotation behavior, especially following one week of exposure. Dilution of these effects occurred following longer exposure periods. It is possible that these bivalves perceive changing temperature as a stress and respond accordingly in the short-term, and then acclimate to the new environmental conditions. The response patterns suggest that increasing water temperatures could stimulate *C. fluminea* population expansion.

Keywords: *Corbicula fluminea;* Dispersal; Temperature; Invasive species; Climate change; Mucous drogue-line

2.2. Introduction

Over the past decades there has been a growing interest in studying biological responses of aquatic organisms to increasing temperature. This interest is linked to attempts to envision effects of climate change with the expected increase in water temperature (e.g. Larsen and Riisgard 2009, Rahel and Olden 2008). In addition to issues related to climate change, other human activities, such as the growing investment in the construction of reservoirs and canals and the consequent change of the thermal regimes of altered systems, contribute to the interest on the effect of temperature in biological responses (Robinson and Childs 2001). Temperature is a decisive environmental variable that greatly determines metabolic rates of organisms (Allan and Castillo 2008). This is even more critical in animals exhibiting poikilothermic ectothermy, such as most invertebrates (Purves et al. 2001). In the specific case of aquatic invertebrates, it has been proven that water temperature indeed influences important biological features, including feeding and digestion (e.g. Viergutz et al. 2006) and reproduction (e.g. Galbraith and Vaughn 2009, Moyo 2011).

The metabolic changes associated with temperature regimes are likely to translate into variations in population fitness and ultimately, at a macro-ecological scale, species distribution. In fact, the spatial distribution and dispersal patterns of a given species is often bound by temperature and thermal tolerance ranges and physiological optima (Muller and Baur 2011). Since thermal regimes and temperature changes can influence species distribution, a fundamental interest has been growing in correlating this information with invasive species. Understanding the physiological thermal range of an invasive species in turn can help us to understand their current and potential dispersal ranges and patterns. Such knowledge can also assist in predicting invasive potential of a new habitat and consequently support the design and implementation of preventive measures that can avoid or reduce the negative outcomes of the invader's establishment. This perspective attains even higher relevance when applied to biofouling invaders, e.g. some freshwater bivalves. Invasive bivalves can accumulate in artificial low-flow areas (e.g. pipes and filters), adding negative economic impacts to the freshwater-dependent industry (Elliott et al. 2005, Rosa et al. 2011). In addition, some of these industries use heat treatment as a highly efficient enhancement agent in pest control either alone or in combination with chemical treatment (Jenner et al. 1998, Mackie and Claudi 2010). It is worth investigating how heated water relates to dispersal patterns adjacent (see e.g. the water temperature increase in the vicinities of thermal power plants as suggested by French and Schloesser (1996)) and within the facilities to better understand the

potential role of human-induced thermal change on the effective dispersal prowess of these highly migratory species.

An important biological invader that concomitantly is a relevant industrial biofouler is the freshwater bivalve *Corbicula fluminea* (Müller, 1774), commonly known as the Asian clam (DAISIE , Rosa et al. 2011). This species is native to Southeast Asia, but underwent a massive global range expansion over the last century (Araujo et al. 1993). The specific Asian clam life-cycle features contribute to its success as an invasive species. Besides showing rapid growth rates and a short life span (Kraemer and Galloway 1986), this is an early-maturing hermaphroditic species that can self-fertilize (Britton and Morton 1982) delivering high fecundity rates (up to 600-700 juveniles/day; (Aldridge and McMahon 1978)); offspring are generally released as pediveligers with about 230 µm length weighing as low as 10 µg dw (Kraemer and Galloway 1986). Its introduction in non-native habitats and subsequent dispersal have also been frequently attributed to human activities, e.g. channelization, navigational dredging, commercial and recreational boating, food sourcing, marketing as fish bait (Brancotte and Vincent 2002, Britton 1982, Cherry et al. 1980). Other authors suggest dispersal also through passive transport by waterfowls, attached to feet or feathers (Britton 1982), or carried in fish gastrointestinal tracts (Cantanhêde et al. 2008). However, there is little actual evidence supporting these non-human transport routes as primary means of dispersal (Britton 1982, Cantanhêde et al. 2008, McMahon 1983). Additionally, both downstream and upstream dispersal were claimed to occur (Voelz et al. 1998). If downstream transport with the water flow seems very likely to occur particularly in earlier life-stages due to the pediveligers and juveniles with reduced size and weight, upstream dispersion seems less obvious.

The discovery of the Asian clam's mucilaginous drogue line (Prezant and Chalermwat 1984) suggests the mucous-assisted flotation behavior to be an important mode of dispersal. The authors showed that long mucous drogue lines are produced by modified cells (mucocytes) packed along the inner demibranchs of the ctenidia at least in juvenile and small adult clams (up to 14 mm shell length). These drogue lines, together with other behavioral mechanisms, assist drift of the clam through flotation in response to water currents (Prezant and Chalermwat 1984). Stressful conditions, including increased water temperature, were already suggested to induce stochastic entrainment and dispersal or flotation behavior by the Asian clam (McMahon 1983, Prezant and Chalermwat 1984). However, to date, no experimental evidences have confirmed this hypothesized relationship. Indeed, increasing temperature affects several fitness parameters on *C. fluminea,* including body mass, shell length, mortality and reproduction rate (Vohmann et al. 2009, Weitere et al. 2009). Moreover,

temperature seems to influence the activity of biological processes in other bivalves that assist attachment via byssus to previously unoccupied surfaces, thus promoting colonization of novel habitats and consequently the species spread. For example, this is the case in *Mytilus edulis* (Young 1985), *Dreissena polymorpha* (Clarke and McMahon 1996, Rajagopal et al. 1996), *Sinonovacula constricta* (Wang and Xu 1997), and *Modiolus philippinarum* (Rajagopal et al. 1999). Therefore, it is possible that temperature plays a role in the dispersal mechanisms at a physiological level in other bivalves such as the Asian clam through changes in cellular structures that also promote dispersal.

This study was motivated by the identified gap in the literature on the effects of temperature over the physiological basis of the Asian clam's dispersal patterns. Specifically, this study assesses the effects of increasing water temperature in the production of the mucous drogue line by *C. fluminea*, as well as examines whether temperature-induced physiological changes actually translate into variations in the flotation behavior. The information reported and discussed here constitute fundamental knowledge that can be used (i) as a proxy of the effects of global climate change in the dispersal patterns of this invasive bivalve, and (ii) as a starting point towards the development of more efficient temperature-dependent control methods in freshwater-dependent industries affected by the Asian clam.

2.3 Material and methods

2.3.1. Corbicula fluminea *collection and maintenance*

During early spring 2011, clams (shell length below 11 mm) were collected from Six Mile Run, a shallow creek located in Somerset County, New Jersey, USA (N40º28.166' / W74º32.620'). At the moment of the clam's collection the water temperature was 10.1 ºC, with pH of 8.73. The creek has a well-established *Corbicula fluminea* population with densities of about 400 individuals m^{-2} with a mean shell length of 16.43 mm; the dominant substratum is coarse sand with 2.42 % organic content, and turbidity, dissolved oxygen and EPT (Ephemeroptera-Plecoptera-Trichoptera) index here were recorded at 3.8, 8.7 mg L^{-1} and 3, respectively (Vazquez 2010).

Clams were transported to Montclair State University in plastic buckets filled with creek water. Four clams were immediately sacrificed and treated as histological samples (see the histology section below for details) to provide a field reference in data analysis. All other clams were acclimated for 1 week in glass aquaria: nine glass aquaria holding 16 clams, each animal inside a crystallizing dish (10.5 cm diameter, 4.5

cm height) placed at the bottom of each aquarium; the aquaria were filled with 75 L field water, which was gradually replaced with dechlorinated tap water along the acclimation period. An externally driven recirculation and filtration system was set in the aquaria to ensure water quality during the acclimation period and the temperature in the culturing room was kept at 22 ± 1 ºC with a controlled photoperiod of $16h^{L}$:$8h^{D}$. An equivalent set of animals was acclimated following the same protocol, the differences being that each crystallizing dish contained a bottom sediment layer (ca. 2 cm thickness of coarse sand brought from the field). These clams were further used for a parallel assessment of flotation behavior (see below for details). The crystallizing dishes allowed the necessary moving of the clams before the observation periods along the experiments with minimal disturbance, so that records on behavioral parameters relate only to the established stimulus of increased water currents (see below for details) rather than reflect effects of direct animal handling.

An additional water sample was collected in the field to serve as a microalgae inoculum grown in the laboratory to feed the clams. The water sample was acclimated for 5 days in the laboratory and 1 L was then added to a sterilized Erlenmeyer vessel filled with 1 L of Mauro's 3M culture medium (Kratz and Myers 1955). The microalgae were left to grow under permanent aeration, continuous illumination at 22ºC, for 10 days.

2.3.2. Exposure conditions

Clams were exposed to different temperatures in aquaria filled with 75 L dechlorinated tap water. The same aquaria and crystallizing dishes used in acclimation were used in experiments in order to avoid additional stress on the clams. The test aquaria were kept under a water recirculation system as previously described maintenance and water volume was kept constant in the aquaria along the exposure period by periodic re-filling with dechlorinated tap water as necessary. The photoperiod was maintained in $16h^{L}$:$8h^{D}$ and each aquarium was added with a common heater to establish three independent temperature treatments of 20, 25 and 30 ºC with 3 replicates each. The tested temperatures are within the tolerance limits described for the Asian clam (upper temperature limit: 36-37ºC) (Karatayev et al. 2005) and reflect actual variations in the field at the site where the clams were collected, which has been regularly monitored (R. Prezant, personal communication; US Geological Survey database - http://waterdata.usgs.gov/nj/nwis/current/?type=qw&group_key=basin_cd, at Mill Brook at Route 10 or Pequannock River at Oak Ridge), as well as summer conditions in other invaded systems (e.g. Boltovskoy et al. 1997, Joy 1985, Welch and Joy 1984). Additionally these temperature ranges can be found especially in the vicinities of power

plants using cooling waters from abutting streams and discharging thermal effluents (Cherry et al. 1980, Morgan et al. 2003). Temperature was recorded four to five times daily throughout the exposure period for monitoring purposes. The 16 clams in the crystallizing dish placed at the bottom of the aquarium constituted each experimental unit. To control potential interference of size-dependent effects in the results, clams were grouped according to established size classes so that each replicate contained 8 clams less than 5 mm, 4 clams 5-8 mm and 4 clams 8-11 mm shell length. Larger clams were excluded from the experimental setup since production of the mucous drogue line has, to date, only been documented for clams smaller than 14 mm shell length (Prezant and Chalermwat 1984). Unlimited food resources were provided to the clams by periodic infusion of microalgae into aquaria at least twice per week (see previous section).

2.3.3. Histological procedures and microscopic analysis

Microscopic inspection of ctenidial mucocytes distributed in the inner demibranchs of the experimental clams was performed. Samples composed of 4 clams (2 with less than 5 mm, 1 with 5-8 mm and 1 with 8-11 mm shell length) were brought from the field (see above for details) and taken from each replicated crystallizing dish following each exposure period (one, two and three weeks of exposure to different temperatures). Clams were opened through gentle forcing between valves with a blunt needle and their soft tissues (whole body) were fixed in Zenker's fluid overnight. The fixed samples were washed overnight in running tap water and dehydrated through an increasing concentration series of ethanol and toluene solutions. After dehydration, tissues were carefully embedded in paraffin wax (Paraplast©, 60ºC m.p.) so that antero-posterior sections could be made in the organisms corresponding to longitudinal cuts of the demibranchs. Blocs were sectioned using a microtome set to 5-7 µm cut thickness. Sections were stained with sodium borate buffered aqueous toluidine blue.

Slides containing demibranchs of each individual were located and the first 9 sequential sections were selected for further analysis. Light micrographs were taken from the best cut within each ribbon using an electronic image acquisition system (Olympus SC30 digital camera; analySIS getIT) coupled to an Olympus CKX41 inverted microscope. In order to reduce the potential interference in the results of intra-demibranch spatial variability, the observed demibranch images were artificially divided into three sections and mucocyte size and number were assessed within each section. A linear segment of ca. 970 µm was established over each section following a longitudinal direction across the ctenidial lumen and all mucocytes located within this distance were counted. Data yield from this analysis were expressed as number of

mucocytes per µm. Five mucocytes were randomly measured within each section thus 15 measured cells per individual accounted for the dataset. Micrographs were analysed using the open-source software ImageJ, Image Processing and Analysis in Java (available at http://rsbweb.nih.gov/ij/index.html; accessed Jan 2012).

2.3.4. Flotation behavior

Clam flotation behavior was observed following one, two and three weeks of exposure to different temperatures. Four observation periods of 10 minutes each were made during the last day of each defined exposure period and before collecting clams for histological procedures. Following the approach by (Prezant and Chalermwat 1984), each replicated crystallizing dish containing the clams was re-positioned in the test aquaria under a gentle water current produced by the aquarium filtration system (maximum flow rate of 13.25 L min^{-1}) to stimulate clam flotation. When submitted to the water flow, the clams would distend their exhalant siphon and foot and gently lifting off the substratum, and would drift into the water column. Such an integrated sequence of movements occurring within each 10-min observation period was recorded as a drift. Data were expressed as relative frequency of drifts observed in each replicate per day. Given the infaunal character of this bivalve species and provided the suggested association between stressful conditions and drifting, we hypothesized that sediment could play a protective role hence inhibiting flotation. Thus, the exposure and drifting record protocol was repeated using clams contained in the crystallizing dishes added with a bottom sediment layer (ca. 2 cm thickness of coarse sand brought from the field).

2.3.5. Statistical analysis

Mucocyte size and number were addressed as independent experimental variables. Mean values within individuals that composed each replicate were calculated and used to further determine the product between size and number as an additional variable to complement the analysis. The mean values between individuals composing each replicate were used in the statistical analysis (n = 3). Detailed graphical exploration of the datasets was run following Zuur et al. (2010) and Quinn and Keough (2002) to check the assumptions required for parametric statistics. Slight deviations were found mostly to normality but occasionally also to homocedasticity, and data transformation was applied for improvement whenever necessary.

A repeated-measures (RM) ANOVA approach was followed for each histological variable considering temperature as the between-subjects factor and time (week) as the within-subjects factor. Whenever a significant interaction between time

and temperature was present, simple main effects of temperature or week were analyzed separately (Quinn and Keough 2002) as follows. The main effect of temperature was addressed by running one-way ANOVA using the within-cells error term calculated from the former RM ANOVA output as denominator of the test F; the same applied to the main effect of time (week) but using rather one-way RM ANOVA with week computed as a within-subjects factor and using the overall (within subjects) error term as denominator of the test F. Whenever deviations from sphericity were found as measured by ε, the degrees of freedom were adjusted in the RM ANOVA procedures using the most conservative Greenhouse-Geisser estimate (Quinn and Keough 2002). A Tukey *post-hoc* test followed whenever applicable to determine significant differences between treatments of time or temperature. The Bonferroni procedure was used to conservatively adjust the associate significance level of the family-wise type I error. A total of three comparisons applied for each variable (three temperature or three exposure periods) thus a significance level of 0.017 was used in the analyses. Explorative Pearson correlation analysis was run to assess whether mucocytes size is related to cell number within each combination of timepoint and temperature.

Clam flotation behavior was quantified as the ratio of the number of recorded drifts by total number of clams (drift ratio) within each replicate at each observation timepoint (last day of the first, second and third week of exposure). Differences between temperature treatments were assessed through a RM ANOVA approach using observation time (week) as the within-subjects factor and temperature as the between-subjects factor. The sphericity assumption, as well as simple main effects of temperature or week, was addressed as described above. Explorative Pearson correlation analysis to assess whether each of the mucocyte variables related to drifting behavior as temperature changed within each exposure period was also performed.

2.4. Results

Temperature was the main factor tested for its influence on the production of the ctenidial mucilaginous threads by the Asian clam *Corbicula fluminea*. Low variation in temperature was generally found within treatments along the test period. The test organisms were exposed to three temperature treatments set to 20, 25 and 30ºC. Appreciable deviation of the lowest controlled temperature to the set point was observed: 22 ±1.1ºC (mean ± standard deviation). The actual temperatures measured

for the other two treatments were 25 ± 0.5ºC and 30 ± 0.9ºC. Because of the noticed difference between the 20º C set temperature and the corresponding mean temperature monitored during the exposure, the lowest temperature treatment is henceforth considered to be of 22ºC. No mortality was recorded during the test period in any experimental treatment.

The graphical trends in Fig. 2.1, representing the variation of ctenidial mucocytes in clams exposed to 22, 25 and 30 ºC for distinct exposure periods (one, two and three weeks of exposure), suggest that exposure time has an important role in modeling the response of ctenidial mucocytes to increasing temperature. This pattern was confirmed by the significant interaction between temperature and week found for cell number and the product between cell number and cell size (Table 2.1). Main time-dependent effects were observed, with special emphasis on ctenidial mucocytes size. Significantly larger cells were found following the first week of exposure to all temperatures as compared to the records obtained following two and three weeks of exposure, with changes statistically significant for 22ºC (Fig. 2.1.a; Table 2.2.a). This pattern remained unchanged as temperature increased, which is consistent with the lack of significant differences in mucocyte size of clams exposed to different temperatures within each exposure period (Fig. 2.1.a; Table 2.2.b). Cell number was also significantly affected by the exposure duration, at 22ºC. At this temperature, significantly fewer mucous cells were found in clams following the first week of exposure as compared to the third week of the test (Fig. 2.1.b; Table 2.2.a). Cell size was multiplied by the corresponding cell number to produce a new variable for analysis. To some extent this additional variable represents normalized changes in mucocytes assisting the proper interpretation of the main variables (cell size and cell number). For example, an increase in cell number may only mean that cells divided if accompanied by the proportional reduction in cell size. The product between cell size and number following one week of exposure to 22ºC was significantly lower than that recorded following three weeks of exposure similarly to the observed pattern for the number of mucocytes (Fig. 2.1.c; Table 2.2.a).

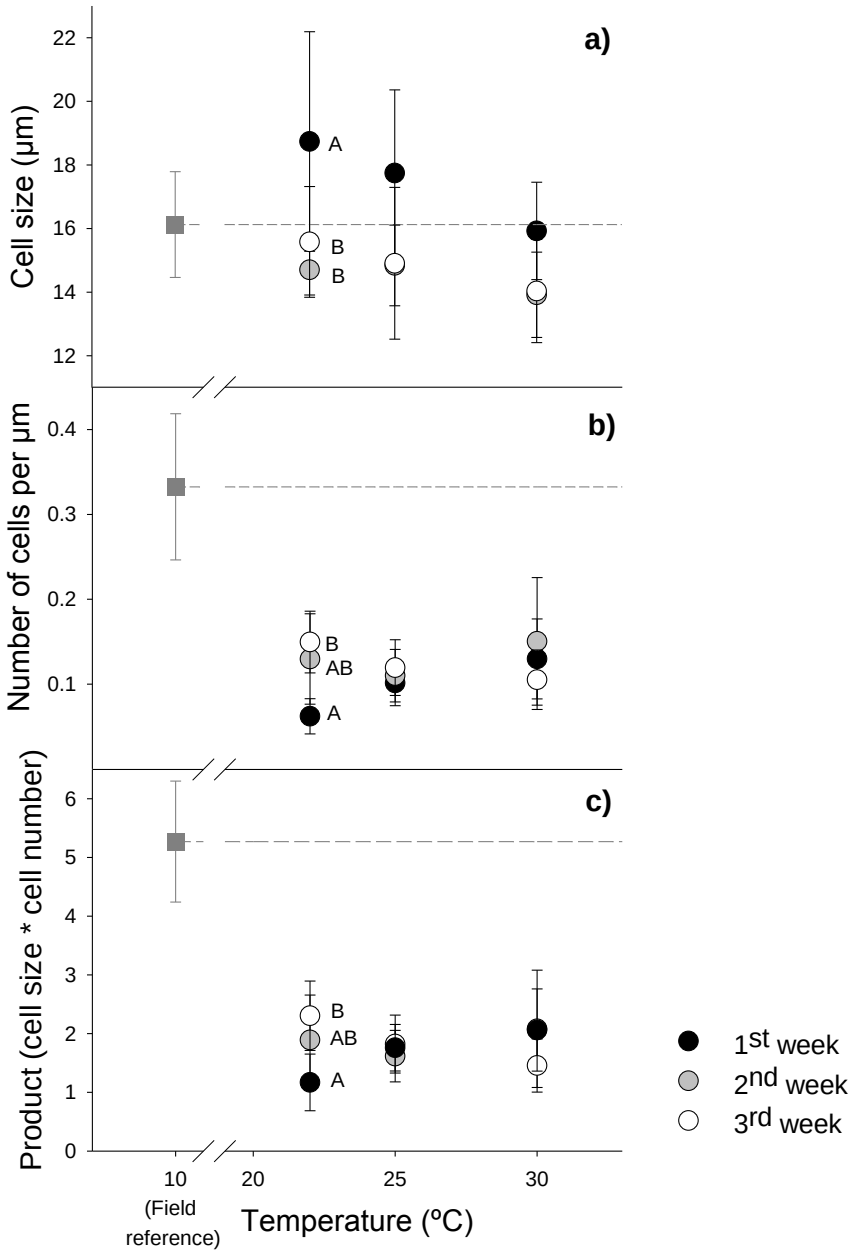

Fig. 2.1. Variation of ctenidial mucocytes in clams exposed for one, two, and three weeks to different temperatures. **(a)** Mean mucocyte size; **(b)** Mean mucocyte number; **(c)** Product between corresponding mucocyte size and number. Field reference corresponds to untreated clams and is represented by the square grey mark and dashed line. Letters placed next to points denote differences between groups of exposure period at each exposure temperature following the *post-hoc* Tukey test. Error bars represent standard deviation.

Table 2.1. Summary of repeated measures ANOVA applied to address the effect of time and temperature in mean ctenidial mucocytes size and number, as well as corresponding product (mucocyte size x number). Significant effects (α = 0.05) are marked in bold.

	Cell size				Cell number				Product (number*size)			
	df	MS	F	p	df	MS	F	P	Df	MS	F	p
Within-subjects												
Week	2	25.786	29.30	**<0.001**	2	0.003	5.30	**0.022**	2	0.172	1.772	0.212
Week*Temperature	4	0.904	1.027	0.432	4	0.002	4.49	**0.019**	4	0.602	6.204	**0.006**
Residual	12	0.880			12	0.001			12	0.097		
Between-subjects												
Temperature	2	7.564	7.64	**0.022**	2	0.001	2.15	0.198	2	0.105	0.958	0.435
Residual	6	0.990			6	0.001			6	0.110		

Fig. 2.1 and Table 2.1 (see cell size in particular) suggest temperature-dependent effects within exposure period. However, these were not statistically significant as main effects were analyzed even within the first week of the test, where the most consistent changes could be noticed with temperature (Table 2.2.b). Nevertheless, mucocyte size decreased consistently while the number and product increased consistently with increasing temperature following one week of exposure (Fig. 2.1.a). Other general graphical trends could not be statistically confirmed, including the record of fewer mucocytes and lower product between cell size and number with increasing temperatures following three weeks of exposure (Fig. 2.1.b and 2.1.c).

An inversely proportional relationship between cell size and cell number is clear following one week of exposure to increasing temperatures, with more but smaller cells being produced (Fig. 2.1.b as compared to 2.1.a). This is supported by a significant negative correlation between the number of mucocytes cells and their size (Pearson coefficient = -0.442; p = 0.016). However, this pattern was not confirmed through time, with non-significant correlations being found following two and three weeks of exposure (Pearson coefficient = -0.201, p = 0.271; and Pearson coefficient = 0.020, p = 0.920, respectively). Furthermore, the graphical pattern of the product between cell size and number (Fig. 2.1.c) seems to resemble that shown for cell number (Fig. 2.1.a) rather than provide an intermediate picture between the potentially related endpoints.

The ctenidial mucocytes were also examined immediately after clam collection from the field in order to provide a reference value for untreated organisms (Fig. 2.1; “field reference”). Although cell size in exposed clams is within the same range of that recorded for field clams, treated clams had fewer cells showing similar size to those of

field clams (Fig. 2.1.b as compared to Fig. 2.1.a). The duration of exposure plays an important role on how temperature drives the changes relatively to the field reference if this is taken as a surrogate for the basal stage of the organisms. Indeed, following one week of exposure, all endpoints tended to converge to the corresponding field record as temperature increases, while following three weeks of exposure the opposite occurred. This contrasts with an increasing distance from the unexposed reference to the records in clams exposed for three weeks as temperature rises.

Table 2.2. Summary of **(a)** repeated measures ANOVA and **(b)** one-way ANOVA applied to address the main effects of time or temperature, respectively, in mean ctenidial mucocytes size and number, as well as corresponding product (mucocyte size x number). Significant effects (α = 0.017) are marked in bold.

(a)	**22ºC**				**25ºC**				**30ºC**			
	df	MS	F	p	df	MS	F	p	df	MS	F	p
Cell size												
Week	2	13.903	17.62	**0.010**	1.1	16.04	7.00	0.104	1	9.136	8.34	0.102
Residual	4	0.789			2.3	2.29			2	1.095		
Cell number												
Week	2	0.007	7.00	**0.010**	1.4	0	0	1.000	1.2	0.001	1.00	0.337
Residual	12	0.001			12	0.001			12	0.001		
Product (cell number*cell size)												
Week	2	1.115	11.49	**0.002**	1.9	0.036	0.37	0.554	1.3	0.348	3.59	0.083
Residual	12	0.097			12	0.097			12	0.097		
(b)	**Week #1**				**Week #2**				**Week #3**			
	df	MS	F	p	df	MS	F	p	df	MS	F	p
Cell size												
Temp	2	6.077	3.91	0.082	2	0.709	3.30	0.108	2	2.587	2.64	0.151
Residual	6	1.554			6	0.215			6	0.981		
Cell number												
Temp	2	0.004	2.05	0.210	2	0.001	0.65	0.555	2	0.001	0.50	0.630
Residual	6	0.002			6	0.002			6	0.002		
Product (cell number*cell size)												
Temp	2	0.771	1.77	0.250	2	0.166	0.381	0.699	2	0.372	0.853	0.472
Residual	6	0.436			6	0.436			6	0.436		

Time was the main factor explaining the variation in the clams' drifting behavior (Table 2.3) with the highest proportion of drift-responsive clams being observed in the

first week of testing (Fig. 2.2). Following the first week of exposure there were significantly more drifts than following two and three weeks of exposure (Fig. 2.2; Table 2.4), at the higher temperature treatments of 25 and 30ºC (Fig. 2.2; Table 2.4). Following two weeks of exposure to any of the tested temperatures clam flotation behavior was very rarely observed and following three weeks of exposure clams did not drift at all. The slight differences in the drift records between these two latter time-points were not confirmed statistically (Fig. 2.2; Table 2.4). In these experiments, temperature did not statistically affect drifting behavior (Table 2.4), although following the first week the clams flotation behavior clearly increased as the 22ºC compares to 25ºC (Fig. 2.2). The parallel experiment run with clams that were given sediment protection yielded very definitive results. No drift was observed in any of the temperature treatments regardless the exposure period considered.

Correlation analysis to assess whether each of the mucocyte-related variables could be linked to drifting behavior revealed no significant associations (data not shown).

Source of variation	df	MS	F	P
Within-subjects				
Week	2	0.379	51.757	**<0.001**
Week*Temperature	4	0.045	6.146	**0.006**
Error	12	0.007		
Between-subjects				
Temperature	2	0.31	2.635	0.151
Error	6	0.12		

Table 2.3. Repeated-measures ANOVA referring to mean clam flotation following one, two and three weeks of exposure to different temperatures. Significant effects (α = 0.05) are marked in bold.

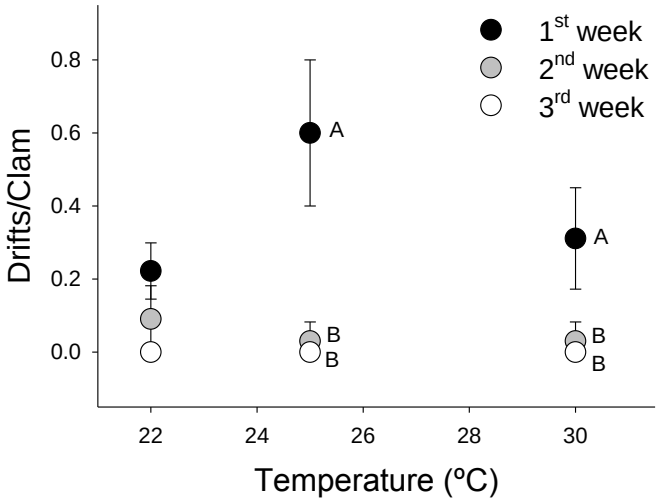

Fig. 2.2. Mean clam flotation following exposure to different temperatures for one, two and three weeks. Letters placed next to points denote differences between groups of exposure period at each exposure temperature following the *post-hoc* Tukey test. Error bars represent standard deviation.

Table 2.4. Summary of **(a)** repeated measures and **(b)** one-way and ANOVA applied to address the main effects of time and temperature, respectively, in mean clam flotation. Significant effects (α = 0.017) are marked in bold.

(a)	**22ºC**				**25ºC**				**30ºC**			
	df	MS	F	p	df	MS	F	P	df	MS	F	p
Week	2	0.037	5.28	0.023	1.1	0.623	89	**<0.001**	1	0.173	24.71	**<0.001**
Error	12	0.007			12	0.007			12	0.007		

(b)	**Week#1**				**Week#2**			
	df	MS	F	p	df	MS	F	P
Temperature	2	0.117	0.88	0.462	2	0.004	0.03	0.970
Error	6	0.133			6	0.133		

2.5. Discussion

Bivalves are able to acclimatize to cope with changing environmental conditions or minimally show variation in macrostructural or microstructural form in response to these changes, e.g. in shell morphology (Peyer et al. 2010, Trichkova et al. 2008), behavioral traits, such as predator avoidance strategies (Flynn and Smee 2010), or valve movements (Tran et al. 2011). There are studies reporting a wide-range in shell plasticity of populations of the Asian clam *Corbicula fluminea* inhabiting geographically adjacent habitats that show distinct environmental conditions (e.g. Prezant and Tiu 1987, Renard et al. 2000, Sousa et al. 2007). Other evidence of *C. fluminea* phenotypic plasticity is its proven ability to regulate body mass while facing long starvation periods as a final outcome of an efficient management of energy budgets and allocation to different body compartments (Vohmann et al. 2009). This suggests that this bivalve has a large range for physiological adjustment to cope with potentially stressful conditions. The present study provides additional evidence on the ability of the Asian clam to adjust physiology and behavior as a response to changing environmental conditions. The results show that changes in water temperature, at least at high ranges (above 20º C), affect the production of the mucous drogue line by *C. fluminea* and its drifting behavior, thus possibly influencing dispersal patterns with consequences on population expansion. It should be noticed here that drifting by the Asian clam may not relate at least directly to physiological changes occurring in the ctenidial mucocytes. However, it should be kept in mind that changes with temperature were recorded consistently

following the first week of exposure both in mucocyte cells and in flotation behavior. Although a relationship between the physiological and behavioral parameters was not confirmed statistically with the particular design employed in this study, this latter evidence seems to point towards an association between the production of the mucous drogue line and clam's drifting driven by temperature.

Increasing water temperature, particularly to levels closer to the species upper tolerance limit (36-37º C (Karatayev et al. 2005)), can produce physiological stress. At less extreme conditions, increase in temperature generally relates to an increase in metabolic rates, particularly evident in ectotherms, with consequential changes in resources exploitation (Gillooly et al. 2001, Larsen and Riisgard 2009, Lui and Leung 2004, Vohmann et al. 2009). The data recorded on ctenidial mucocytes in clams exposed to high-range temperatures seem consistent with this metabolic shift. In fact, increasing temperature apparently triggered an acceleration of the mucocytes life cycle thus favoring higher cell division rates (Campbell 1996). As a consequence more but smaller cells were produced, especially after one week of exposure. On the other hand, the data collected on the reference sample (10ºC) did not support this hypothesis as particularly evidenced by its higher number of mucocytes as compared to treated samples. This seems to indicate distinct mucocyte cycle patterns at low-range temperatures that would be worth to explore in future studies. Indeed, the literature is not consensual as to the effect of increasing temperature in mucocyte-like cells, making it even more speculative to advance with an explanation for the phenomenon. Studies on other secretory cells that produce mucin substances support our results for high-range temperatures, e.g. Gammert et al. (1988) showed that the number of goblet cells of the nasal mucosa of rats increased with increasing temperature. Conversely, other studies demonstrate the opposite, e.g. in mucous cells of the epidermis of catfish (Quiniou et al. 1998). Along with these reported inconsistencies, the present study provides contradictory results when considering shorter (1 week) *versus* longer exposure periods. Specifically, the pattern of increasing cell division rates with increasing temperatures was not evident for longer exposure periods. Thus it is likely that the observed changes in mucocytes relate to an immediate response to a perceived environmental stress rather than reflecting a longer term shift in metabolic rates.

Prezant and Chalermwat (Prezant and Chalermwat 1984) suggested that the production of the mucous thread and consequent drifting capability could be more readily induced when clams face stressful conditions. *C. fluminea* drift as quantified in the present study reflected, in particular, in the clams' response to induced stress after one week of exposure. This is supported by the effects of temperature treatments on

mucocytes size and number following the first week of exposure as compared to longer exposure periods. It is possible that the first week of increasing temperature acted as a critical stress that, in turn, induces mucocyte production that could more readily "allow" mucous drogue line and ultimately flotation behavior. As the higher temperature exposure continues, individuals might have adjusted their metabolism to the new environmental conditions thus acclimatizing to the new thermal regime. When clams were supplied with a sediment layer, no drift was recorded throughout the exposure period. Apparently, at least in this current experiment, the sediment plays an important protection, perhaps stabilizing, role that seems to compensate for the stress induced by increased temperature. Provided the infaunal habit of *C. fluminea*, the type of sediment has been argued to represent a relevant constraint to the species distribution in natural systems such as creeks and rivers (e.g. Bodis et al. 2012). However, the clam has also been reported to proliferate massively in underwater structures and channels of freshwater-dependent industries (Rosa et al. 2011), hence studies such as the present addressing the species dispersal abilities in systems lacking sediment should not be disregarded. Since a direct relationship between mucocyte activity and drifting behavior was initially hypothesized, the role of sediment was only assessed using drift ratios as an endpoint. Further histological studies on the effects of temperature in mucous production in test systems supplied with sediment would help clarify the extent of its protective role, and ultimately the role of temperature as an actual stress agent.

It was already proven that *C. fluminea* mucous cells residing in the inner demibranchs produce the mucilaginous drogue line, which has an active role in the species' dispersal ability (Prezant and Chalermwat 1984). In this way, and following the suggestions by McMahon (1983) on the eventual effect of increasing temperature as a promoter of clam's lift-off into the water column, it seems reasonable to assume that our results indicate that increasing temperature at high-ranges could enhance the Asian clam dispersal ability through the increased production of the mucous drogue line as an immediate response. Although this seems a reasonable prediction for scenarios where the water temperatures range within those tested here (22º to 30ºC), it should be stressed out that the data obtained with the field reference (untreated clams facing 10ºC water temperature) did not corroborate it, highlighting the need for future studies that address mucous production and drift behavior under low-range temperature changes. Temperature also influences byssus production in other bivalves. For example, Young (1985) and Clarke and McMahon (1996) found that increasing temperature leads to increasing byssus thread number in *Mytilus edulis* and *Dreissena polymorpha*. While byssal thread and the mucous drogue line both play key roles in relevant species dispersal, these structures are ontogenetically,

phylogenetically, and physiologically distinct: byssal threads form from a complex multiglandular byssal gland releasing a quinine tanned protein with a mucin and collagen component and emerge via a pedal ventral grove (Prezant 1990, Waite et al. 1998). Despite these differences, a behavioral parallel can be traced that suggests a link between temperature and dispersal potential in byssus-producing species and mucoid drogue line dispersing species.

With water temperature alteration, other water properties such as viscosity are affected. Increasing temperature causes decreased viscosity and can contribute to decreased buoyancy of floating particles. This could have consequences on bio-mechanical activities such as the drifting or swimming efficiency of aquatic organisms (Larsen and Riisgard 2009, Larsen et al. 2008). Thus, decreased viscosity with increasing temperatures may eventually help explain the observed increase in flotation behavior between clams exposed to 22ºC and those exposed to higher temperatures during the first week. However, a monotonic trend for drift intensification with increasing temperature could not be recorded. Other authors have not been able to demonstrate a straight relationship between changes in temperature and viscosity-driven changes in drift. For example, Wang and Xu (1997) concluded that with decreasing temperature there is a more frequent drifting behavior in bivalve veliger larvae, and Williams (1990) found that different invertebrate species showed no consistent drifting responses as temperature changed. This suggests that clam drifting (and equivalent behavior in other organisms) results from a complex interaction of several factors including physical parameters (e.g. viscosity), inherent physiology, and environmental challenges. Knowledge on how this complexity of factors enhances or inhibits dispersal is of obvious relevance when considering invasive species such as *C. fluminea*. Furthermore, it contributes to the understanding of the ecological dynamics of benthic communities, which is strongly influenced by invertebrate drift and settlement, either by the continuous loss of clams into the water column that reduces population density in a given location, or by its continuous settling out with important colonizing implications (Lancaster et al. 1996, Townsend and Hildrew 1976).

The temperatures tested in this study are within the tolerance limits described for the Asian clam (see Karatayev et al. 2005) and can also be found in the field especially in the vicinities of power plants using cooling waters from abutting streams and discharging thermal effluents (Cherry et al. 1980). Adding to the potential dispersal shifts induced by temperature changes that might occur in lotic and lentic habitats of Asian clam populations are the potential implications and consequences of global climate change, and the alteration of thermal regimes due to dam and canal construction. Thus, the results constitute a valuable add-on to the baseline information

that is required to predict environmentally-driven changes in dispersal patterns of the Asian clam. It is also important to consider the fact that the higher efficiency in control methods relying in high water temperature applied for short periods may not be as straightforward as presumed as we consider that a concomitant stimulation of dispersal within affected areas can occur representing a drawback in the method. Further research still applies to clarify temperature-dependent effects on *C. fluminea* dispersal. For example, in an ongoing study, we recorded remarkable differences in the interlamellar epithelium (totally absent or incipient ctenidial mucocytes) of *C. fluminea* demibranchs in individuals from a population established in a Portuguese channel as compared to the Somerset, New Jersey, USA population studied in the current experiments. Genetic typing (RFLP analysis and mtCOI sequencing) revealed no clear distinction between this population and the Somerset population examined in the present study i.e. both exhibit the same haplotype. It is reasonable to hypothesize that local adaptation may constrain general conclusions on the influence of environmental change in the species dispersal patterns. Furthermore, some authors (e.g. Byrne et al. 2000, Morton 1977) suggested that the mucous produced in the demibranchs may nourish the developing embryos and/or assist the release of juveniles out of the gills. To tease apart potential issues of seasonality or geographic localities or difference induced by localized populations, further studies are being conducted addressing seasonal variation in ctenidial mucocytes and any correlation to drifting behavior. Many questions remain on how different environmental parameters constrain the dispersal of *C. fluminea*. Additional fundamental knowledge on how temperature regulates physiological and behavioral changes in the clams with consequences in dispersal ability has been demonstrated in the present study. The results indicate that increasing temperature at high-ranges can lead to population expansion in *C. fluminea* and might be a proxy that helps predict changes in dispersal patterns correlated with change in water temperature.

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the experiments. No endangered or protected species were disturbed or involved in the present study.

2.7. References

Aldridge DW, McMahon RF. 1978. Growth, fecundity, and bioenergetics in a natural population of asiatic freshwater clam, *Corbicula manilensis* Philippi, from north central Texas. Journal of Molluscan Studies 44: 49-70.

Allan JD, Castillo MM. 2008. Stream ecology - Structure and function of running waters. Dordrecht: Springer. 94-103 pp.

Araujo R, Moreno D, Ramos MA. 1993. The Asiatic clam *Corbicula fluminea* (Müller, 1774) (*Bivalvia: Corbiculidae*) in Europe. American Malacological Bulletin 10: 39-49.

Bodis E, Sipkay C, Toth B, Oertel N, Nosek J, Hornung E. 2012. Spatial and temporal variation in biomass and size structure of *Corbicula fluminea* in Danube River catchment, Hungary. Biologia 67: 739-750.

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 Fur Hydrobiologie 138: 483-507.

Brancotte V, Vincent T. 2002. The invasion of french hydrographic system by the molluscs *Corbicula* spp details of settlement and main role of ship canals. Bulletin Francais De La Peche Et De La Pisciculture: 325-337.

Britton JC. 1982. Biogeography and ecology of the Asiatic clam, *Corbicula*, in Texas. Pages 21-30 in Davis JR, ed. Proceedings of the Symposium on Recent Benthological Investigations in Texas and Adjacent States. Austin, Texas: Aquatic Sciences Section, Texas Academy of Science.

Britton JC, Morton B. 1982. A dissection guide, field and laboratory manual for the introduced bivalve *Corbicula fluminea*. Malacological Review Supplement 3: 1-82.

Byrne M, Phelps H, Church T, Adair V, Selvakumaraswamy P, Potts J. 2000. Reproduction and development of the freshwater clam *Corbicula australis* in southeast Australia. Hydrobiologia 418: 185-197.

Campbell NA. 1996. Biology. Menlo Park (CA), USA: Cummings Publishing. 1297 pp.

Cantanhêde G, Hahn NS, Gubiani ÉA, 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.

Cherry DS, Rodgers Jr. JH, Graney RL, Cairns Jr. J. 1980. Dynamics and control of the Asiatic clam in the New River, Virginia. Virginia: Virginia Water Research Center, Virginia Polytechnic Institute and State University.

Clarke M, McMahon RF. 1996. Effects of temperature on byssal thread production by the freshwater mussel, *Dreissena polymorpha* (Pallas). American Malacological Bulletin 13: 105-110.

DAISIE – European Invasive Alien Species Gateway. www.europe-aliens.org. Acessed 2012.

Elliott P, Aldridge DC, Moggridge GD, Chipps M. 2005. The increasing effects of zebra mussels on water installations in England. Water and Environment Journal 19: 367-375.

Flynn AM, Smee DL. 2010. Behavioral plasticity of the soft-shell clam, *Mya arenaria* (L.), in the presence of predators increases survival in the field. Journal of Experimental Marine Biology and Ecology 383: 32-38.

French JRP, Schloesser DW. 1996. Distribution and winter survival health of Asian clams, *Corbicula fluminea*, in the St Clair River, Michigan. Journal of Freshwater Ecology 11: 183-192.

Galbraith HS, Vaughn CC. 2009. Temperature and food interact to influence gamete development in freshwater mussels. Hydrobiologia 636: 35-47.

Gammert C, Felix H, Weihe WH. 1988. The influence of climatic factors on the nasal mucosa of rats. Rhinology (Utrecht) 26: 41-50.

Gillooly JF, Brown JH, West GB, Savage VM, Charnov EL. 2001. Effects of size and temperature on metabolic rate. Science 293: 2248-2251.

Jenner HA, Whitehouse JW, Taylor CJL, Khalanski M. 1998. Cooling water management in European power stations - Biology and control of fouling. Hydroécologie Appliquée 10: 1-225.

Joy JE. 1985. A 40-week study on growth of the asian clam, *Corbicula fluminea* (Müller), in the Kanawha river, West Virginia. Nautilus 99: 110-116.

Karatayev AY, Burlakova LE, Padilla DK. 2005. Contrasting distribution and impacts of two freshwater exotic suspension feeders, *Dreissena polymorpha* and *Corbicula*

fluminea. Pages 239-262 in Dame RF, Olenin S, eds. The Comparative Roles of Suspension-Feeders in Ecosystems, vol. IV. Earth and Environmental Sciences. Dordrecht, The Netherlands: Springer.

Kraemer LR, Galloway ML. 1986. Larval development of *Corbicula fluminea* (Müller) *(Bivalvia, Corbiculacea*) - An appraisal of its heterochrony. American Malacological Bulletin 4: 61-79.

Kratz WA, Myers J. 1955. Nutrition and growth of several blue green algae. American Journal of Botany 42: 282-287.

Lancaster J, Hildrew AG, Gjerlov C. 1996. Invertebrate drift and longitudinal transport processes in streams. Canadian Journal of Fisheries and Aquatic Sciences 53: 572-582.

Larsen PS, Riisgard HU. 2009. Viscosity and not biological mechanisms often controls the effects of temperature on ciliary activity and swimming velocity of small aquatic organisms. Journal of Experimental Marine Biology and Ecology 381: 67-73.

Larsen PS, Madsen CV, Riisgard HU. 2008. Effect of temperature and viscosity on swimming velocity of the copepod *Acartia tonsa*, brine shrimp *Artemia salina* and rotifer *Brachionus plicatilis*. Aquatic Biology 4: 47-54.

Lui KKY, Leung KMY. 2004. Sand elimination by the Asiatic hard clam *Meretrix meretrix* (L.): Influences of temperature, salinity and season. Journal of Shellfish Research 23: 421-427.

Mackie GL, Claudi R. 2010. Monitoring and control of macrofouling mollusks in fresh water systems. Boca Raton, FL: CRC Press. 508 pp.

McMahon RF. 1983. Ecology of an invasive pest bivalve, *Corbicula* in Russel-Hunter WD, ed. The Mollusca, vol. 6. London: Academic Press, Inc.

Morgan DE, Keser M, Swenarton JT, Foertch JF. 2003. Population dynamics of the Asiatic clam, *Corbicula fluminea* (Müller) in the Lower Connecticut River: Establishing a foothold in New England. Journal of Shellfish Research 22: 193-203.

Morton B. 1977. Freshwater fouling bivalves. Pages 1-14. First International *Corbicula* Symposium. Fort Worth, Texas, USA: Texas Christian University. 1-14 pp.

Moyo NAG. 2011. An investigation into the factors affecting the natural reproduction of *Opsaridium peringueyi*. Physics and Chemistry of the Earth 36: 876-880.

Muller O, Baur B. 2011. Survival of the invasive clam *Corbicula fluminea* (Müller) in response to winter water temperature. Malacologia 53: 367-371.

Peyer SM, Hermanson JC, Lee CE. 2010. Developmental plasticity of shell morphology of quagga mussels from shallow and deep-water habitats of the Great Lakes. Journal of Experimental Biology 213: 2602-2609.

Prezant RS. 1990. Form, function and phylogeny of bivalve mucins. Pages 83-95 in Morton B, ed. The Bivalvia - Proceedings of a memorial symposium in honour of Sir Charles Maurice Yonge. Edinburgh: Hong Kong Universiy Press.

Prezant RS, Chalermwat K. 1984. Flotation of the bivalve *Corbicula fluminea* as a means of dispersal. Science 225: 1491-1493.

Prezant RS, Tiu AT. 1987. Spiral crossed-lamellar shell growth in *Corbicula* (*Mollusca, Bivalvia*). Transactions of the American Microscopical Society 105: 338-347.

Purves WK, Sadava D, Orians GH, Heller HC, eds. 2001. Life - The Science of Biology. 6th Edition ed. USA: Sinauer Associates, Inc. 1044 pp.

Quiniou SMA, Bigler S, Clem LW, Bly JE. 1998. Effects of water temperature on mucous cell distribution in channel catfish epidermis: a factor in winter saprolegniasis. Fish & Shellfish Immunology 8: 1-11.

Quinn GP, Keough MJ, eds. 2002. Experimental design and data analysis for biologists New York: Cambridge University Press. 537 pp.

Rahel FJ, Olden JD. 2008. Assessing the effects of climate change on aquatic invasive species. Conservation Biology 22: 521-533.

Rajagopal S, Van der Velde G, Nair KVK, Jenner HA. 1999. Response of the tropical mussel *Modiolus philippinarum* (Hanley) to heat treatment: An experimental study for antifouling application. Marine and Freshwater Behavior and Physiology 32: 239-253.

Rajagopal S, Van der Velde G, Jenner HA, Van der Gaag M, Kempers AJ. 1996. Effects of temperature, salinity and agitation on byssus thread formation of zebra mussel *Dreissena polymorpha*. Netherlands Journal of Aquatic Ecology 30: 187-195.

Renard E, Bachmann V, Cariou ML, Moreteau JC. 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.

Robinson AT, Childs MR. 2001. Juvenile growth of native fishes in the Little Colorado River and in a thermally modified portion of the Colorado River. North American Journal of Fisheries Management 21: 809-815.

Rosa IC, Pereira JL, Gomes J, Saraiva PM, 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.

Sousa R, Freire R, Rufino M, Mendez J, Gaspar M, Antunes C, Guilhermino L. 2007. Genetic and shell morphological variability of the invasive bivalve *Corbicula fluminea* (Müller, 1774) in two Portuguese estuaries. Estuarine, Coastal and Shelf Science 74: 166-174.

Townsend CR, Hildrew AG. 1976. Field experiments on the drifting, colonization, and continuous redistribution of stream benthos. Journal of Animal Ecology 45: 759-772.

Tran D, Nadau A, Durrieu G, Ciret P, Parisot J-P, Massabuau J-C. 2011. Field chronobiology of a molluscan bivalve: How the moon and sun cycles interact to drive oyster activity rhythms. Chronobiology International 28: 307-317.

Trichkova TA, Kozuharov DS, Hubenov ZK, Botev IS, Zivkov MT, Cheshmedjiev SD. 2008. Characteristics of zebra mussel (*Dreissena polymorpha*) populations in infested reservoirs, northwest Bulgaria. Journal of Natural History 42: 619-631.

Vazquez KE. 2010. Range extension parameters of the opportunistic bivalve *Corbicula fluminea* (Müller, 1774) in New JerseyMontclair State University, Montclair.

Viergutz C, Kathol M, Norf H, Arndt H, Weitere M. 2006. Control of microbial communities by the macrofauna: a sensitive interaction in the context of extreme summer temperatures? Oecologia 151: 115-124.

Voelz NJ, McArthur JV, Rader RB. 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.

Waite JH, Qin XX, Coyne KJ. 1998. The peculiar collagens of mussel byssus. Matrix Biology 17: 93-106.

Wang WX, Xu ZZ. 1997. Larval swimming and postlarval drifting behavior in the infaunal bivalve *Sinonovacula constricta*. Marine Ecology-Progress Series 148: 71-81.

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

Welch KJ, Joy JE. 1984. Growth rates of the asiatic clam, *Corbicula fluminea* (Müller), in the Kanawha river, West Virginia. Freshwater Invertebrate Biology 3: 139-142.

Williams DD. 1990. A field-study of the effects of water temperature, discharge and trout odor on the drift of stream invertebrates. Archiv Fur Hydrobiologie 119: 167-181.

Young GA. 1985. Byssus-thread formation by the mussel *Mytilus edulis* - Effects of environmental factors. Marine Ecology Progress Series 24: 261-271.

Zuur AF, Ieno EN, Elphick CS. 2010. A protocol for data exploration to avoid common statistical problems. Methods in Ecology and Evolution 1: 3-14.

CHAPTER 3

Dispersal of *Corbicula fluminea*: Factors influencing the invasive clam's drifting behavior

Dispersal of *Corbicula fluminea*: Factors influencing the invasive clam's drifting behavior

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3.1. Abstract

Corbicula fluminea, commonly known as the Asian clam, is one of the most successful invasive species in fresh and brackish waters worldwide. Dispersal is one of the most determinant steps in the invasive process, and the full understanding of the mechanisms involved in this step is critical for adequate pest management both in the wild and in industries affected by this species' biofouling activity. A mucous drogue line produced by mucocytes packed along the inner demibranchs of the clams' gills seem to play an important role in assisting drifting and hence dispersal. Two Asian clam populations geographically separated (one in the USA and the other in Portugal), investigated at different times of the year, were reported to differ in terms of mucous drogue line production and floating responses (these were only present for the American population). In this study, genetics and seasonality effects were hypothesized to explain the difference between the populations. To test these hypotheses, the two populations were genetically compared, and the Portuguese one was followed for 14 months to record the animals' mucous drogue line production and flotation capabilities and locate the population reproductive periods. Our results signal a possible scenario of microevolution with consequences on the production of the clams' mucilaginous drogue line.Although some authors advocate a link between mucous threads formation and reproduction events, such a relationship was not observed in this study. By contributing to the understanding of a physiological trait of the Asian that is important for dispersal, this study may be of practical relevance for pest monitoring and control.

Keywords: Asian clam; Mucous drogue line; Flotation behavior; Life-cycle traits; Invasion

3.2. Introduction

Invasive species are a major worldwide threat due to the serious ecological and economic impacts they are responsible for (Barinova et al. 2010, Pimentel et al. 2005). Lakes, estuaries and rivers are particularly prone to invasion (Ricciardi and MacIsaac 2000) because of the large number and variety of transport vectors and anthropogenic disturbance agents affecting these systems (Cohen and Carlton 1998). Dispersal into a new habitat is a determinant stage in the invasion process (Davis 2010), and hence the full understanding of the mechanisms involved in this stage is a key asset to manage the pests.

Amongst the most successful aquatic invaders is the freshwater bivalve *Corbicula fluminea* (Müller, 1774), commonly known as the Asian clam (DAISIE 2012). Several life-cycle traits contribute to its success as an invader, including high growth rates, short life spans, high fecundity rates (up to 600-700 juveniles day^{-1}; Aldridge and McMahon 1978) and hermaphroditism sometimes associated to self-fertilization (Britton and Morton 1982, Kraemer and Galloway 1986). These traits combined with increased waterborne traffic resulted in the massive expansion of *C. fluminea* from its native distribution range in Southeast Asia to vast regions in Europe (Araujo et al. 1993) and North (Phelps 1994) and South America (Ituarte 1994) over the last century.

A particular life-cycle trait of this species that has been considered to contribute to its dispersal abilities is the production of a mucilaginous drogue line by modified cells (mucocytes) packed along the inner demibranchs of the ctenidia of juvenile and young adult clams. This drogue line was proven to assist clam flotation (in clams up to 14 mm shell length) in response to water currents, thus promoting drifting into new locations and favoring the species dispersal (Prezant and Chalermwat 1984).

Although mucilaginous drogue line production may play an important role in the species' dispersal, being potentially relevant from the pest management point of view, little is known about this physiological feature. In a previous study, Rosa et al. (2011) reported marginal industrial biofouling effects and mild invasion severity by *C. fluminea* in Portugal even though the species entered the country more than 30 years ago (Mouthon 1981). As a possible explanation for this unexpected observation, it was hypothesized that environmental conditions could constrain the production of the mucilaginous drogue line thus affecting the species' dispersal. In a subsequent study (Rosa et al. 2012), conducted in early spring (April), a USA population of *C. fluminea* was examined and temperature was shown to influence this trait. When the authors investigated the mucilaginous drogue line production and drifting behavior of a Portuguese Asian clam population later in summer (between June and August),

implementing similar methodologies, neither mucocytes nor any flotation-like event were observed. Here a follow-up study is reported, with two major hypotheses being investigated to clarify the inconsistency observed amongst the two clam populations.

One of the hypotheses tested was that the two populations could represent different haplotypes of *C. fluminea* (Hypothesis 1). Given that the *Corbicula* genus shows high genetic variability and significant phenotypic plasticity has been attributed to the species (Glaubrecht et al. 2003, Park and Kim 2003, Pigneur et al. 2011, Sousa et al. 2007), different haplotypes could translate into physiological differences regarding drogue line production and hence drifting abilities. To test this hypothesis, the American and Portuguese clam populations were genetically characterized and compared. In parallel, the hypothesis that mucilaginous drogue line production could vary seasonally, which would explain the different behaviors observed in spring and summer, was also investigated (Hypothesis 2). Some authors (e.g. Byrne et al. 2000, Morton 1977) suggested, although providing no clear supporting evidence, that the mucous threads in *C. fluminea* have an additional role in nourishing the embryos, also developing in the inner demibranchs, and/or in assisting the release of juveniles out of the gills. This being the case, an annual variation of mucous production, concomitant with the animals' reproductive cycle, could be expected. To assess this hypothesis, the Portuguese clam population was followed for 14 months as a study model. The animals' flotation behavior and the production of mucilaginous threads by ctenidial mucocytes were analyzed throughout the test period. The population's reproductive period(s), potentially connected with mucous production, were also located by following the seasonal changes in the clams' body condition (as dry tissue weight) and the population growth dynamics, complementing with morphological examination of the clams' gills to identify the presence of incubating progeny.

Because *C. fluminea* is a serious aquatic invader, with both ecological and industrial impacts, it is important to enlarge the body of knowledge on the species' biology. The study of the Asian clam dispersal mechanisms, namely by characterizing possible seasonal patterns and the life-cycle traits linked to the process, may contribute to the design of more effective monitoring strategies and improved control methods for the nuisance.

3.3. Materials and methods

3.3.1. Assessing Hypothesis 1: genetic typing of the two Corbicula fluminea *populations*

The genetic characterization of both the American (see Rosa et al. 2012 for details on this population) and the Portuguese populations (see below for details on the collection site) was done through restriction fragment length polymorphism (RFLP) analysis of the mitochondrial cytochrome *c* oxidase subunit I gene (mtCOI) plus sequencing of the gene (Renard et al. 2000). Twenty clams (shell length above 22 mm) were collected from each population. The animals' valves were opened by gently forcing them with a blunt needle, and each individual was placed in a vial filled with absolute ethanol PA at 4 ºC in order to preserve DNA integrity. Total DNA was extracted from approximately 30 mg of each individual using the E.Z.N.A.® Mollusc Isolation Kit as indicated by the manufacturer. A 710 bp fragment of the mtCOI gene was amplified by polymerase chain reaction (PCR) using the primers designed by (Folmer et al. 1994): LCOI490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAA AATCA-3'). A negative control (no DNA template) was used in the reaction. Amplification of 15 ng DNA occurred in a total volume of 25 µL and the reaction mixture also included 1 µM of each primer, 0.2 mM of each dNTP, 1 mM of $MgCl_2$, 0.025 U/µL adjusted to 1 U/sample of Taq DNA Polymerase (Fermentas, Lithuania) plus the recommended buffer. The PCR protocol consisted in an initial denaturation step of 60 s at 94 ºC, 35 annealing/elongation cycles of 60 s at 94 ºC, 60 s at 4 0ºC and 90 s at 75 ºC, and a final elongation step at 72 ºC for 5 min. The PCR product was then digested by the restriction enzyme Sac I (Takara, China): 5 µL of the PCR product with 5 U of the restriction enzyme plus the corresponding buffer as recommended by the enzyme manufacturer were incubated overnight at 37 ºC; 5 U of restriction enzyme were further added to prolong digestion for 3 h; the reaction was terminated by adding 1 µL of loading buffer to each mixture. An agarose gel (1.5 % (w/v)) electrophoresis was run in order to confirm whether all individuals could be identified as *C. fluminea* (one band of 710 bp or two bands of 200 and 500 bp; Renard et al. 2000). In order to further find the haplotype(s) present in the samples, the PCR product was purified (ExoSAP-IT®; Affymetrix, USA) and sequenced in a certified laboratory according to ISO 9001:2008, using the oligo LCOI490. The obtained sequences were compared with sequence data from the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov) using BLASTn homology search. Similar sequences were

aligned with the clams' sequences using CLUSTALW2 (EMBL-EBI) and the differences between the sequence of nucleotides were then assessed.

3.3.2. Assessing Hypothesis 2

3.3.2.1. Study site and clam collection details

C. fluminea individuals were collected in Casal de São Tomé, Mira, Portugal (N40°25'06.90''/W8°44'13.18''), from a sandy muddy shallow creek, which is connected to other brooks, forming a network of small canals. Clams were collected monthly from October 2011 to December 2012 and twice a month between May and September 2012, the latter being expected to cover the breeding period. Two different sampling strategies were employed depending on the subsequent analysis. Qualitative sampling was used to obtain organisms for histological studies and analysis of drifting behavior as detailed below. Clams were collected by using a shovel to drag sediment into a bag with 1 mm mesh size, used to roughly sieve the sample. Clams with shell length in the range 9 to 14mm, corresponding to mature animals that were already proven to be able to produce a mucilaginous drogue line (Ituarte 1985, Prezant and Chalermwat 1984), were then selected. Quantitative sampling was used to address the seasonal variation of the clams' body condition and obtain cohort frequencies (see below for details). Clams were collected with a Van Veen grab (15 x 32 x 19.5 cm) from three sampling points along a transect established in the creek. The sediment collected was dragged into a 1-mm mesh size bag to roughly sieve the sample, which was then transferred to a 20-L bucket filled with ca. 15 L of field water for transportation to the laboratory.

Temperature, pH, conductivity and dissolved oxygen contents were measured *in situ* with a multiparameter field probe (WTW-Multi3430). Flow speed was estimated by examining the traveling time of a floating plastic cylinder. Water samples (ca. 4 L) were collected and vacuum filtered (GF/C 1.5-µm pore filters) for further determination of turbidity through the calculation of the absorption coefficient (m^{-1}; Brower et al. 1997). The filtration residue was used to quantify the total suspended solids (mg L^{-1}; APHA 1995) and photosynthetic pigments (µg L^{-1} Chl *a* Lorenzen 1967). Sediment was also collected to quantify the loss-on-ignition organic matter contents (% (w/w); ASTM 2000).

3.3.2.2. Analysis of the seasonal changes in clams' flotation behavior and mucilaginous drogue line production

Clams' flotation behavior was observed *in situ* and in the laboratory. In the field, 10 clams (shell length in the range 9 to 14 mm) were randomly drawn from the qualitative sample previously collected (see above for details) and placed into a plastic container with 35 x 25 x 6 cm. The clams were subjected to the field water flow by keeping the container fully flooded during 4-5 minutes and their flotation activity (if any) was recorded. In addition, as clams were observed to be actively siphoning, a few drops of 5 % (w/v) toluidine blue were placed near the siphons to assess whether a mucous drogue line was being produced. The clams were then transported to the laboratory in plastic containers filled with field water, which was then gradually replaced by dechlorinated municipal water for acclimation purposes. The sample was kept under continuous aeration at constant temperature (20 ± 2 ºC) and photoperiod ($16h^{L}$:$8h^{D}$). After 24 hours, the clams were transferred into a 15-L aquarium, containing 14 L of dechlorinated municipal water, and placed inside a submerged crystallizing dish to continue the acclimation period for another 24 h. An externally driven recirculation and filtration system was set in the aquarium to ensure the water quality. Following the approach suggested by Prezant and Chalermwat (1984) and Rosa et al. (2012), the clams were submitted to a gentle water flow and their flotation behavior (if any) was recorded over a 30-min period.

Microscopic inspection of the ctenidial mucocytes distributed in the inner demibranchs of clams sampled qualitatively was performed to get additional insight into the mucilaginous drogue line production and support the interpretation of the flotation data. Ten clams (shell length ranging from 9 to 14 mm) were transported in plastic containers filled with field water to the laboratory, where they were immediately opened through gentle forcing between valves with a blunt needle. Their soft tissues (whole body) were fixed in Zenker's fluid overnight. The fixed samples were treated as detailed by Rosa et al. (2012). Briefly, they were washed and dehydrated, and then the tissues were carefully embedded in paraffin wax (Paraffin mp 56-58 ºC, Merck KGaA) so that antero-posterior sections could be made in the organisms, corresponding to longitudinal cuts of the demibranchs. The blocs were sectioned at 5-7 µm cut thickness. The sections were stained with sodium borate buffered aqueous toluidine blue. The first eight sequential sections of the demibranchs were selected for microscopic examination to assess the presence of mucous cells (Olympus CKX41 inverted microscope).

3.3.2.3. Identification of the population's reproductive period(s)

Data on both the seasonal variation of the clam's body condition and the population dynamics, complemented by the morphological examination of the clams' gills, were employed to locate the population's reproductive periods.

The clams were sorted from the quantitative samples brought from the field (see above for details). The total clam numbers were counted and the animals' shell lengths were measured with a digital caliper to the nearest 0.1 mm. Sub-samples of 30 clams covering the whole samples' size range were taken to establish allometric relationships between biomass (dry weight of the soft tissues obtained by drying to constant mass at 60-80 ºC) and shell length. The model $DTW = a.L^b$, where DTW and L represent clams' dry tissue weight (mg) and shell length (mm), respectively, was fitted to the data by plotting ln DTW against ln L. Data showing a Cook's distance greater than one were eliminated since they can be considered influential (Quinn and Keough 2002). Whenever the probability distributions of ln DTW at each ln L were normal, a Model I regression was applied (ordinary least squares). In cases where normality was not observed (November and December 2011, and January and March 2012), a Model II regression was applied (ranged major axis regression) (Legendre and Legendre 1998). Coefficients of determination (r^2) were determined in order to estimate the proportion of the total variation in ln DTW that was explained by the allometric relationship with ln L (Quinn and Keough 2002). ANCOVA followed by the Tukey multiple comparison test was applied to compare the slopes of the regression models obtained over the study period using ln L as a covariate (Quinn and Keough 2002). Regression lines determined by the Model II regression approach were excluded from this analysis as the extension of this model to ANCOVA cannot be applied (Quinn and Keough 2002).

Ten clams (shell length in the range 9 to 14 mm) were randomly collected from each qualitative sample (see above for details on sampling) to examine the presence of juveniles in the gills. This evaluation was used to infer *C. fluminea* maturation state and to morphologically identify the onset of the reproductive period(s). After collection, the clams' valves were immediately opened by forcing them with a blunt dissection needle and the whole organism was preserved in 96% (v/v) ethanol. At the laboratory, extemporaneous microscopic slides were prepared by gently smashing and smearing the gills into the slide with the cover slip. Whole homogenized alcohol samples were also observed to ensure that any juvenile released during preservation could be tracked. The slides were observed under an inverted light microscope (Olympus CKX41) and the presence/absence of juveniles was recorded.

3.4. Results

3.4.1. Assessing Hypothesis 1: genetic typing of the two Corbicula fluminea populations

Genetic typing of the Portuguese and the American clam populations revealed that there is no clear distinction between them as both exhibit the same haplotype (*COI* haplotype I; GenBank: AF269090-3).

3.4.2. Assessing Hypothesis 2

3.4.2.1. Analysis of the seasonal changes in clams' flotation behavior and mucilaginous drogue line production

No clam drifting was observed *in situ* or in the laboratory throughout the entire study period. Also, no toluidine blue staining was observed in the field, indicating that the mucous drogue line was not being produced by the clams observed. The clams, both *in situ* and in the laboratory, distended their exhalant siphon and foot when exposed to the water flow, but only small movements with no clear lifting off the substratum for further drifting into the water column were observed. Microscopic inspection of the clams' inner demibranchs generally revealed no ctenidial mucocytes (Fig. 3.1.a). The histological screening showed mucocytes in the demibranchs of individuals sampled in the first two months of the study (October and November 2011). Overall, mucocytes were observed in 15 out of the 20 individuals sampled in those months. However, such mucocytes seemed to stand in an incipient state as clearly shown in Fig. 3.1.b by comparison with the "swollen" cells shown in Fig. 3.1.c.

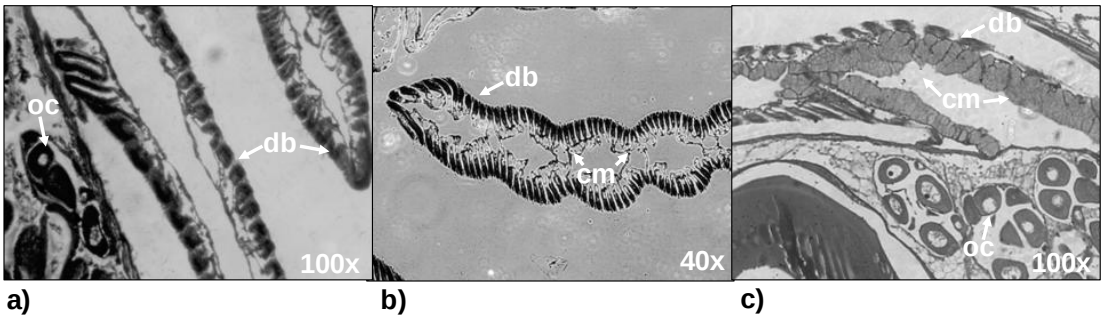

Fig. 3.1. Overview of the visceral mass of *C. fluminea* focusing oocytes (oc) and inner demibranchs (db) **(a)** without ctenidial mucocytes (cm), **(b)** with incipient mucocytes, and **(c)** with abundant mucocytes. Panels **a)** and **b)** refer to the Portuguese clam population while panel **c)** refer to the American *C. fluminea* population (collected from Sommerset, NJ) analyzed by Rosa et al. (2012).

3.4.2.2. Identification of the population's reproductive period(s)

Fig. 3.2 shows how physical and chemical parameters characterizing field water and sediment changed along the study period. As expected, water temperature was lower in autumn and winter months and higher during the spring and summer. Lower values of dissolved oxygen were observed during summer and early autumn while higher values were recorded during winter and spring; technical problems prevented the reliable measurement of dissolved oxygen values in January 2012. Water pH underwent no pronounced variation over the study period, ranging from 7.14 in May 2012 to 8.37 in September 2012. Conductivity was also generally constant, except for the value recorded in January 2012 (916 µS cm^{-1} against an average conductivity of 468 µS cm^{-1} over the study period), which is possibly related to an undetected probe malfunction. Turbidity was generally lower during the cold seasons, corresponding to heavy-rain periods; the absorption coefficient fluctuated between 2.99 m^{-1} and 6.44 m^{-1} during the warm seasons. The water flow was generally stable during the study period, with an atypical peak in March 2012 (0.973 m s^{-1} compared to an average flow of 0.111 m s^{-1}). No clear pattern was observed for the total suspended solids, which oscillated between 0.815 and 9.077 g L^{-1}, nor for chlorophyll *a* contents, which were generally below 1.50 mg L^{-1}. Sediment's organic matter contents were low, with values oscillating between 0.300 and 2.948 % (w/w) throughout the sampling period.

The general allometric equation relating dry tissue weight and shell length was successfully fitted to the several data sets obtained throughout the study period ($p < 0.05$ in all cases; Table 3.1 and Fig. 3.3). Such gradients were significantly affected by the collection date (ANCOVA: $F = 12.46$; d.f. = 15, 469; $p < 0.05$), with each allometric model differing from at least two other equations characterizing distinct data sets (*post-hoc* Tukey test; $p < 0.05$). Fig. 3.4, obtained from the allometric models (Table 3.1), shows the seasonal change of dry tissue weight of two hypothetical standard organisms: a juvenile with 8 mm shell length and an adult with 25 mm shell length. The model juvenile is characterized by generally constant physiological condition throughout the sampling period although its dry tissue weight undergoes a slight increase during spring, followed by a decrease in early summer, which are compatible with incipient reproduction. As for the model adult clam, the variation of the allometric models translates into a clear and more pronounced annual pattern of body condition: the dry tissue weight of such an individual increases from late winter over spring and suffers a marked drop in late spring; over summer the body condition keeps generally

lower than in spring, fluctuating in a series of peaks, with tendency to stabilize in late autumn. This seasonal pattern of body condition is consistent with a primary release of offspring in late spring, followed by multiple spawning events over summer until mid-autumn.

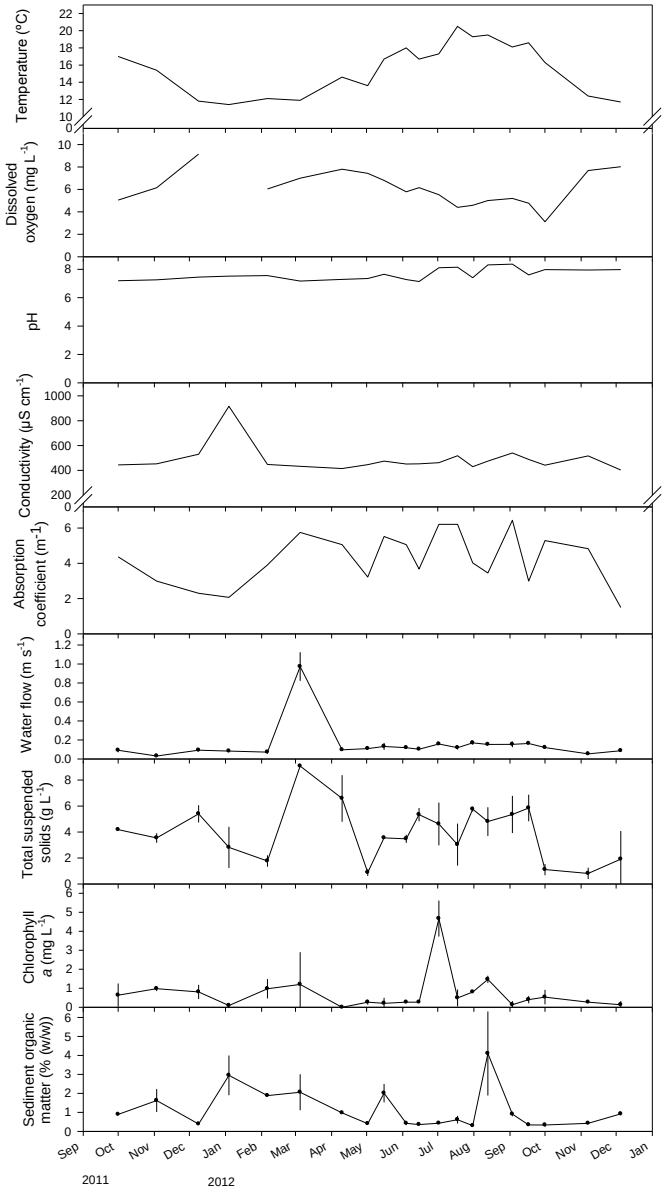

Fig. 3.2. Variation of the water quality parameters and organic matter content in the sediment of the study site during the sampling period. The absorption coefficient was used as an indication of water turbidity (see Material and methods section for details). For parameters where replicated determinations were made, the points represent mean values (of two replicates for absorption coefficient total suspended solids, chlorophyll a and sediment organic matter; of three replicates for water flow) and vertical lines represent standard deviation.

Sampling date	a x 10^2	b	r^2	Number of distinct regressions
01/Oct 2011	1.11	2.66	0.943	5
03/Nov 2011	1.62	2.51	0.917	-
09/Dec 2011	1.53	2.52	0.814	-
04/Jan 2012	1.65	2.49	0.888	-
06/Feb 2012	3.54	2.27	0.821	7
05/Mar 2012	1.17	2.62	0.827	-
10/Apr 2012	7.73	2.04	0.856	10
02/May 2012	21.17	1.65	0.840	6
16/May 2012	14.22	1.79	0.865	7
04/Jun 2012	3.88	2.18	0.925	2
15/Jun 2012	2.57	2.28	0.918	6
02/Jul 2012	3.30	2.23	0.914	3
18/ Jul 2012	4.24	2.16	0.907	3
31/ Jul 2012	1.32	2.49	0.906	9
13/Aug 2012	1.59	2.53	0.931	4
03/Sep 2012	2.18	2.33	0.903	7
17/Sep 2012	3.14	2.19	0.918	8
01/Oct 2012	3.24	2.26	0.851	4
07/Nov 2012	4.20	2.11	0.908	5
05/Dec 2012	7.28	1.94	0.801	4

Table 3.1. Estimated parameters of the allometric model DTW = a . L^b (DTW in mg and L in mm) and respective coefficients of determination (r^2). The number of allometric equations statistically differing from that found for each sampling date (ANCOVA followed by the Tukey *post-hoc* test; $p < 0.05$) is also presented; allometric models based on Model II regression approach were excluded from this analysis (see Material and methods section for details), being denoted by -.

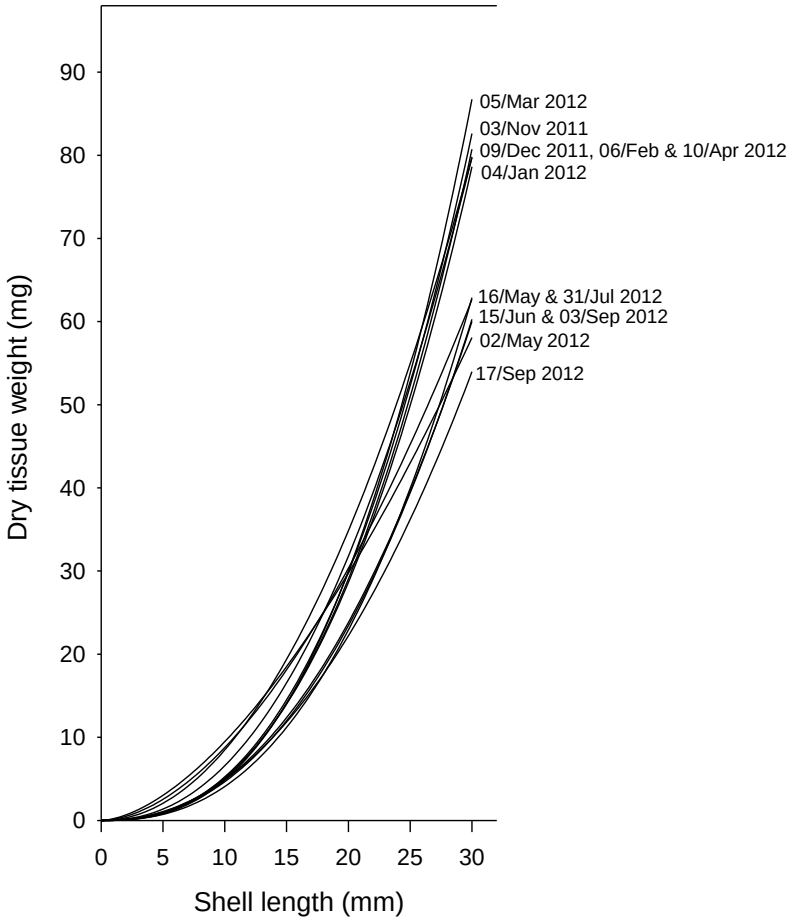

Fig. 3.3. Allometric models characterizing *C. fluminea* body condition on selected occasions between October 2011 and December 2012. The curves were drawn using the parameters presented in Table 3.1. For clarity purposes, only models statistically differing from at least six other equations (ANCOVA followed by the *post-hoc* Tukey test; $p < 0.05$) and models found through Model II regression were represented in the figure.

The difference, in absolute terms, of the body condition of clams collected in equivalent periods of 2011 and 2012 (October to December) is worth noticing, with the animals collected in 2012 having significantly lower dry tissue weight (but following a similar trend in both periods). Such a difference can be related to different environmental conditions in the two periods; the lower water temperature in October to December 2012 as compared to the equivalent period in 2011 (Fig. 3.2) should have played its role in this context.

Morphological examination showed that the presence of juveniles in clams' inner demibranchs scattered across the study period as shown by the shadowed areas in Fig. 3.4, signaling a more continuous-like breeding behavior for the population, consistent with multiple spawning as mentioned above. Progeny under incubation was found in parents' gills even in November and December 2012.

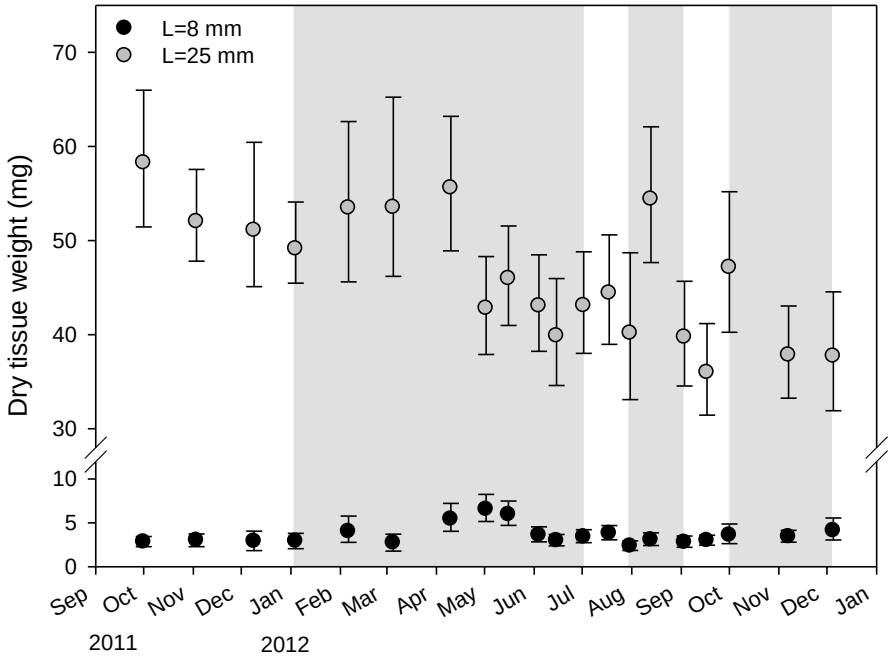

Fig. 3.4. Seasonal variation of dry tissue weight of two standard clams with 8 and 25 mm shell length during the sampling period. The underlying allometric models are presented in Table 3.1 and Fig. 3.3. Error bars represent 95 % confidence intervals. Shadowed areas represent the periods where morphological examination showed incubating progeny in the parents' gills.

Fig. 3.5 shows the clam population density and mean shell length in the study site over the sampling period. Overall, clam density was (mean ± SD) 3247 ± 1122 clams m^{-2}, with a maximum peak of over 6000 clams m^{-2} recorded in August 2012 and minimum densities of less than 1500 clams m^{-2} reached in April, May and December 2012 (Fig.3. 5). The highest values of mean shell length were recorded in April and May 2012 (mean ± SD: 17.59 ± 3.80 mm and 17.40 ± 3.57 mm, respectively), while on average the clams were smallest in November 2012 with a mean shell length of (mean ± SD) 14.00 ± 2.74 mm (Fig. 3.5). In June and in the beginning of August, clam's mean shell length was also low as compared to the rest of the year (mean ± SD of 14.44 ± 3.60 mm and 14.12 ± 3.34 mm, respectively) (Fig. 3.5).

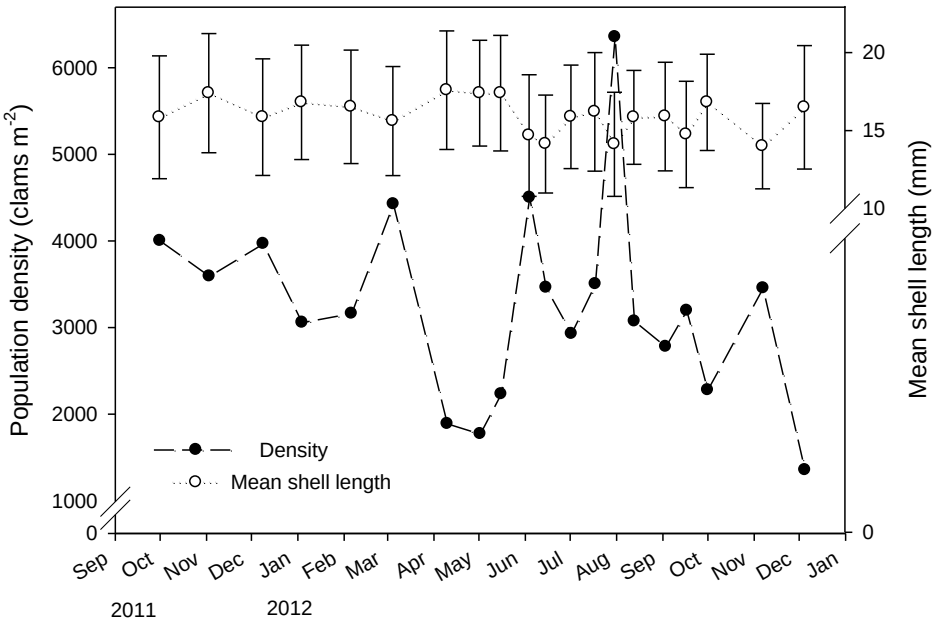

Fig. 3.5. Seasonal variation of the clam population density (left yy' axis) and mean shell length (right yy' axis) during the sampling period. Error bars represent standard deviation. Population density was represented as a single value after pooling of the three replicated samples taken at each sampling point.

Fig. 3.6, where size frequency distributions are presented, further documents the dynamics of the population structure over the study period. Young adults with shell length between 11.5 and 17.5 mm tended to be the most abundant size class, dominating the population size structure. The abundance of juveniles (shell length up to 10 mm; Cataldo and Boltovskoy 1998, Ituarte 1985, Mouthon 2001) in the population was always low compared to that of adults. Juveniles noticeably increased over June 2012, decreasing in the beginning of the following month. Then, it peaked again in late

July 2012, and juveniles kept being found until mid-September 2012. A final increase of juvenile abundance was observed in November 2012. Such a variation of the population size structure is consistent with multiple spawning events occurring from late spring through summer until mid-autumn. The recruitment periods identified in summer and autumn (Fig. 3.6) consistently occurred 1-2 months after decreases in the clams' body condition indicative of spawning (Figs. 3.3 and 3.4). An earlier, less significant recruitment event seemed to happen in March 2012 (Fig. 3.6), 5 months after the previous spawn in October 2011 (Figs. 3.3 and 3.4), with the offspring released in autumn having lower growth rate over winter as compared to the individuals released in the warm months.

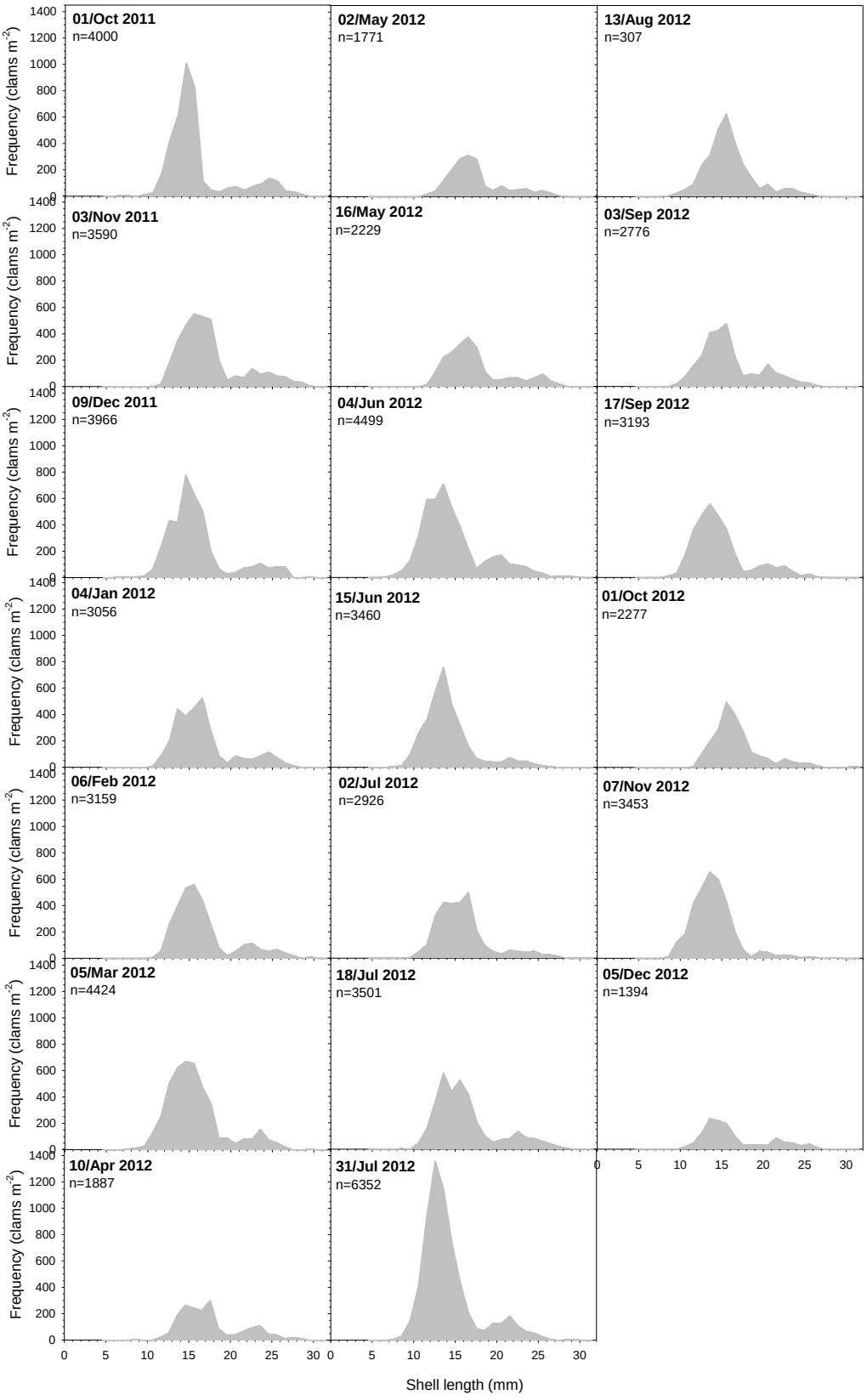

Fig. 3.6. Size frequency distribution of the clam population during the sampling period (October 2011 to December 2012). n represents the total clam density (clams m^{-2}).

3.5. Discussion

Understanding the process through which the Asian clam produces a mucilaginous drogue line that promotes its flotation and drifting into new areas may contribute to more effective pest management. As this process was investigated in two *Corbicula fluminea* populations geographically separated (one in Sommerset, NJ, USA and the other in Portugal), at different times of the year, unexpected differences were observed – mucous threads and floating responses, reported for the American clam population (Rosa et al. 2012) were completely absent amongst the specimens collected from the Portuguese population. It was hypothesized that such differences could be due to genetic differences between the two populations and/or seasonal effects, and these two hypotheses were hence investigated in this study.

The taxonomy of the *Corbicula* genus is generally confuses and yet unsolved because of the recognized high phenotypic variation in shell morphology, color and ornamentation, contrasting reproductive strategies and ecological differences (Glaubrecht et al. 2003, Park and Kim 2003, Pigneur et al. 2011, Sousa et al. 2007). It was shown here that there is no clear genetic distinction between the American and the Portuguese *Corbicula* populations, both exhibiting the same haplotype – haplotype I as described by Renard et al. (2000) in Europe, which corresponds to *C.* sp. (Form A) described by Lee et al. (2005) in the USA and *C. leana* described by Park & Kim in Asia (2003). It should be noticed that androgenesis is a common feature of the *Corbicula* genus (Byrne et al. 2000, Ishibashi et al. 2003, Komaru and Kawagishi 1998), which compromises analysis relying only on mitochondrial sequences as conducted in this study i.e. distinct nuclear lineages can be grouped in the same mitochondrial cluster (Hedtke et al. 2008, Park and Kim 2003, Pigneur et al. 2011). Further genetic studies, combining nuclear and mitochondrial data along with detailed morphological examination, should thus be carried out for a definite genetic comparison of the two populations of interest.

Some authors (Byrne et al. 2000, Morton 1977, Williams and McMahon 1987) suggested that the mucous produced in *C. fluminea* demibranchs play an important role in reproduction, namely by assisting the nourishment of the developing embryos and the release of the progeny out of the parents' gills. The mucilaginous drogue line production and associated drifting behavior could thus be expected to vary seasonally concomitantly with the species reproductive cycle.

In this study, data on the animals' body condition seasonal pattern, gills' morphological examination and population size structure dynamics were integrated to locate the reproductive period(s) of the Portuguese clam population. These data

consistently indicated that breeding occurred from late spring until mid-autumn, with multiple spawns in a more continuous-like reproductive pattern over this period. Major recruitment events in June, August-September and November were identified based on the population dynamics analysis. Reproduction of *C. fluminea* is known to vary greatly with location (Table 3.2), which suggests a strong influence of environmental and genetic factors in life-cycle related events. Although one recruitment episode (from early summer to early autumn) or two episodes (one in spring/early summer and one in late summer/early autumn) are most commonly reported for Asian clam populations, the pattern observed in this study is in agreement with many other works that also show recruitment occurring in mid/late Autumn (Table 3.2).

Despite the reproductive patterns observed in the clam population, no mucocyte cells could generally be found in the tested animals' gills (in the cases where mucocytes were observed, the cells were in an incipient state) over the 14-month study. This result does not definitely contradicts the link between mucous production and reproduction-related events – in populations where mucous is actually produced, mature parents' developing embryos and progeny release from the parents' gills may benefit from it as defended by some authors (Byrne et al. 2000, Morton 1977, Williams and McMahon 1987). However, the reported data prove that mucous production is not a necessary condition for successful clam reprodution to occur. Previous studies on mucocytes showed that the mucous threads are produced by juvenile and small adult clams (up to 14 mm shell length), and, together with other behavioral mechanisms, assist the drift of the clams through flotation in response to water currents (Prezant and Chalermwat 1984, Rosa et al. 2012). The absence of mucocytes cells in the inner demibranchs of the tested clams is thus consistent with the absence of drifting, both in the field and in the laboratory, throughout the sampling period.

As the American and the Portuguese clam populations were proven to be genetically similar and season-driven effects were ruled out, the question why the two populations have different mucous production and drifting capabilities remains unanswered. It can be speculated that, while corresponding to the same species and haplotype, the two populations show distinct physiological features due to a high rate of genotypic and/or phenotypic plasticity. It is possible that the colonization of different habitats triggered microevolutive events towards local adaptation (Ashley et al. 2003, Sousa et al. 2007), which may explain the inhibition of the drogue line production and drifting behavior in the Portuguese clam population. Further studies are necessary to assess this hypothesis.

Table 3.2. Summary of shell length, clam density and recruitment events for several *C. fluminea* populations. The study site location, sampling date and water temperature range are also shown. This summary result from a thorough literature search and only studies reporting data on at least 4 of the columns were considered.

Study	Study site location	Sampling date	Water temperature range (ºC)	Shell length range (mm)	Clam density range (clams m^{-2})	Recruitment events	
Aldridge and McMahon 1978	Lake Arlington, USA	Sep 1974 – Jan 1976	12.2-32.2	0.2-40	17.7-94.6	2	Apr-Jul & Aug-Dec
Ituarte 1985	Punta Atalaya, Argentina	Nov 1982-Apr 1985	~11-27	~3 - ~30	36-1132	1	September
McMahon and Williams 1986	Trinity River, USA	Sep 1980 – Dec 1982	4.8-29	2.6- ~45	305-16198	2	Mar-Jul & Aug-Nov
Hornbach 1992	Mechums River, USA	Oct 1982-Oct 1983	0- ~26	0.4-19	173-1495	1	July
French and Schloesser 1996	St. Clair River, USA	1988-1990	0.5-12.5	1.8– 5.3	18-187	-	-
Cataldo and Boltovskoy 1998	Paraná River Delta, Argentina	Oct 1995-Oct 1996	10-29	0.2-33	379-2609	1	Oct-Nov
Rajagopal et al. 2000	Lek River, Netherlands	Aug 1991-Jan 1993	~2-~24	-	-	2	May/Jun & Sep
Mouthon 2001	Saone River, France	Sep 1986-Dec 1999	0.5-27.2	1.5- ~29	~120-934	2	Jun/Jul & Aug/Sep
Mouthon 2001	Rhône River, France	Sep 1996-Dec 1999	3-23.5	~0.5 - ~29.3	~300-5266	1	Jul-Sep
Morgan et al. 2003	Connecticut River, USA	Aug 1993-Nov 1994	-1.7-30.6	-	52-114	1	Jun-Sep
Mouthon and Parghentanian 2004	Loire River, France	Dec 2001-May 2003	[Ice]-25	0.5- ~34	88- <4000	2	Jan-Feb & May-Oct
Sousa et al. 2006	Lima River, Portugal	Aug 2004 & Aug 2005	20.1-22.9	13.0-51.6	60	-	-
Schmidlin and Baur 2007	Alrhine River, Switzerland	Mar 2003-Oct 2003	7-24	1-24	83-339	1	Jun-Jul
Sousa et al. 2008	Minho River, Portugal	Jan 2005- Aug 2006	6.7-23.1	1.85-41.83	92-2152	-	-
Franco et al. 2012	Mondego Estuary, Portugal	Dec 2007-Dec 2008	10.1-25.4	0.92-37	419	Continuous	-
Present study	Casal de São Tomé, Portugal	Oct 2011-Dec2012	11.8-20.5	6-32	1353-6352	2	Jun-Sep & Nov

Overall, this study confirms that mucocytes development is directly related to Asian clam drifting behavior and indicates that microevolutionary events may condition these processes. It thus contributes to the understanding of a physiological trait of the species that is meaningful to the species dispersal efficiency and hence relevant from the pest management point of view.

3.6. Aknowledgements

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3.7. References

Aldridge DW, McMahon RF. 1978. Growth, fecundity, and bioenergetics in a natural population of asiatic freshwater clam, *Corbicula manilensis* Philippi, from north central Texas. Journal of Molluscan Studies 44: 49-70.

APHA APHA. 1995. Standard methods for the examination of water and wastewater. 19th ed.; 1995. Washington.

Araujo R, Moreno D, Ramos MA. 1993. The Asiatic clam *Corbicula fluminea* (Müller, 1774) (*Bivalvia: Corbiculidae*) in Europe. American Malacological Bulletin 10: 39-49.

Ashley MV, Willson MF, Pergams ORW, O'Dowd DJ, Gende SM, Brown JS. 2003. Evolutionarily enlightened management. Biological Conservation 111: 115-123.

ASTM. 2000. Standard test methods for moisture, ash, and organic matter of peat and other organic soils.

Barinova SS, Yehuda G, Nevo E. 2010. Comparative analysis of algal communities in the rivers of northern and southern Israel as bearing on ecological consequences of climate change. Journal of Arid Environments 74: 765-776.

Britton JC, Morton B. 1982. A dissection guide, field and laboratory manual for the introduced bivalve *Corbicula fluminea*. Malacological Review Supplement 3: 1-82.

Brower JE, Zar JH, von Endem CN, eds. 1997. Field and laboratory methods for general ecology. 4 ed. Boston, USA: WCB McGraw-Hill.

Byrne M, Phelps H, Church T, Adair V, Selvakumaraswamy P, Potts J. 2000. Reproduction and development of the freshwater clam *Corbicula australis* in Southeast Australia. Hydrobiologia 418: 185-197.

Cataldo D, Boltovskoy D. 1998. Population dynamics of *Corbicula fluminea* (Bivalvia) in the Paraná River Delta (Argentina). Hydrobiologia 380: 153-163.

Cohen AN, Carlton JT. 1998. Accelerating invasion rate in a highly invaded estuary. Science 279: 555-558.

DAISIE – European Invasive Alien Species Gateway. www.europe-aliens.org. Acessed 2013.

Davis MA. 2010. Invasion Biology. Oxford: Oxford University Press.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. 1994. DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. Molecular marine biology and biotechnology 3: 294-299.

Franco JN, Ceia FR, Patricio J, Modesto V, Thompson J, Marques JC, Neto JM. 2012. Population dynamics of *Corbicula fluminea* (Müller, 1774) in mesohaline and oligohaline habitats: Invasion success in a Southern Europe estuary. Estuarine Coastal and Shelf Science 112: 31-39.

French JRP, Schloesser DW. 1996. Distribution and winter survival health of Asian clams, *Corbicula fluminea*, in the St Clair River, Michigan. Journal of Freshwater Ecology 11: 183-192.

Glaubrecht M, von Rintelen T, Korniushin AV. 2003. Toward asystematic revision of brooding freshwater corbiculidae in Southeast Asia (*Bivalvia, Veneroida*): On shell morphology, anatomy and molecular phylogenetics of endemic taxa from islands in Indonesia. Malacologia 45: 1-40.

Hedtke SM, Stanger-Hall K, Baker RJ, Hillis DM. 2008. All-male asexuality: Origin and maintenance of androgenesis in the Asian clam *Corbicula*. Evolution 62: 1119-1136.

Hornbach DJ. 1992. Life history traits of a riverine population of the Asian clam *Corbicula fluminea*. American Midland Naturalist 127: 248-257.

Ishibashi R, Ookubo K, Aoki M, Utaki M, Komaru A, Kawamura K. 2003. Androgenetic reproduction in a freshwater diploid clam *Corbicula fluminea* (*Bivalvia:Corbiculidae*). Zoological Science 20: 727-732.

Ituarte CF. 1985. Growth dynamics in a natural population of *Corbicula fluminea* (Bivalvia Sphaeriacea) at Punta Atalaya, Rio de La Plata, Argentina. Studies on Neotropical Fauna and Environment 20: 217-225.

Ituarte CF. 1994. *Corbicula* and *Neocorbicula* (*Bivalvia: Corbiculidae*) in the Paraná, Uruguay, and Rio de la Plata Basins. The Nautilus 107: 129-135.

Komaru A, Kawagishi T. 1998. Cytological evidence of spontaneous androgenesis in the freshwater clam *Corbicula leana* Prime. Development Genes and Evolution 208: 46-50.

Kraemer LR, Galloway ML. 1986. Larval development of *Corbicula fluminea* (Müller) (*Bivalvia, Corbiculacea*) - An appraisal of its heterochrony. American Malacological Bulletin 4: 61-79.

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

Legendre P, Legendre L. 1998. Numerical Ecology. Amsterdam: Elsevier.

Lorenzen CJ. 1967. Determination of chlorophyll *a* and phae-pigments: Spectrophotometric equations. Limnology and Oceanography 12: 343-346.

McMahon RF, Williams CJ. 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*). Proceedings of the Second International Corbicula Symposium: American Malacological Bulletin - Special edition no.2.

Morgan DE, Keser M, Swenarton JT, Foertch JF. 2003. Population dynamics of the Asiatic clam, *Corbicula fluminea* (Müller) in the Lower Connecticut River: Establishing a foothold in New England. Journal of Shellfish Research 22: 193-203.

Morton B. 1977. Freshwater fouling bivalves. Pages 1-14. First International *Corbicula* Symposium. Fort Worth, Texas, USA: Texas Christian University.

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

Mouthon J. 2001. 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. 2001. 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, 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 Fur Hydrobiologie 161: 267-287.

Park JK, Kim W. 2003. Two *Corbicula* (*Corbiculidae : Bivalvia*) mitochondrial lineages are widely distributed in Asian freshwater environment. Molecular Phylogenetics and Evolution 29: 529-539.

Phelps HL. 1994. The asiatic clam (*Corbicula fluminea*) invasion and system-level ecological change in the Potomac River estuary near Washington, DC. Estuaries 17: 614-621.

Pigneur L-M, Marescaux J, Roland K, Etoundi E, Descy J-P, Van Doninck K. 2011. Phylogeny and androgenesis in the invasive *Corbicula* clams (*Bivalvia, Corbiculidae*) in Western Europe. Bmc Evolutionary Biology 11.

Pimentel D, Zuniga R, Morrison D. 2005. Update on the environmental and economic costs associated with alien-invasive species in the United States. Ecological Economics 52: 273-288.

Prezant RS, Chalermwat K. 1984. Flotation of the bivalve *Corbicula fluminea* as a means of dispersal. Science 225: 1491-1493.

Quinn GP, Keough MJ, eds. 2002. Experimental design and data analysis for biologists New York: Cambridge University Press.

Rajagopal S, van der Velde G, de Vaate AB. 2000. Reproductive biology of the Asiatic clams *Corbicula fluminalis* and *Corbicula fluminea* in the river Rhine. Archiv Fur Hydrobiologie 149: 403-420.

Renard E, Bachmann V, Cariou ML, Moreteau JC. 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.

Ricciardi A, MacIsaac HJ. 2000. Recent mass invasion of the North American Great Lakes by Ponto-Caspian species. Trends in Ecology & Evolution 15: 62-65.

Rosa IC, Pereira JL, Gomes J, Saraiva PM, Goncalves 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.

Rosa IC, Pereira JL, Costa R, Gonçalves F, Prezant RS. 2012. Effects of upper-limit water temperatures on the dispersal of the Asian Clam *Corbicula fluminea*. PLoS ONE 7: e46635.

Schmidlin S, Baur B. 2007. Distribution and substrate preference of the invasive clam *Corbicula fluminea* in the river Rhine in the region of Basel (Switzerland, Germany, France). Aquatic Sciences 69: 153-161.

Sousa R, Antunes C, Guilhermino L. 2006. 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, Freire R, Rufino M, Mendez J, Gaspar M, Antunes C, Guilhermino L. 2007. Genetic and shell morphological variability of the invasive bivalve *Corbicula fluminea* (Müller, 1774) in two Portuguese estuaries. Estuarine, Coastal and Shelf Science 74: 166-174.

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

Williams CJ, McMahon RF. 1987. Annual variation of tissue biomass and carbon nitrogen content in the freshwater bivalve *Corbicula fluminea* relative to downstream dispersal. Canadian Journal of Zoology 67: 82-90.

CHAPTER 4

The Asian clam *Corbicula fluminea* in the European freshwater-dependent industry: A latent threat or a friendly enemy?

The Asian clam *Corbicula fluminea* in the European freshwater-dependent industry: a latent threat or a friendly enemy?

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4.1. Abstract

While the biofouler *Corbicula fluminea* (Müller, 1774) is known to cause great economic losses in North America, studies reporting the problem in Europe are much scarcer. This paper explores the industrial effects of the species in Portugal, a gateway by which the bivalve entered Europe around 30 years ago. National waterworks, major power stations, cement plants, pulp and paper mills and irrigation systems were surveyed. The industrial impacts of the pest were shown to remain relatively mild; irrigation systems are those that seem to be facing more significant economic losses due to infestation. Possible reasons for the apparent discrepancy between this result and the species dispersal in natural ecosystems are discussed, and recommendations on adequate responses to the latent threat are provided. This study may assist the implementation of integrated pest management policies in countries at risk of invasion or recently invaded, and contribute to an understanding of the species' progression in industrial environments.

Keywords: Biofouling; *Corbicula fluminea*; Distribution; Freshwater-dependent industry; impacts; Invasive species

4.2. Introduction

Since the groundbreaking work of Elton (1958) on invasive species, the interest of the scientific community and governmental agencies in the topic has been steadily augmenting. Amongst such pests is the freshwater infaunal bivalve *Corbicula fluminea* (Müller, 1774), commonly known as the Asian clam. This species, native to Southeast Asia, underwent massive global range expansion over the last century (Araujo et al. 1993). One of the first records of *C. fluminea* in North America dates back to 1924 (Beran 2006), while in Europe it was initially reported by Mouthon in France (Basse Dordogne) and Portugal (Tejo River estuary) in the early 1980s (Mouthon 1981). At present, the species colonises vast regions of Europe, North and South America (Beasley et al. 2003), and it is still a spreading pest, being classified as one of the 100 worst biological invaders (DAISIE 2008).

Invasive bivalves are a serious ecological and economic problem due to their effects on natural ecosystems and damaging impacts on man-made structures (Claudi and Mackie 1994, Minchin et al. 2002, Pimentel et al. 2005, Vitousek et al. 1996). The latter are mainly related to their biofouling activity – by growing and establishing dense populations on underwater structures and equipments, the molluscs impair and degrade them. Industrial facilities whose operation depends on the intensive use of water drawn from natural waterbodies, such as drinking water treatment plants and power stations, are especially vulnerable to the pests' biofouling activity (e.g. Johnson et al. 1986, LePage 1993, McMahon 1977, Sinclair 1964). Some of the problems experienced by these facilities as a result of bivalves' infestations include: pipe and equipment blockage, reduced efficiency of water cooling systems, increased corrosion, safety hazards when systems such as fire protection units are affected, and plant operation disturbance associated with the need for biofouling removal. Damage caused by invasive bivalves and their control costs several billion dollars each year in the US alone (Pimentel et al. 2005).

Understanding the way infestations are established and progress in the facility, how the bivalves enter the plant, how resident populations develop, which systems are likely to be affected, how are they going to be affected and at which stage of the infestation, constitutes crucial information when it comes to design and prioritise effective programmes to monitor and control industrial biofouling. In this context, documented infestation incidents and related practices are a very useful tool, especially to those that face invasive bivalves for the first time.

As far as *C. fluminea* is concerned, the literature reporting industrial infestations is somewhat limited. Several studies describing the impacts of the species on North

American plants, including power stations, drinking water treatment plants, sand and gravel companies and irrigation canals do exist (e.g. Ingram 1959, Johnson et al. 1986, McMahon 1977, Prokopovich and Herbert 1965, Sinclair 1964). However, a large number of these studies are not of easy access, in many cases corresponding to private internal reports (e.g. Cherry et al. 1980). Furthermore, equivalent studies addressing the problem in the European scenario are much scarcer (Jenner et al. 1998). Differences between the North American Asian clam populations and those that more recently colonised and spread across Europe may be expected in aspects such as growth rates, physiological tolerance, population densities and annual reproductive cycles (Mackie and Schloesser 1996). These features of the species biology and ecology determine to a large extent the pattern of industrial infestations, and are thus critical for the implementation of effective monitoring and control measures. Therefore, although European pest managers can obviously take advantage of the documented experience of their American counterparts, reports on *C. fluminea* infestation episodes and mitigation practices in European freshwater-drawing industries should constitute a more concrete working basis for them.

One further issue that sometimes may confound those searching the literature for information on problems caused by the Asian clam is related to a generalisation, somewhat abusive, of the industrial impacts of the zebra mussel *Dreissena polymorpha.* This species is a particularly severe freshwater biofouler bivalve due to its exceptional dispersal capacity and epifaunal mode of life, and thus the literature addressing this pest is considerably vaster (Claudi and Mackie 1994, Ludyanskiy et al. 1993). Because the Asian clam also causes significant economic damage, there is sometimes a tendency to exaggerate many aspects of its biofouling activity based on an analogy with those of the zebra mussel to draw attention to the problem. Some of such generalisations are then repeated and assumed proven without practical evidence. Yet the two troublesome bivalves are different in key features of their biology and ecology (Karatayev et al. 2005), and hence the mode and the extent to which they affect industrial facilities are not exactly the same.

From the above discussion, it becomes apparent that comprehensive reports of *C. fluminea* industrial infestations should be encouraged. They are likely to be of great use for pest managers, especially if they refer to experiences in Europe, where the species arrived more recently and possibly the invasive populations stand at a different stage compared to those in most North American habitats (Allendorf and Lundquist 2003, Sakai et al. 2001, Sousa et al. 2006).

The main aim of this paper is thus to provide a detailed, not alarmist, though realistic picture of the current effects of *C. fluminea* on the Portuguese freshwater-dependent industry. The drinking water treatment, thermal power, cement, pulp and paper, and agricultural (irrigation) sectors were considered in this study. The data presented here may be useful for European industrial pest managers dealing with specific infestation risks and episodes. Moreover, the analysis provided in this paper is also relevant at a broader scale. Portugal seems to have been a gateway by which the Asian clam entered Europe around 30 years ago (Mouthon 1981). In spite of the persistent advice of the scientific community on the importance of dealing with the invasion in its early stages (Araujo et al. 1993, Pérez-Quintero 2008, Sousa et al. 2006, Sousa et al. 2008a), nor the responsible governmental agencies, at the national level, nor the industrial facilities, at their own scale, have done much over this period to minimise the spread of this pest and its impacts. In this context, such a holistic, country-scale overview may assist the official entities in countries at risk of invasion or where the species has arrived not long ago, such as Serbia (Paunović 2007), the United Kingdom (Howlett and Baker 1999) and the Czech Republic (Beran 2006), with the implementation of integrated pest management policies before the biological invasion reaches advanced stages.

As a complement to the Asian clam industrial biofouling data, updated information on the species distribution in Portuguese waters is also provided in section 4.3. Such information is critically important for the national freshwater-drawing industrial plants to judge their risk of being infested. It can also be interpreted as the unrestricted spread of the pest at a country-scale within a 30-year window.

4.3. The distribution of *Corbicula fluminea* in Portugal

C. fluminea has been present in Portugal at least since the early 1980s, when it was found in the Tejo River estuary (Mouthon 1981). Since then, the number of studies providing distribution data for the pest in the country, and in the Iberian Peninsula in general, has been increasing significantly (Pérez-Quintero 2008). However, many of them are fragmentary in the sense that they analyse the presence of the species only in specific rivers. For example, Sousa and co-workers documented the Asian clam distribution in the Lima (Sousa et al. 2006) and Minho (Sousa et al. 2008b) Rivers, while Morais et al. (2009) addressed the colonisation of the Guadiana River. In 2006, Reis (2006) provided the first integrated description of the species distribution in the

country. Two years later, Pérez-Quintero (2008) also presented an overview, slightly less exhaustive than that of Reis, of the Asian clam distribution in Portugal.

Fig. 4.1 presents the chronological and current nationwide *C. fluminea* distribution, based on data resulting from a comprehensive compilation of previously published literature (Araujo et al. 1993, Chainho et al. 2006, Morais et al. 2009, Mouthon 1981, Pérez-Quintero 2007, 2008, Reis 2006, Sousa et al. 2006, 2007) as well as updated records obtained throughout this study. Table 4.1 summarises information on the first records of the species for the major Portuguese watersheds. After the first appearance of *C. fluminea* in the country (in Tejo River), northern estuaries of Douro and Minho Rivers were also invaded within a ten-year time interval. From 2000 onwards, the Asian clam was noticed in other estuaries and inland waterways. The spread of the species in the Portuguese territory seems to have accelerated significantly over the past decade. Nowadays the pest is present in at least 11 out of the 15 major watersheds in Portugal, having not been reported yet in Ave, Cávado, Leça and Lis River basins (Table 4.1 and Fig. 4.1).

Table 4.1. First records of *C fluminea* in the Portuguese watersheds already invaded.

Watershed	First record of *C. fluminea*		References
	Year	Location	
Tejo	1980	Tejo River (estuary)	Mouthon 1981
Douro	1988	Douro River (estuary)	Nagel 1989
Minho	1989	Minho River (estuary)	Araujo et al. 1993
Guadiana	2000	Guadiana River (downstream Alqueva dam)	Perez-Quintero 2007
Mondego	2000	Mondego River (estuary)	Chainho et al. 2006
Lima	2002	Lima River (estuary)	Sousa et al. 2006
Vouga	Not known	Vouga River/Corujeira	Reis 2006
Ribeiras do Oeste	Not known	Lizandro River	Reis 2006
Sado	Not known	Sado River	Morais et al. 2009
Mira	2009	Santa Clara dam	Present study
Ribeiras do Algarve	2009	Bravura dam	Present study

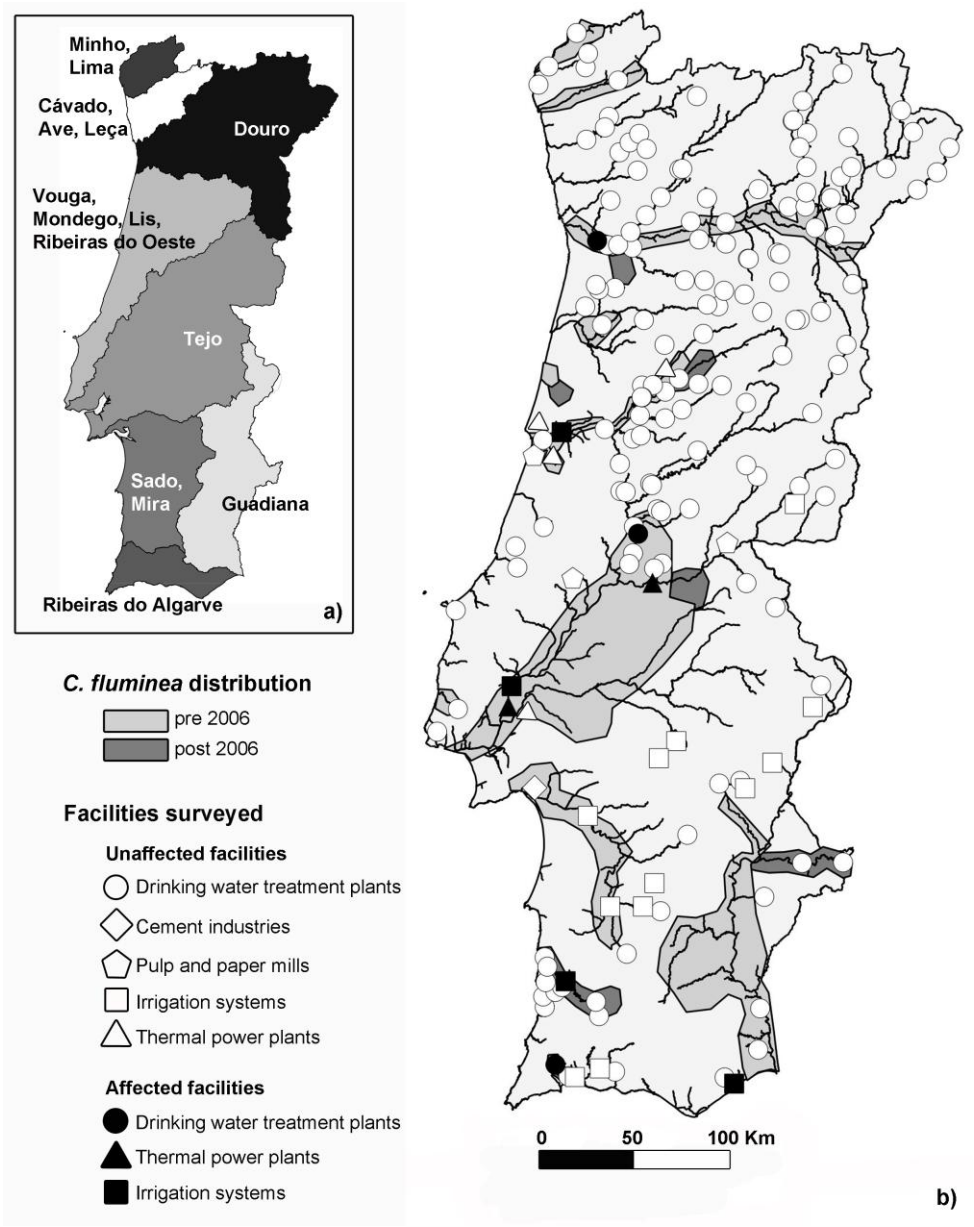

Fig. 4.1. Distribution and industrial impacts of *C. fluminea* in Portugal: areas where the species has been reported and location of the freshwater-dependent industries surveyed for problems related to the pest. In the top-left corner is the map of the administrative hydrographic regions. The symbols represent the surveyed freshwater-dependent industries that both reported Asian clam infestations and have not been affected by the pest yet. In all cases, except the power industry sector, all the national facilities especially prone to infestation (i.e. drawing water from surface waterbodies) have been surveyed. See text for details.

4.4. Methods

The drinking water treatment, thermal power, cement, pulp and paper, and agricultural (irrigation) sectors were considered in the present study. These sectors, which rely on the intensive use of water, were selected due to their relevance to the Portuguese economy.

General information on each sector was first collected mostly from governmental agencies and industrial associations. After listing the major 454 national facilities of the selected sectors (Table 4.2), these were contacted via email and/or telephone in order to identify the respective water source (freshwater vs sea water; surface water, groundwater vs municipal water) – only the facilities drawing from surface waterbodies were considered to be prone to infestation by the Asian clam (Table 4.2). These facilities were then enquired about the occurrence of current or past infestation episodes. Facilities where the pest was reported underwent further analysis. Such plants were visited and site managers were interviewed *in loco* to thoroughly document the infestation episodes and responses given to the problem. The site visits and interviews followed a common surveying guide, previously designed to ensure comprehensiveness and standardisation of data collection. Details on the quality of the raw water and treating procedures, system configuration (e.g. pumps, filter meshes, pipe diameters), type of structures affected by the pest, location of and general conditions (e.g. water flow, water quality, cleaning routines) in such structures and mitigation strategies in place were discussed in depth. Questions concerning the quantitative estimation of the infestation costs were also included in the surveying guide, and this topic was thoroughly discussed in the interviews.

The exact location of all the facilities surveyed was recorded and included in the georeferenced hydrographical map of the Portuguese territory, where information on the species distribution had been previously incorporated (Fig. 4.1). Affected and unaffected industries were distinguished as different layers for spatial analysis.

Table 4.2. Portuguese freshwater-dependent industries surveyed for problems related to *C. fluminea* infestations.

	Drinking water treatment plants	Thermal power plants	Cement plants	Pulp and paper mills	Irrigation systems
Number of major water-drawing facilities in the country	420	6	6	6	16
Number of facilities especially prone to infestation and surveyed	149	6	1	3	16
Number of facilities reporting problems	3	2	0	0	4

4.5. Results

4.5.1. General picture of the effects of C. fluminea *on Portuguese freshwater-dependent industry*

Only 175 of the 454 Portuguese water-drawing facilities considered in this study use surface freshwater, and thus are prone to Asian clam infestations (Table 4.2). The remaining ones use groundwater (as some drinking water treatment plants), municipal water (as happens with many of the cement plants and pulp and paper mills) or sea water (one thermal power plant does so), and were thus excluded from the survey. All the facilities susceptible to infestation were successfully surveyed (Table 4.2).

Overall, only 5 % of the facilities susceptible to infestation reported problems caused by *C. fluminea.* None of the relevant cement plants or pulp and paper mills has been affected yet, whilst irrigation systems and thermal power plants seem to be the structures where the pest causes more concerns, with 25 % and 33% of the surface water-drawing systems reporting the presence of the species, respectively (Table 4.2). Out of the 149 relevant drinking water treatment plants, only three face infestation problems currently (Table 4.2). Another one, which is presently deactivated (located in Northwestern Portugal), also reported problems in the early 1990s. Interestingly, in some facilities, including one irrigation system and four drinking water treatment plants, Asian clams have been seen by operators in the surroundings, but no biofouling problems occurred so far.

4.5.2. Effects of C. fluminea *on drinking water treatment plants*

Fig. 5.2a presents the typical processes implemented by Portuguese waterworks that treat surface water. Raw water is pumped from the source, often a river, through metal grills and screens (mesh of about 1 cm) into reservoirs, where it is temporarily stored. The subsequent treatment steps depend on the raw water quality, which varies greatly across the country. One of the possible treatment configurations involves filtration through rapid gravity filters, followed by disinfection (typically through chlorine injection) that ensures microbiological quality of the final product. Alternatively, the treatment may involve a pre-oxidation stage, preceded by filtration through rapid gravity filters when the raw water turbidity is high. After the pre-oxidation step, flocculation, decantation, filtration and final disinfection follow.

C. fluminea appears to be present in Portuguese drinking water treatment facilities since the early 1990s. It was reported in a plant located in the Douro River basin shortly before its deactivation in 1992. About 1.4 tonnes of clams were removed from the bottom of the storage reservoir in that facility (Fig. 4.2a).

Currently, the species was found to infest three waterworks (Table 4.2), located in Douro River, Tejo River and Ribeiras do Algarve basins (Fig. 4.1), which reported the first occurrence of the species in 2000, 2008 and 2008, respectively. The main structures affected by the pest differ amongst the facilities. In the Douro River basin plant (Fig. 4.1), over 4 tonnes of clams have been removed during scheduled cleaning procedures (which take place every two years). Clogging of the intake screens occurred as well as accumulation in the storage reservoir and in the ozone injection tanks (Fig. 4.2a), but without major damage to the structures. In 2004 a new pre-treatment unit (multilayer sand filter) was installed downstream the raw water reservoir in order to face increased water turbidity. This unit apparently promoted a significant reduction in the amount of clams found downstream. In the Tejo River basin plant (Fig. 4.1), clams have been found on the upper layers of sand filters (Fig. 4.2a). As a result, filter maintenance cycles had to be shortened, and sand has now to be replaced more frequently than before. The last cleaning event involved the discharge of 108 tonnes of sand. In the Ribeiras do Algarve basin plant (Fig. 4.1), clams were only found in a 1.80-meter diameter intake pipeline during a periodic cleaning operation. In the three plants, no operation problems related to *C. fluminea* other than increased complexity and frequency of periodic cleaning and maintenance procedures were mentioned. In particular, alterations in the water odour and taste have not been detected, nor the efficiency of the plant seems to have been significantly affected so far. None of the affected plants implements any specific monitoring programme or control measures to

deal with the pest. Consistently with this picture, at present, the cost associated with Asian clam infestations in Portuguese waterworks, mainly related to system cleaning and maintenance as well as clams disposal (in landfills), are still negligible, corresponding to, on average, less than 0.01% of a plant's operation costs (Table 4.3).

Fig. 4.2. Layout of the typical water systems in Portuguese **(a)** drinking water treatment plants **(b)** thermal power plants, and **(c)** irrigation systems fed by surface waterbodies.

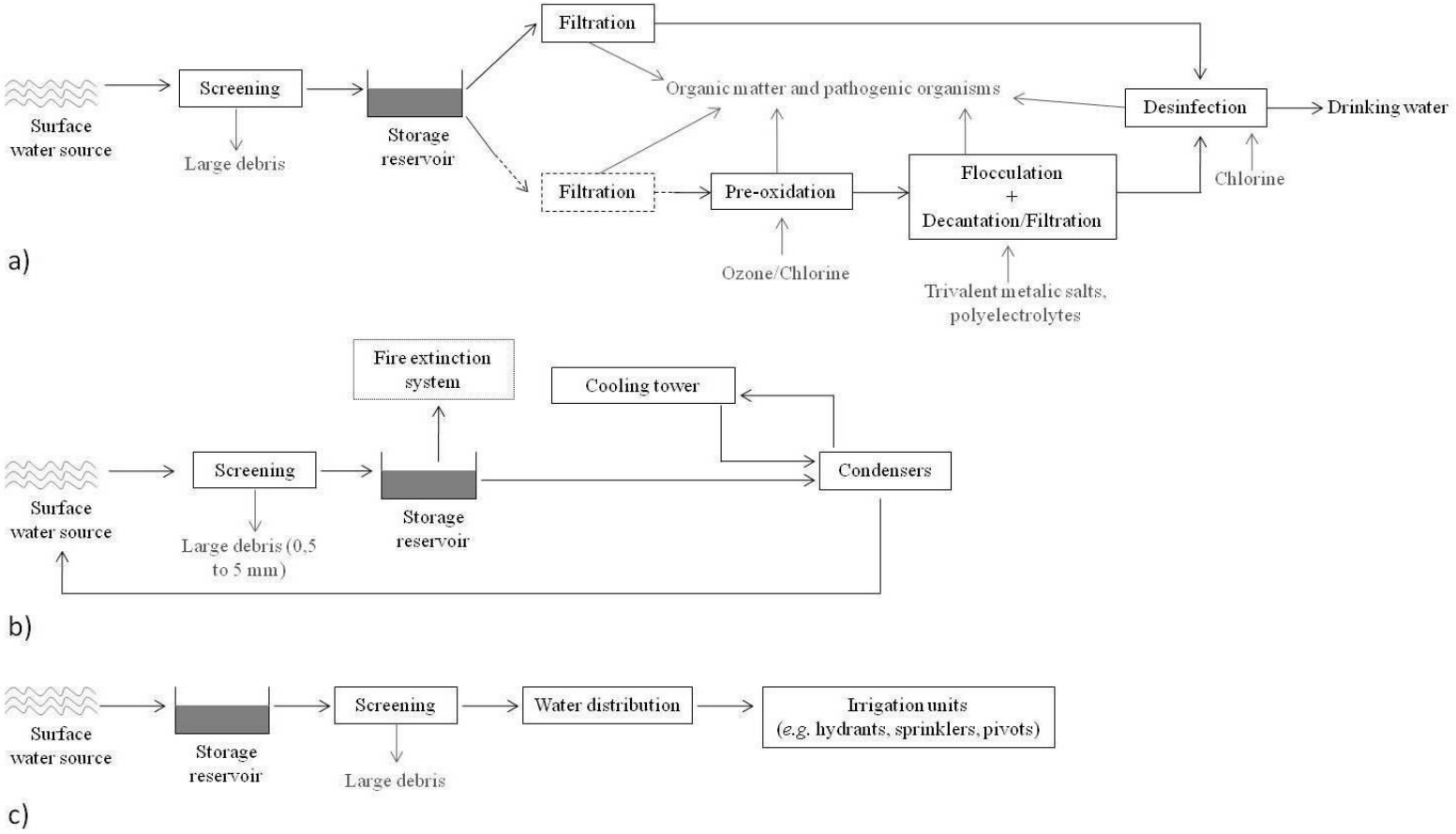

Table 4.3. Summary of the current impacts of *C. fluminea* in the Portuguese freshwater-dependent industry.

	Drinking water treatment plants	Thermal power plants	Cement plants	Pulp and paper mills	Irrigation systems
First industrial infestation reported (date and location)	Early 1990s, Douro River basin	Late 1980s, Tejo River basin	-	-	2005, Mondego River basin
Fraction of the national facilities especially prone to infestation that have already been affected	2 %	33%	0 %	0 %	25 %
Severity of the industrial infestation	Very low	Low	-	-	Moderate
Annual economic losses in the sector due to the pest (€)	1 500	40 000	-	-	150 000

4.5.3. Effects of C. fluminea *on thermal power plants*

In thermal power plants, aimed at electric power generation, massive volumes of water circulate in the cooling systems, required to complete the steam cycle. For economic reasons, the water running in such systems is untreated or barely treated. A simplified layout of typical cooling systems in Portuguese power stations is presented in Fig. 5.2b. Water, drawn from a nearby waterbody, passes through intake screens to a storage reservoir. From here, it goes to the fire extinction system and, most commonly, to the condensers, from which it is then discharged back into the water source. In some cases, a closed refrigeration system exists, with water running between the condensers and cooling towers.

Two of the power stations surveyed, both operating in the Tejo River basin (Fig. 4.1), reported the presence of *C. fluminea* (Table 4.2). One of them experienced an isolated invasion episode in the late 1980s, which was detected during scheduled cleaning procedures (at the time occurring every 4 years). Clams entered the facility and accumulated in a reduced flow rate 1.80-meter width bypass channel. Almost 800 kg of bivalves were washed out and mechanically removed from the channel. Shells were also found in the cooling system pumps as well as in one of the five condensers (Fig. 4.2b), although significant clogging of these structures had not been noticed. Following this infestation episode, metal monitoring boxes were installed in the bypass channel, and the more critical fire protection water system became inspected on a more regular basis.

However, surprisingly, since then only a few clams have been found in the water intake screen and the fire protection system, with no impact on their operation. No further infestations occurred in the plant, even though no proactive control measures were adopted. In the second thermal power plant surveyed, also located in the Tejo River basin, approximately 60 km away from the first one (Fig. 4.1), *C. fluminea* was detected in 1994, during a routine inspection of the condensers (Fig. 4.2b). Since then, clams have been regularly found in the plant during cleaning and maintenance routines as well as other unplanned inspections. Considerable biomass accumulates in the raw water reservoir, while less concerning amounts of clams are typically removed from the cooling tower, condensers and quiescent regions of small diameter (15 to 20 cm) pipes that lead to the cooling tower (Fig. 4.2b). Additionally, some clams are found regularly during the scheduled fire protection system inspections. Despite these occurrences, no significant system efficiency reduction or clogging problems seem to affect the plant. Given the low severity of the infestations faced, none of the power plants implements specific control measures other than normal cleaning routines, in which clams are removed from the plant and transported to a landfill, and more frequent fire protection system inspections. As the first thermal power station surveyed has experienced one single infestation episode, the second is the only one that has current economic losses (related to cleaning routines) due to the Asian clam, which represent around 0.02% of its operation costs (Table 4.3).

4.5.4. Effects of C. fluminea *on irrigation systems*

In Portugal, most of the agricultural land is irrigated with water drawn from surface waterbodies. Fig. 4.2c shows a simplified layout of a typical irrigation system in the country. Water is pumped from the water source into a reservoir. Then it passes through metal grills to remove large debris and gets into the distribution lines to reach the final irrigation structures, which depend on the type of irrigation technique (surface, localised or sprinkler).

Problems related to the presence of *C. fluminea* have been reported in four irrigation systems located in the Mondego, Tejo, Mira and Guadiana Rivers basins (Table 4.2; Fig. 4.1). Clams have been found not only during annual cleaning routines, but also during extraordinary inspections, such as maintenance works following the occurrence of clogging problems.

In the Mondego River basin system (Fig. 4.1), which feeds essentially surface irrigation techniques, Asian clam infestations have been experienced since 2005, when the species was first detected in the main canal during a normal cleaning routine involving its full drainage. Several tonnes of clams were removed from the canal then as well as more recently when the next cleaning routine took place (early 2010). In spite of that, the

species seems to be causing no major problems in this irrigation system other than the need for removing and disposing the accumulated clams. In particular, there has been no need to increase the frequency of the cleaning events. In contrast, the impacts of the pest are more significant in the irrigation systems located in the Tejo, Mira and Guadiana Rivers basins (Fig. 4.1), especially in the sprinkler irrigation structures that they feed, where it has been found since 2008, 2009 and 2007, respectively. One of the main problems related to the presence of *C. fluminea* in these systems is the clogging of pivot pipelines and hydrants (Fig. 4.2c), which reduces the water flow and often ceases irrigation. As a result, much more frequent cleaning of these units is necessary. For instance, in the system fed by the Tejo River (Fig. 4.1), around 20 kg of clams have to be removed daily from each pivot pipeline. Clams accumulation in the pumping chambers and storage reservoirs (Fig. 4.2c), resulting in harder cleaning, were also reported. While increased cleaning requirements in these three irrigation systems represent significant additional costs related to labour, clams disposal and water losses, the most feared consequence of infestations is that agricultural cultures are affected by prolonged periods with no irrigation due to system blockage and subsequent cleaning and restoring. The Portuguese irrigation systems are struggling to implement effective control methods for the pest, and the respective management entities are very interested in collaborative research that may help them to cope with the Asian clam. Presently, the economic losses in national mechanised irrigation systems due to *C. fluminea* infestations already sum up to 2% of the respective operating costs, which is two orders of magnitude higher than the corresponding percentages observed in the drinking water treatment and power sectors (Table 4.3).

4.6. Discussion

4.6.1. Problems encountered with C. fluminea *in Portuguese freshwater-dependent industry*

The ability of the Asian clam to foul industrial water systems has been well documented in North America, but studies reporting the problem in European facilities are much scarcer, hence motivating the contribution provided by this study.

Results indicate that, compared to the situation in North America (section 4.2), the impacts of this pest in the Portuguese freshwater-dependent industry remain relatively mild, both in terms of the number of facilities affected and the severity of the problems experienced (section 4.5.1; Tables 4.2 and 4.3). No cement plants or pulp and paper mills have reported any infestations yet. In drinking water treatment plants and thermal power

stations (sections 4.5.2 and 4.5.3), the first infestation episodes have not been dramatic, being detected only during routine inspections. Furthermore, regular cleaning of the respective water systems seems to be good enough to prevent noticeable clogging and efficiency issues due to clams accumulation. Therefore, the losses caused by *C. fluminea* in these sectors are still low and far from others reported in the literature (Table 4.3; Pimentel et al. 2005). In contrast to this general trend, the irrigation sector appeared as an exception, with a significant proportion of the national systems, especially those feeding sprinkler irrigation structures, reporting significant impairment and increasing economical losses due to the presence of the Asian clam (section 4.5.4; Tables 4.2 and 4.3).

Overall, the estimated annual cost of *C. fluminea* infestations to the Portuguese freshwater-dependent industry may be up to € 200 000. In absolute terms, this value may not seem very high, especially if compared to, for example, the economic impact of the pest in North America, reported as $ 1 billion/year in the 1980s (Isom 1986, Pimentel et al. 2005), or the € 1.2 million the zebra mussel costs to the English waterworks each year (Elliott et al. 2005). However, such a value should be regarded taking into consideration the relative scale of the affected facilities, countries and respective economies. For example, an extra expenditure of a few thousand of euros due to Asian clam infestations has a considerable impact on the annual budget available for the management of a typical Portuguese irrigation system. Additionally, it is important to emphasise that, at the present and even more so in the future, the industrial losses due to the Asian clam are likely to be only one of the facets of the economic damage caused by the pest (Lovell and Stone 2005). In fact, in general, a more comprehensive, systematic and integrated method to estimate this sort of economic damage is still to be developed (Lovell and Stone 2005).

The Asian clam has been reported in Portuguese waters since the early 1980s (Mouthon 1981). Having a strong invasive character, the species is now present in many waterbodies across the country (section 4.3; Fig. 4.1, Table 4.1). Such spread probably resulted from the action of several vectors (Counts 1986), including the transport by waterfowl and human-mediated transport, in particular through recreational boats. Byssus drifting-related transport (Prezant and Chalermwat 1984) and unassisted upstream movement (Voelz et al. 1998) are also likely to have played a significant role in the Asian clam dispersal. Given the current species distribution, the relatively low incidence and severity of industrial infestations in national freshwater-dependent plants can be somewhat surprising. However, such a picture is not unique. For instance, in Serbia, nine years after *C. fluminea* was recorded for the first time, industrial biofouling problems had not been reported yet (Paunović 2007). Also, in North America, the Asian clam was widely reported for over 20 years before causing noticeable industrial problems (Bidwell et al. 1995).

The following two main hypotheses can be posed to explain the apparent discrepancy between the observed Asian clam dispersal in natural ecosystems and the pest industrial impacts in Portugal.

Often, successful biological invasions evolve from introduction to full establishment in a newly colonised habitat through a phase during which the species persists at low densities in a restricted area. This has been termed lag time phase and may last up to decades (Byers et al. 2002, Sakai et al. 2001, Shigesada and Kawasaki 1997). Over the lag time phase, nonindigenous populations face the challenge of adapting to the novel environmental conditions. One first plausible hypothesis to explain the mild industrial impacts described in this study is that the Asian clam populations in the Portuguese waterbodies are mostly at the lag time phase with deficient recruitment, meaning that reduced spawning and/or high juvenile mortality lead to low growth rates of the populations established in the industrial plants, ultimately resulting in tolerable and easily manageable degrees of biofouling. In fact, the invasive *C. fluminea* populations of at least one Portuguese river have been suggested to be at the lag time phase (Sousa et al. 2006, Sousa et al. 2008b).

Another possible explanation for the moderate Asian clam infestations in Portuguese industries is the fact that the species is prone to die-offs, occurring mostly in the summer (Cooper et al. 2005, Phelps 1994, Scheller 1997, Sickel 1986, Sousa et al. 2007, Vohmann et al. 2009). Such mass mortality episodes, from which the population affected may take long to recover, can limit the progress of the populations in the plants' water systems. This explanation is particularly consistent with the isolated infestation episode in the late 1980s reported by one of the power stations surveyed (section 4.5.3), especially if associated with literature data reporting the decay of *C. fluminea* populations after a rapid initial growth (Phelps 1994). The reasons for *C. fluminea* die-offs are not fully understood. Some authors suggest that they may be caused by decreased water flow, high organic matter concentration, elevated temperature as well as reduced dissolved oxygen level and redox potential (Cherry et al. 1980, Cooper et al. 2005, French and Schloesser 1996, Ilarri et al. 2011, Scheller 1997, Sickel 1986, Sousa et al. 2007). Vohmann et al. (2009) argue that increased water temperature and oxygen depletion alone do not seem to explain this phenomenon. They suggest that the low food concentrations occurring in the summer cause the animals to significantly starve, resulting in increased mortality. This theory is supported by the fact that, in the summer, Asian clams' metabolic demands tend to be higher due to the increased individuals' growth rate and reproductive effort and there is augmented competition for space and nutrients, which result in considerably higher food requirements (Sickel 1986). Episodic events of contamination by toxic substances, epidemics, parasite infestations or temperature

variation may also induce clams' mass mortalities (Sickel 1986, Vohmann et al. 2009). Sickel (1986) noted that *C. fluminea* die-offs may reflect a natural response of a dense population that exceeds a sustainable biomass level or in which an overpopulated cohort reaches its age limit, rather than being a direct result of any environmental changes. Rapid die-offs have been observed in Asian clam populations in at least one Portuguese waterbody (Minho River) (Ilarri et al. 2011).

Given the mild severity of current industrial impacts of *C. fluminea* in Portugal, and possibly in other countries, the question is whether in Europe this pest may be considered as a friendly enemy and its threat disregarded. The more sensible answer is no, because it may be only a matter of time and natural invasion progress until more dramatic consequences are experienced. Although differences between the North American and European populations may exist (Mackie and Schloesser 1996), it is important that pest managers in Europe, where the species arrived only 30 years ago, bear in mind the experience of North American plants, which have been dealing with the Asian clam for much longer. For example, in the United States, in 1981, around 50 years after the first report of the species in the country, a series of infestations in nuclear power stations lead the United States Nuclear Regulatory Commission to instruct all the national plants to inspect their systems for *C. fluminea* (Williams and McMahon 1986). In this context, while most of the Portuguese facilities affected find their normal cleaning routines to be a cost-effective control measure for the moment, and perhaps proactive mitigation strategies (Claudi and Mackie 1994), in general, are not justified yet, the conservative recommendation should be that systematic monitoring programmes are put into practice to anticipate the possible sudden increase of the pest industrial spread and impacts. Such programmes should be regarded as an investment that may prevent much greater losses in the future. It is very likely that Europe is facing a latent industrial problem related to *C. fluminea,* in addition to the ecological one (Ilarri et al. 2011, Sousa et al. 2008b), to which policy makers should pay immediate attention. In particular, the dissemination of information on the species dispersal, impacts and control amongst freshwater-dependent plants as well as assistance with the implementation of industrial monitoring and mitigation programmes are strongly recommended. Systematic bioeconomic risk analysis (e.g. Leung et al. 2002), planned as future research, will most likely build on these considerations to provide more quantitative guidelines on the allocation of resources to preventive measures.

4.6.2. Comparative analysis of C. fluminea *and* D. polymorpha *industrial biofouling activities*

The zebra mussel *D. polymorpha* is perhaps the most prominent freshwater biofouler bivalve due to its enormous dispersal capacity and epifaunal character (Claudi and Mackie 1994). Therefore the impacts of this species are very well documented and the design of monitoring and control programmes to deal with the pest in industrial environments has received much interest (Claudi and Mackie 1994, Ludyanskiy et al. 1993, Sprecher and Getsinger 2000). In an attempt to draw attention to *C. fluminea* biofouling activity there is sometimes a tendency to generalise the problems and practices associated with the zebra mussel. Although the industrial impacts and management of both species do share some common traits, they are different in key features of their biology and ecology, which have direct implications in the way they affect industrial facilities and ultimately in their control.

One major difference between *D. polymorpha* and *C. fluminea* infestations is related to the distinct species' mode of life. The zebra mussel is an epifaunal bivalve, attaching to non-toxic hard surfaces by means of byssal threads, produced by a gland housed in the foot. An adult mussel of 25 mm shell length produces up to 500 byssal threads (Bidwell et al. 1995, Claudi and Mackie 1994). *D. polymorpha* individuals attached to intake units, the inner surface of pipes and equipments or other system components are difficult to remove. The byssal threads tend to remain even after the shells are removed. Through them, the metals' integrity is affected due to increased corrosion underneath the points of attachment (Claudi and Mackie 1994). Moreover, the byssal threads disturb the laminar flow of water in pipes (Claudi and Mackie 1994). Zebra mussels also attach to each other, forming thick layers that may break off under turbulence and gravity, increasing downstream fouling. In contrast, the Asian clam is an infaunal biofouler bivalve that primarily accumulates at the bottom of the structures rather than attaching to the surfaces. The absence of strong byssal threads in the adult stage greatly facilitates the physical removal of the invading populations.

Another important difference between *D. polymorpha* and *C. fluminea* refers to the species reproductive and life cycles. The zebra mussel is unusual amongst freshwater bivalves in the sense that it reproduces through a long free-swimming larval stage (Ackerman et al. 1994). For this reason, the foremost source of plant infestation are parent populations established well upstream the water intake system, because the veligers require from 3 to 5 weeks to develop to the settling stage. The veligers that enter or remain in the facility at this stage are those of much concern from the industrial effects point of view (Claudi and Mackie 1994). Adult mussels established in the plant will

marginally contribute to the infestation because the water residence time tends to be such that the veligers they produce pass through the system much before reaching the settling stage. In the case of the Asian clam, the planktonic development stage after release from the progenitors is much shorter (on the scale of hours) (Ackerman et al. 1994, King et al. 1986, Kraemer and Galloway 1986), meaning that populations that are established closer upstream the facility should constitute an important infestation source. Moreover, contrary to zebra mussels, Asian clams settle to take up an infaunal existence, and it is thus plausible that unattached young individuals in the immediate surroundings of the intake points are more easily sucked into the system. Also, in view of the infaunal character of *C. fluminea*, it is not completely clear whether the individuals born in the plant may contribute to the population established there. While a planktonic development stage is identified in the species life cycle, the process through which settlement occurs does not seem to be fully understood, and it is possible that newly-born individuals are able to remain inside the plant (McMahon 1983). The role played by and the relative contribution of individuals originated from moderately distant parent populations, very young juveniles sucked into the system and the population established in the plant itself as an infestation source is worth investigating.

One major direct implication of the differences discussed above in the mitigation of the two pests is concerned with chemical control strategies. Basically, there are two major types of such strategies: reactive and proactive treatments (Claudi and Mackie 1994). Reactive strategies are targeted at adults already established in the facility, being suitable for systems that can tolerate some degree of biofouling. Proactive strategies are designed to prevent the settlement of the bivalves in the plant. In the case of zebra mussel infestations, reactive chemical treatment is applied to facilitate the removal of the firmly attached adults as well as preventing large individuals, which increase clogging of small cross section structures, to grow in the system. Taking into account that the Asian clam do not attach strongly to surfaces, proactive chemical dosage prior to clams removal does not result in much benefit in terms of the complexity of the cleaning procedure. However, it still prevents the adults to grow large, and may prove to be further advantageous if one can confirm that the resident populations play a significant role as an infestation source.

4.7. Conclusions

The study presented here revealed that, somewhat surprisingly, the impacts of the biofouler *C. fluminea* in the Portuguese freshwater-dependent industry are relatively mild, although the species has been reported in the country since the early 1980s. While, in

general, the effects of the pest do not justify control measures (e.g. chemical control or the physical alteration of the water systems) other than periodic cleaning for the moment, the sensible advice is that information on the species characteristics and behaviour should be widely disseminated amongst the national plants and systematic monitoring strategies should be implemented. Based on the North American experience, a sudden increase of losses due to the pest in industrial environments may occur.

Amongst all the sectors surveyed, irrigation systems are currently the most affected structures, experiencing increasing annual losses that already sum up to 2 % of the overall operating costs. The respective managers are thus very keen to implement more effective pest mitigation programmes. Controlling invasive bivalves in irrigation systems is not an easy task, mainly due to their configuration, the massive volumes of water processed and the need to guarantee plants survival and development, but it is certainly a challenge that is worth being addressed by the scientific community.

As no specific measures have been implemented so far in order to minimise the dispersal of the Asian clam in Portugal and its biofouling activity, the results presented here provide a holistic picture of the unrestricted industrial infestation process within a 30-year window. They may thus assist official entities in countries at risk of invasion or where the *C. fluminea* has recently arrived with the implementation of integrated pest management policies. A repeated survey of the impacts of the species on national freshwater-dependent plants within a reasonable length of time and comparison with the present study may contribute to an understanding of the progression of the species in industrial environments.

The control of this aquatic pest will certainly benefit from an ample understanding of the infestation process. In this context, studies relating populations' recruitment rates in industrial facilities to the clams settlement and dispersal, focussing in particular the role played by the mucilaginous byssal thread formed in the early life stages, are welcome.

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4.9. References

Ackerman JD, Sim B, Nichols SJ, Claudi R. 1994. Review of the early-life history of zebra mussels (*Dreissena polymorpha*) - comparisons with marine bivalves. Canadian Journal of Zoology-Revue Canadienne De Zoologie 72: 1169-1179.

Allendorf FW, Lundquist LL. 2003. Introduction: Population biology, evolution, and control of invasive species. Conservation Biology 17: 24-30.

Araujo R, Moreno D, Ramos MA. 1993. The Asiatic clam *Corbicula fluminea* (Müller, 1774) (*Bivalvia: Corbiculidae*) in Europe. American Malacological Bulletin 10: 39-49.

Beasley CR, Tagliaro CH, Figueiredo WB. 2003. The occurrence of the Asian clam *Corbicula fluminea* in the lower Amazon Basin. Acta Amazonica 33: 317-324.

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

Bidwell JR, Farris JL, Cherry DS. 1995. Comparative response of the zebra mussel, *Dreissena polymorpha*, and the asian clam, *Corbicula fluminea*, to DGH/QUAT, a nonoxidizing molluscicide. Aquatic Toxicology 33: 183-200.

Byers JE, et al. 2002. Directing research to reduce the impacts of nonindigenous species. Conservation Biology 16: 630-640.

Chainho P, Costa JL, Chaves ML, Lane MF, Dauer DM, Costa MJ. 2006. Seasonal and spatial patterns of distribution of subtidal benthic invertebrate communities in the Mondego River, Portugal – A poikilohaline estuary. Hydrobiologia 555: 59-74.

Cherry DS, Rodgers Jr. JH, Graney RL, Cairns Jr. J. 1980. Dynamics and control of the Asiatic clam in the New River, Virginia. Virginia: Virginia Water Research Center, Virginia Polytechnic Institute and State University.

Claudi R, Mackie GL. 1994. Practical manual for zebra mussel monitoring and montrol. Boca Raton: Lewis Publishers.

Cooper NL, Bidwell JR, Cherry DS. 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.

Counts CL. 1986. The zoogeography and history of the invasion of the United States by *Corbicula fluminea* (*Bivalvia: Corbiculidae*). Proceedings of the Second International *Corbicula* Symposium, American Malacological Bulletin - Special edition no.2.

DAISIE – European Invasive Alien Species Gateway. www.europe-aliens.org. Acessed 2008.

Elliott P, Aldridge DC, Moggridge GD, Chipps M. 2005. The increasing effects of zebra mussels on water installations in England. Water and Environment Journal 19: 367-375.

Elton C. 1958. The ecology of invasions by animals and plants. USA: The University of Chicago Press.

French JRP, Schloesser DW. 1996. Distribution and winter survival health of Asian clams, *Corbicula fluminea*, in the St Clair River, Michigan. Journal of Freshwater Ecology 11: 183-192.

Howlett D, Baker R. 1999. *Corbicula fluminea* (Müller): New to U.K. Journal of Conchology 36: Only abstract.

Ilarri MI, Antunes C, Guilhermino L, Sousa R. 2011. Massive mortality of the Asian clam *Corbicula fluminea* in a highly invaded area. Biological Invasions 13: 277-280.

Ingram WM. 1959. Asiatic clams as potential pests in California water supplies. Journal American Water Works Association: 363-370.

Isom B. 1986. Historical review of Asiatic clam (*Corbicula*) invasion and biofouling of waters and industries in the Americas in Britton J, ed. Proceedings of the Second International *Corbicula* Symposium: American Malacological Bulletin - Special edition no.2.

Jenner HA, Whitehouse JW, Taylor CJL, Khalanski M. 1998. Cooling water management in European power stations Biology and control of fouling. Hydroécologie Appliquée 10: 1-225.

Johnson KI, Henager CH, Page TL, Hayes PF. 1986. Engineering factors influencing *Corbicula* fouling in nuclear service water systems. American Malacological Bulletin special edition nr.2: 47-52.

Karatayev AY, Burlakova LE, Padilla DK. 2005. Contrasting distribution and impacts of two freshwater exotic suspension feeders, *Dreissena polymorpha* and *Corbicula fluminea*. Pages 239-262 in Dame RF, Olenin S, eds. The Comparative Roles of

Suspension-Feeders in Ecosystems, vol. IV. Earth and Environmental Sciences. Dordrecht, The Netherlands: Springer.

King CA, Langdon CJ, Counts CL. 1986. Spawning and early development of *Corbicula fluminea* (*Bivalvia, Corbiculidae*) in laboratory culture. American Malacological Bulletin 4: 81-88.

Kraemer LR, Galloway ML. 1986. Larval development of *Corbicula fluminea* (Müller) (*Bivalvia, Corbiculacea*) - An appraisal of its heterochrony. American Malacological Bulletin 4: 61-79.

LePage WL. 1993. The impact of *Dreissena polymorpha* on waterworks operations at Monroe, Michigan: a case history. Pages 333-358 in Nalepa TF, Schloesser DW, eds. Zebra mussels: biology, impacts and control. Boca Raton: Lewis Publishers.

Leung B, Lodge DM, Finnoff D, Shogren JF, Lewis MA, Lamberti G. 2002. An ounce of prevention or a pound of cure: bioeconomic risk analysis of invasive species. Proceedings of the Royal Society of London Series B-Biological Sciences 269: 2407-2413.

Lovell SJ, Stone SF. 2005. The economic impacts of aquatic invasive species: A review of the literature. NCEE Working Paper Series 05-02.

Ludyanskiy ML, McDonald D, Macneill D. 1993. Impact of the zebra mussel, a bivalve invader - *Dreissena polymorpha* is rapidly colonizing hard surfaces throughout waterways of the United States and Canada. Bioscience 43: 533-544.

Mackie GL, Schloesser DW. 1996. Comparative biology of zebra mussels in Europe and North America: An overview. American Zoologist 36: 244-258.

McMahon RF. 1977. Shell size frequency distributions of *Corbicula manilensis* Philippi from a clam fouled steam condenser.

McMahon RF. 1983. Ecology of an invasive pest bivalve, *Corbicula* in Russel-Hunter WD, ed. The Mollusca, vol. 6. London: Academic Press, Inc.

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

Morais P, Teodósio J, Reis J, Chícharo MA, Chícharo L. 2009. The Asian clam *Corbicula fluminea* (Müller, 1774) in the Guadiana River Basin (Southwestern Iberian Peninsula): Setting the record straight. Aquatic Invasions 4: 681.684.

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

Paunović M. 2007. Distribution of Asian clams *Corbicula fluminea* (Müller, 1774) and *C. fluminalis* (Müller, 1774) in Serbia. Aquatic Invasions 2: 99-106.

Pérez-Quintero JC. 2007. Diversity, habitat use and conservation of freshwater molluscs in the lower Guadiana River basin (SW Iberian Peninsula). Aquatic Conservation: Marine and Freshwater Ecosystems 17: 485-501.

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

Phelps HL. 1994. The asiatic clam (*Corbicula fluminea*) invasion and system-level ecological change in the Potomac River estuary near Washington, DC. Estuaries 17: 614-621.

Pimentel D, Zuniga R, Morrison D. 2005. Update on the environmental and economic costs associated with alien-invasive species in the United States. Ecological Economics 52: 273-288.

Prezant RS, Chalermwat K. 1984. Flotation of the bivalve *Corbicula fluminea* as a means of dispersal. Science 225: 1491-1493.

Prokopovich NP, Herbert DJ. 1965. Sedimentation in the Delta-Mendota Canal. Journal AWWA: 375-382.

Reis J. 2006. Atlas dos bivalves de água doce de Portugal Continental. Lisboa: ICN.

Sakai AK, et al. 2001. The population biology of invasive species. Annual Review of Ecology, Evolution and Systematics 32.

Scheller JL. 1997. The effect of die-offs of asian clams (*Corbicula fluminea*) on native freshwater mussels (*Unionidae*). Faculty of the Virginia Polytechnic Institute and State University of Blacksburg, Blacksburg.

Shigesada N, Kawasaki K. 1997. Biological Invasions - Theory and Practice. Oxford: Oxford University Press.

Sickel JB. 1986. *Corbicula* population mortalities: factors influencing population control in Prezant RS, ed. Proceedings of the Second International *Corbicula* Symposium, American Malacological Bulletin - Special edition no.2.

Sinclair RM. 1964. Clam pests in Tennessee water supplies. Journal American Water Works Association: 592-599.

Sousa R, Antunes C, Guilhermino L. 2006. 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. 2007. 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, Nogueira A, Gaspar M, Antunes C, Guilhermino L. 2008a. Growth and extremely high production of the non-indigenous invasive species *Corbicula fluminea* (Müller, 1774): Possible implications for ecosystem functioning. Estuarine, Coastal and Shelf Science 80: 289-295.

Sousa R, Rufino M, Gaspar M, Antunes C, Guilhermino L. 2008b. Abiotic impacts on spatial and temporal distribution of *Corbicula fluminea* (Müller, 1774) in the River Minho estuary, Portugal. Aquatic Conservation: Marine and Freshwater Ecosystems 18: 98-110.

Sprecher SL, Getsinger KD. 2000. Zebra mussel chemical control guide. Vicksburg: Environmental Laboratory.

Vitousek PM, Dantonio CM, Loope LL, R. W. 1996. Biological invasions as global environmental change. American Scientist 84: 468-478.

Voelz NJ, McArthur JV, Rader RB. 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.

Williams CJ, McMahon RF. 1986. Power station entrainment of *Corbicula fluminea* (Müller) in relation to population dynamics, reproductive cycle and biotic and abiotic variables. Pages 99-111 in Britton JC, ed. Proceedings of the Second International *Corbicula* Symposium: American Malacological Bulletin - Special edition no.2.

CHAPTER 5

Sensivity of the invasive bivalve *Corbicula fluminea* to candidate control chemicals: The role of dissolved oxygen conditions

Sensivity of the invasive bivalve *Corbicula fluminea* to candidate control chemicals: The role of dissolved oxygen conditions

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5.1. Abstract

The clam *Corbicula fluminea* is amongst the major invasive species in freshwater ecosystems worldwide being responsible for adverse ecological and economic impacts in natural and industrial systems. Thus, it is crucial to maintain the research efforts into the search for improved methods to control the species. In this study we intended to investigate whether hypoxic conditions affect the efficiency of candidate control chemicals that present distinct toxicity mechanisms over the pest's physiology. Exposure of both juvenile and adult clams to the molluscicide niclosamide, the flocculant polyDADMAC, ammonium nitrate, copper sulfate, potassium chloride and dimethoate under normoxic (> 7 mg L^{-1} dissolved O_2) and hypoxic (< 2 mg L^{-1} dissolved O_2) conditions were carried out. Due to unexpected experimental constraints, only the first four chemicals could be addressed in the present chapter and the full dataset will be discussed elsewhere. Preliminary results suggest that hypoxia generally increases the efficiency of these chemicals and that the susceptibility of the clams varies as different life-stages are tested. The assessment recorded in this study provides systematic grounds to infer on the suitability of depressed oxygen conditions to assist chemical treatment applied to this species' management both in infested industrial systems and in invaded open waters.

Keywords: Biofouling; Asian clam; Invasive bivalves' control; Hypoxia; Toxicity

5.2. Introduction

Biological invasions in aquatic ecosystems are currently a major threat worldwide due to their potential to promote deleterious ecological and economic impacts (Barinova et al. 2010, Pimentel et al. 2005). Water-dependent industries can be particularly injured by the fouling activity of invasive species, such as bivalves. These macrofoulers grow and establish dense populations in underwater structures and equipment, frequently inducing severe consequences in the operation and safety of the facilities (Pimentel et al. 2005, Rosa et al. 2011). In the wild, these pests promote damage from the individual to the community level (Sousa et al. 2012). Therefore, the research on potential solutions to manage the nuisances both in natural and industrial environments is of critical relevance.

Corbicula fluminea, commonly known as the Asian clam, is an invasive freshwater macrofouler that underwent a global range expansion in the last century (Araujo et al. 1993, Beasley et al. 2003, Mackie and Claudi 2010). This pest induces serious negative impacts in the invaded ecosystems (e.g. Hakenkamp and Palmer 1999, Williams et al. 2001) and in infested freshwater-dependent industries (e.g. Page et al. 1986, Rosa et al. 2011). Although the Asian clam tolerates a wide range of variation in several environmental conditions, it is amongst the least hypoxia-tolerant freshwater bivalve mollusks (Matthews and McMahon 1999). Hypoxic conditions have also been suggested to increase the susceptibility of these organisms to chemical stressors, including candidates to integrate in pest control programs (Doherty and Cherry 1988, Tran et al. 2001, Tran et al. 2008). This particular characteristic of the species physiology can be viewed as a breakthrough in the development of management solutions for the pest, both in natural and industrial invaded systems. Once hypoxia is a growing threat to freshwater ecosystems worldwide (Diaz 2001), such an environmental condition should be considered when addressing the effects of chemical stressors in natural *C. fluminea* populations, including those that have been suggested as potential candidates for the control of the invasion in open waters (see Sousa et al. 2012 for examples). In industrial environments, hypoxia has been recognized as an efficient control method for the Asian clam (Smithson 1986), although one should bear in mind that the costs of lowering oxygen levels either chemically or physically may not compensate the benefit in pest management for some industries (Johnson and McMahon 1998, Mackie and Claudi 2010); hence, the extent to which oxygen deprivation can improve control methods requires systematic assessment. The efficiency of combinations between oxygen deprivation and different control chemicals targeted at macrofoulers should be analyzed. In fact, the efficiency of some biocides can be increased by synergism or potentiation when used in combination with hypoxia (Doherty and Cherry 1988). If this trend is proven valid for biocides used to

control biofoulers, the potential of applying oxygen deprivation as a complement to chemical treatment solutions increases since reduced chemical quantities and reduced efforts to lower oxygen levels would be required to affect biofouler populations. The present study addressed this rationale by evaluating the changes in the efficiency of different biocides as hypoxia conditions are set.

Several compounds with potential to target macrofoulers such as the Asian clam were selected based on their different mechanism of toxicity. Ammonium nitrate has already been proposed as a biocide to control bivalve biofoulers and it is widely used as a chemical fertilizer in agriculture (Mackie and Claudi 2010). This chemical seems to mainly impair cellular respiration by damaging the gill epithelium, stimulating glycolysis, and uncoupling oxidative phosphorylation (Camargo and Alonso 2006). Polydiallyldimethylammonium chloride (polyDADMAC) is a highly charged density polymer commonly used for removal of suspended solids in many industrial wastewater facilities (Ariffin et al. 2012). Its mechanism of toxicity has been suggested to be compared to that of other polyquaternary amines, i.e. the compound attaches to negatively charged membranes and surfaces and disruption of membrane transfer mechanisms occurs (Post et al. 1997). Potassium salts are toxic to most bivalves (Mackie and Claudi 2010). Valve closure is often prevented following exposure after foot distension and paralysis (Anderson et al. 1976, Daum et al. 1977, Mattice 1983); it was also suggested that potassium destroys the integrity of the gill epithelium leading to asphyxiation of mussels (Fisher and Bernard 1991). Copper sulfate has been used in agrosystems as an effective molluscicide against snails and their eggs (WHO 1965). Copper toxicity has been ascribed to oxidant damage caused by the overproduction of reactive oxygen species (Lushchak 2011). Niclosamide is a molluscicide used in Asia, Canada, New Zealand and the Dominican Republic to control agricultural pests, and in the USA its use is restricted to some governmental services (WHO 2002). Niclosamide induces mitochondria fragmentation and may contribute to apoptotic and autophagic cell death (Park et al. 2011). Dimethoate is an organophosphate insecticide widely used in a large variety of crops (USEPA 2006) acting as an irreversible acetycholinesterase inhibitor, thus preventing the necessary hydrolysis of the neurotransmitter acetylcholine (Beltran and Pocsidio 2009).

The aim of the present study was to investigate whether hypoxia can affect the efficiency of different chemicals that can putatively be used for the control of *C. fluminea*. Concomitantly, we also aimed to gain insight on whether the mechanism of toxicity has a systematic role on the way oxygen deprivation affects the response of the organisms to the chemicals. Finally, the variation in the chemical's efficiency due to clam size was

addressed following former suggestions on distinct sensitivity of *Corbicula* juveniles to different chemical challenges as compared to adults (Belanger et al. 1991, Doherty et al. 1986). Despite setting hypoxia levels (< 2 mg O_2 L^{-1}) sounds unrealistic, particularly given the costs involved in such a procedure in industrial control programs for bivalve biofoulers, the dataset establishes the necessary grounds for further research on more realistic combinations for future application in the control of *C. fluminea* in open waters and industrial environments. In the context of this PhD Thesis, only results on niclosamide, polyDADMAC, ammonium nitrate, and copper sulfate will be presented. Unexpected extreme meteorological events (long-term storms severely affecting the site where clams were collected for the study) compromised the feasibility of the responses given by the organisms in the tests made with the remaining chemicals. Nevertheless, this study is ongoing with the repetition of most of the tests for confirmation and the complete dataset will be presented elsewhere.

5.3. Material and methods

5.3.1. Test organisms

Late juveniles/young adults (hereinafter referred as to juveniles) and older adults (hereinafter referred as to adults) of *Corbicula fluminea* (shell length within 9-17 mm and 18-30 mm, respectively) were collected from a shallow creek in Mira, Portugal (N40°25'06.90''/W8°44'13.18'') that holds a well-established population (density above 2000 clams/m^2). Clams were collected by sieving sediment though a 1-mm mesh bag. Individuals with the intended shell lengths were selected and immediately transported in field water to the laboratory, where they were gradually acclimated. Clams were kept in dechlorinated municipal water (for at least 4 days before testing), at 20 ± 2 °C, under a 16 h^L: 8h^D photoperiod cycle and continuous aeration. The water was fully renewed once a week and the clams were fed ad-hoc with *Pseudokirchneriella subcapitata* suspensions.

5.3.2. Chemicals

Laboratory-grade reagents (> 98% purity w/w) were used to prepare stock solutions of niclosamide, dimethoate, copper II sulphate, potassium chloride and ammonium nitrate, which were then dosed to establish the test treatments. PolyDADMAC was dosed as Floquat FL 4440 (SNF AMBIENTAGUA, Santo Tirso, Portugal) which contains 40% (w/w) polymer.

5.3.3. Experimental design

A static 96-h test design was employed with a blank control (dechlorinated municipal water) plus five concentrations for each chemical under both normoxic and hypoxic conditions. Exposure concentrations were selected based on previous range-finding experiments. The tests were performed separately with juveniles or adults. All treatments were applied in triplicate, each replicate consisting of 10 clams randomly assigned to 500 mL-borosilicate flasks. Clams were kept in the test flasks filled with ca. 500 mL dechlorinated municipal water under continuous aeration for a 7-h acclimation period, over which all clams were confirmed to be actively filtering. After that period, the chemical was dosed under normoxic (> 7 mg L^{-1} dissolved O_2) and hypoxic (< 2 mg L^{-1} dissolved O_2) conditions as described below.

For the tests carried under normoxic conditions, chemical dosage was done in each replicate after filling each test vessel with the necessary amount of dechlorinated municipal water to reach the reference volume of 590 mL; after dosage, the test solution was agitated to homogenise and then a volume of ca. 100 mL was discarded to allow safe aeration. Continuous aeration was set to ensure normoxic conditions during the whole test period. In tests carried under hypoxic conditions, chemicals were dosed into each replicate after filling the test vessels with 590 mL oxygen-depressed dechlorinated municipal water. Nitrogen injection into a 6-L bucket filled with dechlorinated municipal water was used to depress oxygen levels to < 0.5 mg L^{-1} (continuously monitored by a WTW Oxi 315i/SET probe) in each two treatments (6 replicates). The water was then poured into the test flasks (590 mL set volume) and, after dosage, ten clams were added to each replicate. The flasks (of the 6 replicates) were immediately capped and the initial dissolved oxygen concentration was then recorded based on measurements made in an additional flask that was prepared along with each batch of test replicates but left open. Tests were kept at constant temperature (20 ± 2ºC) and photoperiod (16 h^L: 8 h^D). Clam mortality and dissolved oxygen levels were assessed after 48 and 96 h (WTW Oxi 315i/SET). Clams were considered dead if resistance to valve opening was not offered when closed animals were carefully forced to open with a blunt dissection needle.

5.3.4. Data analysis

A repeated measures (RM) ANOVA was performed in order to statistically validate that hypoxia conditions could always be distinguished from normoxia conditions, using dissolved oxygen level (hypoxia versus normoxia) as the between-subjects factor and the measurement time-point (0h, 48h and 96h) as the within-subjects factor. The difference in size between the two groups of clams (all juveniles vs all adults used in the tests) was

statistically confirmed using a student t-test (Quinn and Keough 2002). A Fisher's exact test was performed to compare the mortality of either adults or juveniles in the hypoxia control treatments set in tests made with individuals collected before and after the storm; this allows conclusions on the significance of the effect of the extreme environmental changes on the sensitivity of the clams to dissolved oxygen level (Quinn and Keough 2002). A two-way ANOVA was run to address the effect of dissolved oxygen level (normoxia and hypoxia) and chemical concentration in juveniles or adults mortality. Whenever a significant interaction between oxygen level and chemical concentration was found, simple main effects of chemical concentration within each oxygen level were assessed separately by running one-way ANOVA using the within-groups error term from the former two-way ANOVA output as denominator of the F test (Quinn and Keough 2002). The Dunnet *post-hoc* test was further applied to detect treatments significantly differing from the corresponding control and find LOEC (Lowest Observed Effect Concentration) values. The probit model was adjusted to all dose-response curves (Finney 1971). In order to evaluate the effect of mechanism of toxicity in the responses of the clams to oxygen deprivation, comparison of corresponding hypoxia and normoxia probit models for each chemical was carried out: (i) the normoxia probit model was used to determine the concentration eliciting 20, 40, 60, and 80% mortality (i.e. LC_{20}, LC_{40}, LC_{60}, and LC_{80}, respectively; (ii) the mortality elicited by these concentrations under hypoxia was estimated using the corresponding hypoxia probit model; (iii) the ratio between these defined mortality values was then calculated [(Hypoxia-Normoxia)/Normoxia] in order to build up a curve that represents the increment of the effect noticed under hypoxia relatively to the corresponding effect noticed under normoxia.

5.4. Results

The difference between oxygen treatments was proven statistically significant: dissolved oxygen level across the test period in the hypoxia treatment (mean ± SD: 0.95 ± 0.596) was kept significantly lower than that in the normoxia treatment (mean ± SD: 7.94 ± 1.46) (RM ANOVA: $F_{(1,\ 90)} = 1230.20$; $p < 0.01$). Clams selected as late juveniles were also confirmed to be significantly smaller (mean ± SD: 13.58 ± 1.171) than clams selected as adults (mean ± SD: 23.14 ± 1.810) (t-test: $t = 169.41$; $p < 0.01$).

Clam collection for testing spread along a relatively large time period, during which unexpected extreme climate conditions (storm rain events) occurred. The records on clam mortality in the hypoxia control treatments suggested variation in the sensitivity of the

clams to oxygen deprivation as the response of organisms collected before and after the storms were compared. Statistical comparison between hypoxia control treatments of tests made before and after the storm confirms that mortality of juveniles was significantly lower before the storm (Fisher's exact test: $p < 0.01$) while there seems to exist no significant difference in the sensitivity of adult clams to hypoxic conditions (Fisher's exact test: $p = 0.314$). Based on this analysis, results will be presented and discussed considering two separate groups of chemicals: (i) niclosamide and polyDADMAC, which were tested before the storm, and (ii) copper sulfate and ammonium nitrate, which were tested after the storm.

The concentration-response curves were found to generally fit the probit model (Chi^2: $p > 0.05$) and the curves under hypoxia and normoxia within each chemical mostly present similar shape (Fig. 5.1), which suggests reduced interaction between oxygen level and chemical concentration on the yielded effect. This pattern was corroborated by the statistics (Table 5.1): significant interactions were only found when ammonium nitrate was tested and when niclosamide was tested against adult clams. Although a significant interaction between factors was not frequently noticed, both the concentration and the oxygen level consistently affected clam mortality for all chemicals tested and both clam life stages - note the exception of polyDADMAC tested against adults, where oxygen level did not significantly impair clam survival.

Despite the general lack of significant interactive effect between oxygen level and chemical concentration (Table 5.1), both juveniles and adults were more sensitive to the chemicals under hypoxia compared to normoxia as indicated by the graphical comparison of concentration-curves in Fig. 5.1. This is particularly evident in the response of juvenile clams to copper sulphate and less evident but still noticeable in the response of both juvenile and adult clams to niclosamide (Fig. 5.1). Some specific patterns are worth mentioning as Fig. 5.1 is scrutinized. For example, oxygen level had a relevant role in defining juvenile and adult clam mortality under low ammonium nitrate concentrations, but as the concentration increases the difference between the responses under different oxygen levels decreases. A similar data profile was found for the exposure of adult clams to copper sulphate. Data on juvenile clam mortality exposed to niclosamide seem to indicate the opposite pattern, hence indicating a growing effect of the oxygen level as concentrations increase; the same seems to apply to adults exposed to polyDADMAC although the full concentration-response curve (currently the maximum effect recorded is below 80% mortality) is necessary to confirm the pattern. Juveniles were significantly less susceptible to niclosamide than adults both under normoxia and hypoxia while the opposite was recorded following exposure to polyDADMAC under hypoxia (Fig. 5.1, Table

5.2). Juveniles were significantly more susceptible to copper sulphate under normoxia and to ammonium nitrate under both dissolved oxygen conditions (Fig. 5.1, Table 5.2).

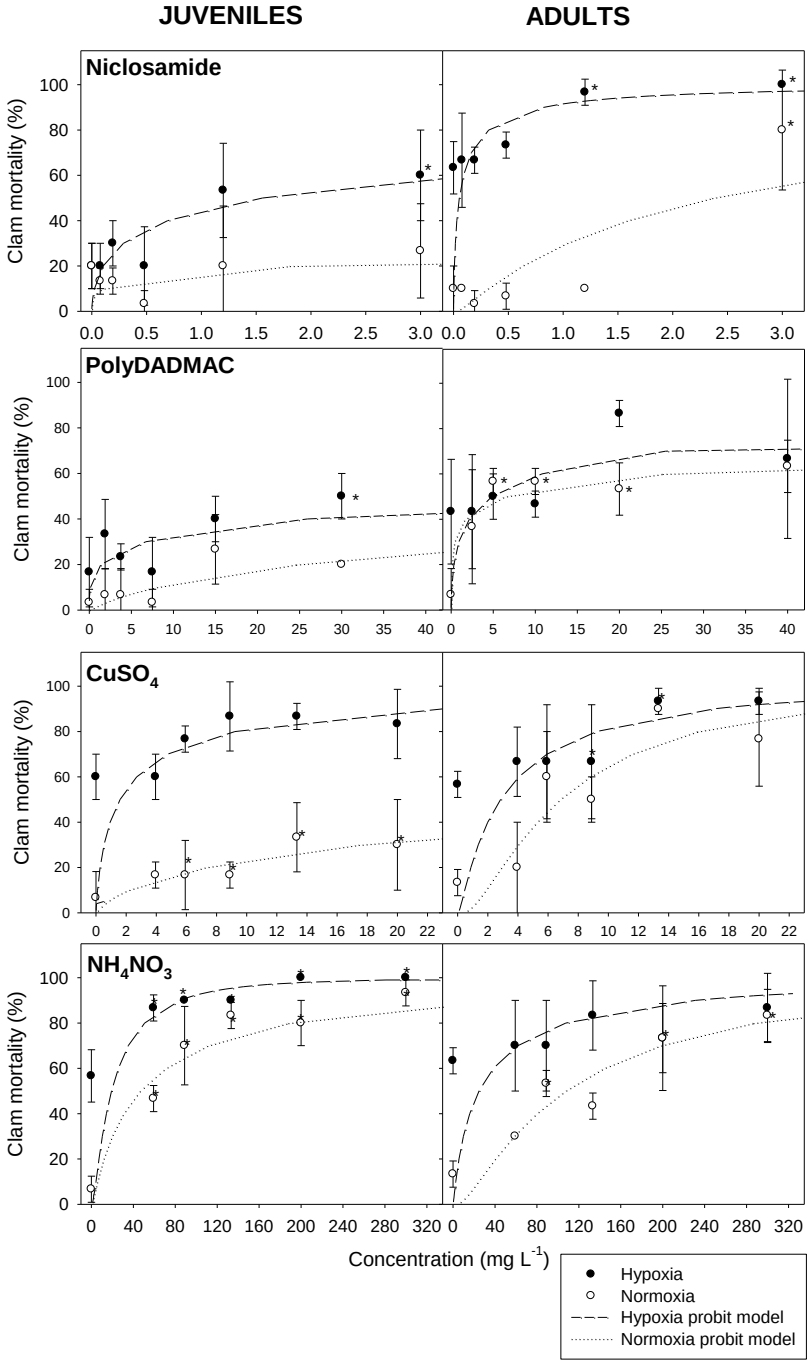

Fig. 5.1. Mortality (%) of juvenile (left-hand panel) and adult (right-hand panel) *C. fluminea* after 96-h exposure to niclosamide, polyDADMAC, copper sulfate and ammonium nitrate under normoxic and hypoxic conditions. The points refer to experimental data (mean ± SD) and the dashed lines represent the probit model adjusted to each dataset. Asterisks (*) mark the chemical treatments where mortality records significantly differ from the corresponding control as indicated by the Dunnet test following one-way ANOVA ($p < 0.05$; Table 2).

Table 5.1. Summary of two-way ANOVAs applied to address the effect of oxygen level (normoxia and hypoxia) and chemical concentration in *C. fluminea* juvenile and adult mortality after 96-h exposure to niclosamide, polyDADMAC, copper sulfate, and ammonium nitrate. Significant effects (significance level = 0.05) are marked in bold.

			Juveniles			Adults		
Chemical	**Source**	**df**	MS	F	P	MS	F	p
Niclosamide	Oxygen level	1	2844.4	12.34	**0.002**	30044.4	235.13	**< 0.001**
	Concentration	5	906.7	3.93	**0.010**	269.1	21.06	**< 0.001**
	Interaction	5	277.8	1.20	0.337	71708	5.62	**0.001**
	Residual	24	230.6			127.8		
PolyDADMAC	Oxygen level	1	3211.1	25.69	**< 0.001**	1002.8	3.28	0.083
	Concentration	5	751.1	6.01	**0.001**	1631.7	5.34	**0.002**
	Interaction	5	84.4	0.68	0.646	596.1	1.95	0.123
	Residual	24	125.0			305.6		
Copper Sulfate	Oxygen level	1	4444.4	17.98	**< 0.001**	27777.8	185.19	**< 0.001**
	Concentration	5	2997.8	12.13	**< 0.001**	651.1	4.34	**0.006**
	Interaction	5	511.1	2.07	0.105	117.8	0.79	0.570
	Residual	24	247.2			150.0		
Ammonium Nitrate	Oxygen level	1	5136.1	88.05	**< 0.001**	5625.0	29.35	**< 0.001**
	Concentration	5	3362.8	57.65	**< 0.001**	1636.1	8.54	**< 0.001**
	Interaction	5	469.4	8.05	**< 0.001**	671.7	3.50	**0.016**
	Residual	24	58.3			191.7		

The variation in clam sensitivity to the different chemicals tested under hypoxia relatively to normoxia generally followed a consistent tendency regardless the focus is on juveniles or adults (Fig. 5.2): at low chemical concentrations, depressed oxygen conditions seem to define the response of the clams; however, as chemical concentration becomes higher, the increase of clam sensitivity under hypoxia relatively to normoxia tended to zero, indicating increased relevance of the chemical rather than oxygen level in determining the effect. For tests with niclosamide (adults), polyDADMAC (juveniles), copper sulfate (juveniles and adults) and ammonium nitrate (adults) there was an accentuated decrease in the increment of clam mortality under hypoxia relatively to normoxia as concentrations increase until reaching a first threshold.

Table 2: Summary of one-way ANOVAs applied to address the main effects of chemical concentration within each oxygen level, in *C. fluminea* juvenile and adult mortality after 96-h exposure to niclosamide, polyDADMAC, copper sulfate, and ammonium nitrate. Significant effects (significance level = 0.05) are marked in bold.

		Juveniles			Adults		
Chemical	**Source**	df	F	p	df	F	p
Hypoxia							
Niclosamide	Concentration	5	4.15	**0.02**	5	6.22	**< 0.001**
	Residual	12			24		
PolyDADMAC	Concentration	5	3.51	**0.035**	5	2.10	0.136
	Residual	12			12		
Copper Sulfate	Concentration	5	2.72	0.072	5	3.89	**0.025**
	Residual	12			12		
Ammonium nitrate	Concentration	5	13.13	**< 0.001**	5	1.23	0.326
	Residual	24			24		
Normoxia							
Niclosamide	Concentration	5	0.87	0.532	5	20.45	**< 0.001**
	Residual	12			24		
PolyDADMAC	Concentration	5	3.06	0.052	5	7.25	**0.002**
	Residual	12			12		
Copper Sulfate	Concentration	5	12.22	**< 0.001**	5	1.65	0.221
	Residual	12			12		
Ammonium nitrate	Concentration	5	52.60	**< 0.001**	5	10.81	**< 0.001**
	Residual	24			24		

Then, the curves evidence a lower slope and finally, as higher concentrations are reached, the tendency to null slopes can be observed denoting very little sensitivity increment under hypoxia relatively to normoxia. A slight variation in this pattern was observed for niclosamide and ammonium nitrate tested against juvenile clams. As polyDADMAC was tested against adult clams, dissolved oxygen level seems to have an almost irrelevant role in promoting adult clam mortality, being chemical concentration the factor that seems to constrain the effect across the whole range, which is consistent with the statistics (Table 5.1).

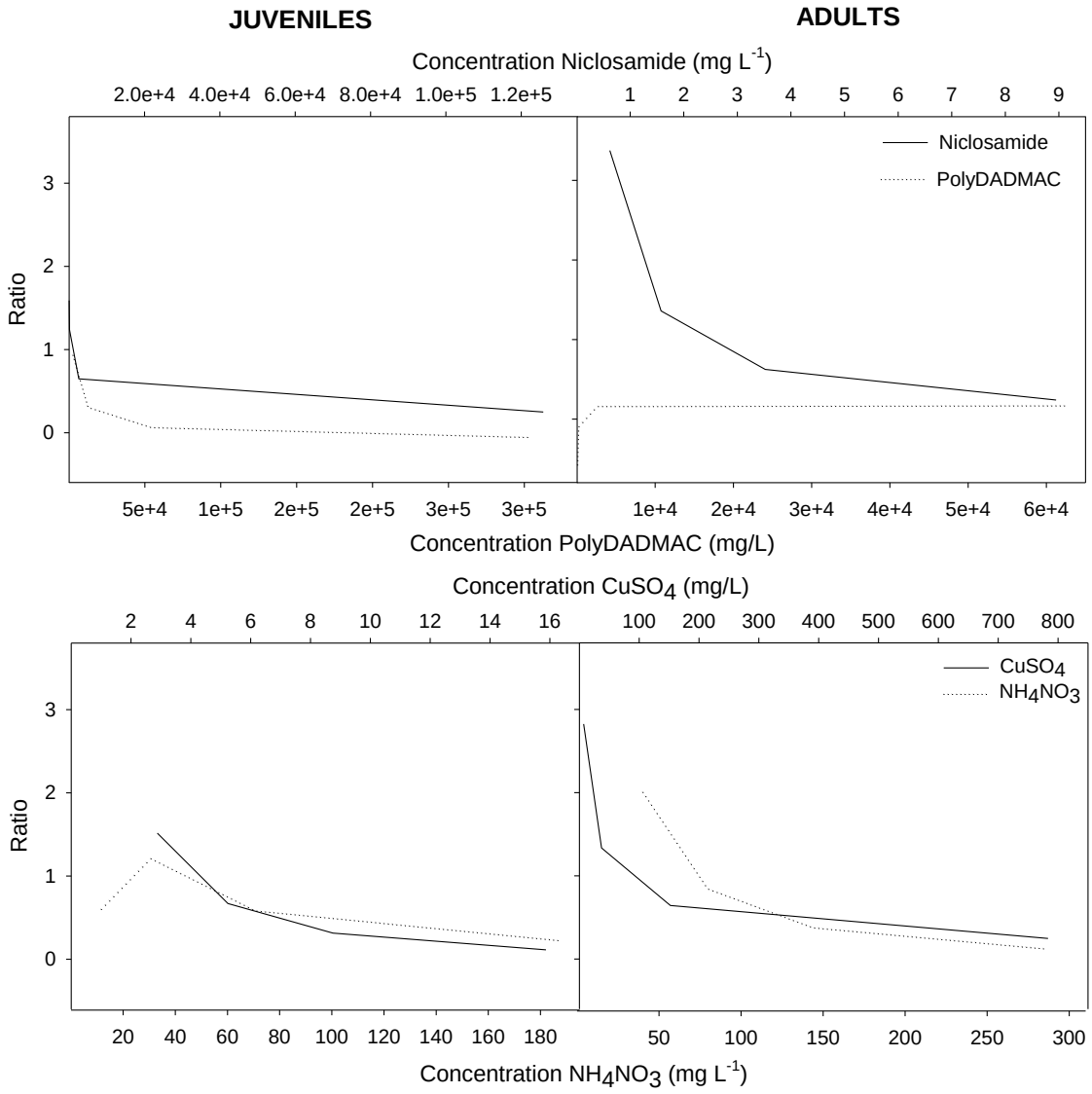

Fig. 5.2. Curves representing the increment in chemical-driven mortality of juvenile (left-hand panel) and adult (right-hand panel) *C. fluminea*. The curves were given by the ratio (H-N)/N, where H and N represents clam mortality under hypoxia and normoxia, respectively (see "Data analysis" for details). The figure groups comparable chemicals considering the organism's collection period (see text for details): niclosamide with polyDADMAC (upper panels) and copper sulfate with ammonium nitrate (lower panels).

5.5. Discussion

Several chemicals have been observed to have greater efficacy to control biofoulers at higher ambient temperatures (Mackie and Claudi 2010). Higher sensitivity to some contaminants under hypoxia has also been proven to occur for some macrofouler species including *C. fluminea* (Tran et al. 2001, Tran et al. 2008). In sites (particularly in lenthic systems) where natural Asian clam populations establish, scenarios of oxygen deprivation together with chemical contamination are likely to occur and tend to be common given the actual context of global climate change and increased man-driven contamination pressuring freshwater ecosystems worldwide (Cloern and Jassby 2012, Diaz 2001). On the other hand, in particular industrial environments, hypoxia can be used to increase chemical efficiency contributing to the improvement of biofouling control measures, although given the costs involved only specific systems would probably consider the use of such a demanding solution. In this context, the present study provides systematic information which allows concluding on the ability of hypoxia conditions to change *C. fluminea* sensitivity to candidate control chemicals with different modes of action. This baseline information allows to infer on whether intermediate levels of dissolved oxygen, which are more common in natural invaded systems or less costly to achieve in infested industrial systems when compared with anoxia, are worth investigating in the future. The results of the present study indicate that, in general, oxygen depletion increases the efficiency of biocides against the Asian clam, considering the mortality of juvenile and adult laboratory populations under short-term exposure conditions.

Both niclosamide and polyDADMAC have been assumed to exert toxicity in biological matrices through the impairment of cellular respiration: niclosamide was proven to affect the respiratory metabolism of snail pests possibly by induction of mitochondria fragmentation (Park et al. 2011, WHO 2002) whereas polyDADMAC is expected to cause disruption of membrane transfer mechanisms which may influence cellular gas exchange processes (Post et al. 1997). Despite the similarities in the chemical's mechanism of toxicity, oxygen level seems to influence their efficiency against the Asian clam at different scales. In fact, the results indicate that hypoxia levels contributed more to the efficiency of niclosamide than to the efficiency of polyDADMAC regardless the concentrations focused. Niclosamide is a molluscicide designed to act over a very specific physiological target while polyDADMAC is a flocculant that is expected to impair the respiratory metabolism by generally constraining gas exchange across biological membranes. The differences in the specificity of the biocides target within the organism may explain the variation in the efficiency of depressed oxygen levels in promoting the biocide effects. Both candidate biocides have great potential to be used integrated in management practices targeted at

the Asian clam. Niclosamide is short-lived in water (WHO 2002) which constitutes a major stimulus to its use; polyDADMAC is already used in the water treatment industry as a flocculant (Ariffin et al. 2012) and thus advantages may arise from its use as a multiple function chemical. However, one should recognise that only high concentrations of polyDADMAC deliver the relevant efficiency levels, even when hypoxia conditions are set in parallel to chemical dosage (concentrations as high as 3000 mg L^{-1} and 4000 mg L^{-1} resulted in less than 40 and 80% mortality for juveniles and adults, respectively, in the present study). According to former data on the environmental selectivity of this flocculant (Gomes et al., submitted), polyDADMAC elicits negative effects in standard indicator species (*Pseudokirchneriella subcapitata* and *Daphnia magna*) at lower concentrations, thus indicating its hazardous potential.

Although both copper sulphate and ammonium nitrate showed increased efficiency under deprived oxygen conditions, the former was effective at concentrations one order of magnitude lower than the latter. The chemical's mechanism of toxicity may also have played its role in distinguishing the chemicals as to their efficiency increase under hypoxia conditions (particularly evident as the response of adult clams is compared). Both copper and hypoxia are inducers of oxidative stress with the following structural, pathological and functional damage occurring frequently (Livingstone 2001, Lushchak 2011). In organisms lacking haemoglobin, which seems to be the case of *C. fluminea* (Tran et al. 2000), the mechanism of toxicity of ammonium nitrate should be coincident with the mechanism of toxicity of unionized ammonia, i.e., damage in the gill epithelium, stimulation of glycolisis, suppression of Krebs cycle, and uncoupling oxidative phosphorylation (Camargo and Alonso 2006). These effects link to the oxygen metabolism but eventually not as directly as the interference in reactive oxygen species balance does, hence the lower efficiency of the treatment with ammonium nitrate compared to copper sulphate.

Copper has a very long history of use as an antifoulant and its dosage is still considered one of the most effective and practical methods of preventing fouling in submerged structures (e.g. by its use as antifouling ink) (Piola et al. 2009). Its high toxicity to the Asian clam has already been registered (Mattice 1983) and testing of its potential as a chemical control method for this biofouler has already been carried out (Ingram 1959). LC_{50} values calculated with the results of the present study (data not shown) and recorded in other studies (Mattice 1983) reveal that concentrations between 0.94 mg L^{-1} and 6.77 mg L^{-1} are effective against adult clams following 96 h of exposure. However, Ingram (1959), refers that, in the field, much higher concentrations (750 mg L^{-1}) are necessary to control *C. fluminea* populations. Therefore, although copper sulfate seems a good candidate to integrate control programs for the Asian clam, further studies

considering larger-scale assessment and accounting to the chemical environmental selectivity should be envisaged. Despite its apparently lower efficiency against the Asian clam, advantages may also come from the use of ammonium nitrate as a biocide particularly in open waters already contaminated with this chemical due to it is use in agricultural activities (Mackie and Claudi 2010)

Because the complete dataset of this study could not be presented here (see the Introduction section), only the clam's responses to chemicals exerting toxicity over the respiratory metabolism can be discussed. Although the effects result from the chemical's action in targets of different physiological specificity, all refer to the same general metabolic pathway, which prevents deeper analysis on whether distinct mechanisms of toxicity play a role in defining the way oxygen conditions affect the responses to the chemical challenges.

Differences between individuals of different life-stages in chemical tolerance have been reported by some studies considering the Asian clam (e.g. Belanger et al. 1991, McMahon and Lutey 1988). Our results were in accordance, with differential sensitivity to the test conditions juveniles/young adults and older clams being consistently found. Different systems may be impacted by different *C. fluminea* life-stages, e.g. industrial strainers only allow the entrance of juvenile clams. In these cases, and facing the differences in sensibility to test conditions, stakeholders must prioritize information on juvenile clams rather than on adults.

Hypoxia in natural waters can be of spontaneous origin (e.g. seasonal stratification), but most of the times it results from human activities (Hattink et al. 2005). When considering industrial environments, oxygen deprivation may be accomplished by adding an oxygen-scavenging chemical into a closed system (e.g. Smithson 1986) or by keeping a static system (e.g. pipeline) for a sufficient time period (Mackie and Claudi 2010). However, industrial methods to achieve hypoxia are not always feasible, may require very long application times, and poor oxygen levels often result in a dramatic increase of sulfate-reducing bacteria which in turn causes corrosion of the materials (Johnson and McMahon 1998, Mackie and Claudi 2010). Therefore, stakeholders must carefully evaluate if the costs and impairments of employing oxygen deprivation compensate the benefits in pest management in each particular case. When dealing with chemical application, other concerns must be taken into account particularly in natural environments or when treated water is to be discharged into neighboring waterways (Mackie and Claudi 2010): chemicals and/or their by-products can be highly toxic to non-target organisms damaging the ecosystem. Environmental safety should hence be always an additional factor to the equation when managing invasive species. Some rules that

apply for the safer use of chemical control include its use in limited settings (i.e. closed systems or under static conditions) and/or conjugated with specific remediation strategies (e.g. application of bentonite clay to transform the active ingredient, thereby rendering it nontoxic) before discharging into the waterways (Fisher et al. 1991 , Mackie and Claudi 2010).

5.6. References

Anderson KB, Thompson CM, Sparks RE, Paparo AA. 1976. Effects of potassium on adult Asiatic clams, *Corbicula manilensis*. Biological Notes. Illinois Natural History Survey 98: 3-7.

Araujo R, Moreno D, Ramos MA. 1993. The Asiatic clam *Corbicula fluminea* (Müller, 1774) (Bivalvia: Corbiculidae) in Europe. American Malacological Bulletin 10: 39-49.

Ariffin A, Razali MAA, Ahmad Z. 2012. PolyDADMAC and polyacrylamide as a hybrid flocculation system in the treatment of pulp and paper mills waste water. Chemical Engineering Journal 179: 107-111.

Barinova SS, Yehuda G, Nevo E. 2010. Comparative analysis of algal communities in the rivers of northern and southern Israel as bearing on ecological consequences of climate change. Journal of Arid Environments 74: 765-776.

Beasley CR, Tagliaro CH, Figueiredo WB. 2003. The occurrence of the Asian clam *Corbicula fluminea* in the lower Amazon Basin. Acta Amazonica 33: 317-324.

Belanger SE, Cherry DS, Farris JL, Sappington KG, Cairns J. 1991. Sensitivity of the asiatic clam to various biocidal control agents. Journal American Water Works Association 83: 79-87.

Beltran KS, Pocsidio GN. 2009. Acetylcholinesterase activity in *Corbicula fluminea* Müller, as a biomarker of organophosphate pesticide pollution in Pinacanauan River, Philippines. Environmental Monitoring and Assessment 165: 331-340.

Camargo JA, Alonso A. 2006. Ecological and toxicological effects of inorganic nitrogen pollution in aquatic ecosystems: A global assessment. Environment International 32: 831-849.

Daum KA, Newland LW, Britton JC, Champagne L, Hagen J. 1977. Responses of *Corbicula* to potassium. First International *Corbicula* Symposium. Fort Worth, Texas, USA: Texas Christian University.

Diaz RJ. 2001. Overview of hypoxia around the world. Journal of Environmental Quality 30: 275-281.

Doherty FG, Farris JL, Cherry DS, Cairns Jr. J. 1986. Control of the Freshwater Fouling Bivalve *Corbicula fluminea* by Halogenation. Archives of Environmental Contamination and Toxicology 15: 535-542.

Doherty FG, Cherry DS. 1988. Tolerance of the asiatic clam *Corbicula* spp. to lethal levels of toxic stressors - a review. Environmental Pollution 51: 269-313.

Finney D. 1971. Probit Analysis. Cambridge, UK: Cambridge University Press.

Fisher SW, Bernard DO. 1991. Methods for evaluating zebra mussel control products in laboratory and field studies. Journal of Shellfish Research 10: 367-371.

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

Johnson PD, McMahon RF. 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.

Lushchak VI. 2011. Environmentally induced oxidative stress in aquatic animals. Aquatic Toxicology 101: 13-30.

Mackie GL, Claudi R. 2010. Monitoring and control of macrofouling mollusks in fresh water systems. Boca Raton, FL: CRC Press.

Matthews MA, McMahon RF. 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.

Mattice JS. 1983. Freshwater macrofouling and control with emphasis on *Corbicula*. Pages 1-30 in Diaz-Tous IA, Miller MJ, Mussalli IG, eds. Symposium on Condenser Macrofouling Control Technologies: The State of the Art. California, USA: Electric Power Research Institute.

Page T, Neitzel D, Simmons M. 1986. Biofouling of power plant service systems by *Corbicula* in Britton JC, ed. Proceedings of the Second International *Corbicula* Symposium: American Malacological Bulletin - Special edition no.2.

Park SJ, Shin JH, Kang H, Hwang JJ, Cho D-H. 2011. Niclosamide induces mitochondria fragmentation and promotes both apoptotic and autophagic cell death. Bmb Reports 44: 517-522.

Pimentel D, Zuniga R, Morrison D. 2005. Update on the environmental and economic costs associated with alien-invasive species in the United States. Ecological Economics 52: 273-288.

Post RM, Lacy JR, Lyons LA, Mueller M, Petrille JC, Shurtz WF (1997). A decade of macrofouling control using non-oxidizing compounds – An industry review. EPRI Condenser Conference, Boston.

Quinn GP, Keough MJ, eds. 2002. Experimental design and data analysis for biologists New York: Cambridge University Press.

Rosa IC, Pereira JL, Gomes J, Saraiva PM, Goncalves 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.

Smithson J. 1986. Development of a *Corbicula* control treatment at the Baldwin Power Station in Britton JC, ed. Proceedings of the Second International *Corbicula* Symposium: American Malacological Bulletin - Special edition no.2.

Sousa R, Novais A, Costa R, Strayer D. 2012. Invasive bivalves in fresh waters: Impacts from individuals to ecosystems and possible control strategies. Hydrobiologia.

Tran D, Boudou A, Massabuau JC. 2001. How water oxygenation level influences cadmium accumulation pattern in the asiatic clam *Corbicula fluminea*: A laboratory and field study. Environmental Toxicology and Chemistry 20: 2073-2080.

Tran D, Massabuau J, Garnierlaplace J. 2008. Impact of hypoxia on hemolymph contamination by uranium in an aquatic animal, the freshwater clam *Corbicula fluminea*. Environmental Pollution 156: 821-826.

USEPA. 2006. Reregistration eligibility decision for dimethoate in USEPA, ed.

WHO. 1965. Molluscicide screening and evaluation. Bulletin of the World Health Organization 33: 567-581.

WHO. 2002. WHO specifications and evaluations for public health pesticides - Niclosamide. Geneva.

Williams EH, Bunkley-Williams L, Lilyestrom CG, Ortiz-Corps EAR. 2001. A review of recent introductions of aquatic invertebrates in Puerto Rico and implications for the management of nonindigenous species. Caribbean Journal of Science 37: 246-251.

CHAPTER 6

Bioremediation of a metal-rich effluent by the invasive bivalve *Corbicula fluminea*

Bioremediation of a metal-rich effluent by the invasive bivalve *Corbicula fluminea*

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6.1. Abstract

Industrial effluents are important sources of contamination of water and sediments, frequently causing serious damage at different levels of biological organization. Management and treatment of harmful industrial wastes is thus a major concern. Metal-bearing effluents, such as acid mine drainage (AMD), are particularly problematic because metals can easily bioaccumulate in organisms and biomagnify across the trophic chain. Several solutions have been proposed to treat AMD, including active methods involving the addition of neutralizing agents, and passive techniques that use natural energy sources for remediation. However, increasing environmental and economic requirements lead the constant search for more sustainable solutions. The present study explores the potential of *Corbicula fluminea*, an invasive freshwater bivalve, as a bioremediation tool using AMD as a model effluent. The study compares unfiltered and bio-filtered effluents at two dilution levels (v/v: 4 % and 10 %) following two distinct approaches: (i) chemical characterization of the metal concentrations in water complemented by determination of the accumulation in the clams' soft tissues and shells; and (ii) ecotoxicity assessment using standard organisms (the bacteria *Vibrio fischeri*, the microalgae *Pseudokirchneriella subcapitata* and the cladoceran *Daphnia magna*). Significant removal of metals from water was recorded for both effluent dilutions, with higher purification levels found for the 4 % effluent. The environmental toxicity of the effluents generally decreased after the bio-filtration treatment. This study thus provides evidence on the potential of *C. fluminea* as a bioremediator for AMD, especially if the treatment can be materialized in a multi-stage configuration system.

Keywords: *Corbicula fluminea*; Bioremediation; Acid Mine Drainage (AMD); Bio-filtration; Effluent treatment

6.2. Introduction

Contamination of water and sediment by industrial effluents, such as those from mining, metallurgical, electronic and pulp and paper industries, occurs worldwide (Zhou et al. 2008). The metal mining industry is one of the largest waste producers in the world, often leading to concerning pollution scenarios (Hudson-Edwards et al. 2011, Lottermoser 2007). Wastes from this industry are heterogeneous, frequently rich in ore minerals such as sulphides or oxides (Lottermoser 2007). These wastes tend to be stored in tunnels, shafts and open pits surrounding the mine, becoming exposed to weathering processes (Alakangas et al. 2010, Christensen et al. 1996, Hudson-Edwards et al. 2011) that result in the oxidation and hydrolysis of sulfides mediated by bacteria. Sulfuric acid is thus produced and leached, promoting the release of metal and semi-metal components from the mineral wastes (Blodau 2006). This highly acidic, metal- and sulphate-rich acid mine drainage (AMD) frequently contaminates neighboring environmental compartments. The very low pH and high metal burden of AMD were already proven to degrade groundwater, surface water, soil and air quality (El Khalil et al. 2008, Huertas et al. 2012) and promote noxious impacts at several levels of biological organization (Peplow and Edmonds 2005).

The mitigation of the adverse physical, chemical, biological and socioeconomic impacts of AMD is one of the major challenges faced by the mining industry worldwide, and many countries have been investing in the development of efficient treatment methods for mining wastewater (Ulrich 1999). Several techniques are available for the treatment of metal-bearing effluents such as AMD. These include active and passive methods, the latter favoring abiotic or biotic approaches. Active methods involve the continuous addition of chemical neutralizing agents, for example lime and limestone (e.g. Lopes et al. 1999a). Several drawbacks are associated to the use of this type of agents, including high costs related to the large amount of chemicals that is necessary (Garcia et al. 2001), the need for continuous maintenance and monitoring (Lottermoser 2007) and poor environmental friendliness due to the production of toxic sludge, which needs to be relocated (Touze et al. 2008). Despite these pitfalls, active methods followed by sludge removal and disposal are the most common approach to treat AMD (Costa and Duarte 2005). On the other hand, passive methods profit from naturally available energy sources, such as gravity, photosynthesis and microbial metabolic energy (Roetting et al. 2008). These methods involve simple equipment and require reduced or no chemical addition and maintenance efforts (Roetting et al. 2008). Good examples of passive methods are anoxic limestone drains (Ulrich 1999) and bioreactors (Touze et al. 2008). Passive alternatives also have some drawbacks,

including short lifetime as a consequence of clogging resulting from the formation of precipitates (Roetting et al. 2008), and the fact that alkaline additives used so far do not meet all the requirements of an ideal reagent (for more details see Perez-Lopez et al. 2011).

The development of treatment methods that use living organisms to remediate AMD and other metal-bearing effluents has been encouraged. These may have much less disadvantages than the abiotic approaches (Akpor and Muchie 2010). Some examples of suggested bioremediators for metal-bearing effluents include bivalves (Gifford et al. 2004, Jana and Das 1997) and sponges (Gifford et al. 2007). In this context, the present study explores the potential of the freshwater bivalve *Corbicula fluminea* (Müller, 1774), commonly known as the Asian clam, as a bioremediation tool for metal-rich effluents, such as AMD.

C. fluminea is an invasive freshwater bivalve native to Southeast Asia that is now spread worldwide, causing important economical (e.g. Page et al. 1986, Rosa et al. 2011) and ecological (e.g. Cherry et al. 2005, Sousa et al. 2008) impacts. Several studies addressed the role of the Asian clam as a sentinel both for potentially toxic metals and organic pollutants (e.g. Beltran and Pocsidio 2009, Doherty 1990). Such a possible use for the bivalve exploits its ability to bioaccumulate chemicals in the tissues along with a fairly large tolerance to the effects resulting from exposure to the contaminants (Colombo et al. 1995, Marie et al. 2006). In addition to the attributes above, the Asian clam has significant filtering capabilities, with filtration rates as high as 33 $ml.h^{-1}.g^{-1}$ (Cohen et al. 1984). The possibility of further exploiting such capabilities for bioremediation purposes has been investigated by some authors. For example, in the Potomac River (USA) during the 1980's, the Asian clam biomass was directly related to fluctuations in water quality, demonstrating the species potential as a bioremediator for eutrophication scenarios (Cohen et al. 1984). Li et al. (2010) also suggested such a use for the species as incorporated in a floating-bed system together with a plant and biofilm carrier.

The main aim of the present study was to evaluate the bioremediation potential of *C. fluminea* using AMD as an experimental model effluent. Such an evaluation was carried out considering both the chemical and the biological perspectives, and thus involved (i) the quantification of metal concentration changes in AMD solutions following filtration by the Asian clam; and (ii) the comparison of the ecotoxicological responses of standard organisms to unfiltered and bio-filtered effluents. The ability of the clams to accumulate chemicals from complex mixtures such as industrial effluents has been poorly studied (see Doherty 1990), which reinforces the novelty and relevance of the data reported here.

6.3. Material and methods

6.3.1. AMD sampling

The AMD samples were collected in São Domingos mine, Mértola, Portugal (N37°40'07.06''/W7°29'54.31''). This is a deactivated cupric and pyrite mining complex that exploited the Iberian Pyrite Belt. As exploitation finished in 1965, the mine presently consists of an open pit permanently flooded together with a network of channels and settlement ponds draining to the final mine effluent (Lopes et al. 1999a). Plans to recuperate the area have been delineated (MINEO 2000), but so far large volumes of AMD still form and remain untreated. The highly concentrated sulphates and metals of the AMD (Table 6.1) severely pollute nearby areas and affect their biological communities (Lopes et al. 1999b, Pereira et al. 2006). The water samples used in the present study were collected in the Caçador settlement pond in February 2012. Conductivity (2.52 mS cm^{-1}) and pH (2.58) were measured *in situ* with a multiparameter field probe (WTW-Multi3430). The water was collected into plastic containers (1.5 L) previously rinsed with 10 % HCl. The samples were transported under refrigeration to the laboratory and stored at -20 ºC until use. Additional samples were collected for hardness (461 mg L^{-1} $CaCO_3$) determination (by colorimetric methods using PC Multi Aqualytic®) and metal quantification. The samples aimed at metal characterization were acidified with nitric acid (65 % PA) to pH < 2 immediately after collection and then stored at 4 ºC until analysis (see below for details on the analytical procedures).

6.3.2. Test organisms

Adult *C. fluminea* individuals were collected in Casal de São Tomé, Mira, Portugal (N40°25'06.90''/W8°44'13.18''). The site is a sandy-muddy shallow creek with a well-established Asian clam population with densities ranging from 2000 to 4000 individuals m^{-2}. The clams were collected using a shovel to drag sediment into a 1-cm mesh size bag, which was then used to roughly sieve the sample and separate the clams. The individuals were transported to the laboratory, where the field water was gradually replaced by dechlorinated municipal water. The animals were maintained under continuous aeration at constant temperature (20 ± 2 ºC) and photoperiod (16 h^L: 8 h^D) until use. The culture water was fully renewed once a week and the clams were fed *ad-hoc* with *Pseudokirchneriella subcapitata* suspensions (see below for culturing procedures). Test organisms with shell length within the range 21-27 mm were

selected from the laboratory culture and not fed for 4 days prior the beginning of the tests.

The ecotoxicological assessment of the effluents was carried out using the standard aquatic species *Vibrio fischeri* (supplied in liophilised form as part of the Microtox® kit), *P. subcapitata* and *Daphnia magna*. The green microalgae *P. subcapitata* has for long been cultured as a nonaxenic batch culture, in synthetic Woods Hole MBL medium (Stein 1973) renewed weekly, at 20 ± 2 ºC and under permanent illumination. Monoclonal bulk cultures of the cladoceran zooplankter *D. magna* had been reared in the laboratory in synthetic ASTM hard water medium (ASTM 1980), supplemented with a standard organic additive (Baird et al. 1989) and vitamins (Elendt and Bias 1990). Temperature and photoperiod were kept constant at 20 ± 2 ºC and 16 h^L: 8 h^D, respectively. The cultures were renewed three times per week and fed with *P. subcapitata* suspensions (3 x 10^5 cells mL^{-1}).

6.3.3. Bio-filtration experiment

To gain insight into the potential effect of effluent concentration on the clams' depuration ability, the AMD was tested at two different dilution levels, 4 and 10 % (v/v), hereinafter AMD-4 and AMD-10, respectively. The effluent was diluted in dechlorinated municipal water. The two dilution levels were selected so that no significant mortality would occur at the same time as the animals were exposed to considerable pollutant contents that could be removed from the water. In a preliminary mortality test, under similar conditions to those in the bio-filtration experiment, AMD-4 and AMD-10 provoked 13.3 ± SD 5.8 and 20.0 ± SD 17.3 % mortality, respectively.

The bio-filtration experiment consisted of a 7-day static exposure to previously filtered (1.5 µm porosity) AMD-4, AMD-10 and a blank control of dechlorinated municipal water (AMD-0). The assay was initiated after a 12-h acclimation period in the test vessels, over which all bivalves were confirmed to be actively filtering. Three replicates were used per treatment, each consisting of 10 clams in 1.5 L of test medium. The vessels were kept continuously aerated and under constant temperature (20 ± 2 ºC) and photoperiod (16 h^L : 8 h^D). Clam mortality was assessed daily, with dead individuals being discarded. The animals were considered alive if showing evident siphoning activity or offering resistance to valve opening as carefully forced with a blunt dissection needle. Water samples were collected at the beginning (pre-filtration effluent; one sample per treatment) and the end (bio-filtered effluent; one sample per replicate) of the bio-filtration experiment. Such samples were aimed at chemical analysis and ecotoxicological assessment (see below for details). The samples used for chemical analysis were acidified (HNO_3 65 % PA) at pH < 2

immediately after collection and then stored at 4 ºC. The samples used in the ecotoxicological tests were preserved at -20 ºC. The water quality was monitored throughout the bio-filtration experiment. Temperature, pH, conductivity and oxygen concentration were measured daily with a multiparameter probe (WTW-Multi3430). Colorimetric methods (PC Multi Aqualytic®) were used to determine ammonium concentration and alkalinity at the beginning, middle (day 3) and end of the test. Hardness at the beginning and end of the experiment was obtained from Ca and Mg concentrations (see 2.5 for details). At the end of the assay, the clams from each vessel were pooled and preserved at -20 ºC for metal quantification in tissues and shells (see 2.5 for details).

6.3.4. Ecotoxicological assessment

V. fischeri luminescence inhibition was assessed by exposing the bacteria for 5, 15 and 30 minutes to pre-filtration and bio-filtered AMD-4, AMD-10 and AMD-0. The commercial Microtox® test kit was used and the standard operational procedure provided by the manufacturer (liquid-phase 81.9 Basic Test) was strictly followed.

Growth inhibition of *P. subcapitata* was assessed following the OECD guideline 201 adapted to 24-well microplate use. Microalgae were exposed to a geometric dilution series (12.5, 25, 50, 75, and 100 %) of pre-filtration and bio-filtered AMD-0, AMD-4 and AMD-10, with the appropriate nutrient supply being added in all cases. A blank control of Woods Hole MBL medium was also set. The treatments were applied in triplicate, each replicate consisting of 990 μL of test solution plus 10 μL of concentrated inoculum (10^6 cells mL^{-1} adjusted based on microscopic cell counting). The microplates were incubated for 72 h at 23 ± 1 ºC, under continuous illumination and agitation (100 rpm). The contents of each well were thoroughly mixed twice a day by repetitive pipetting to prevent cell clumping. At the end of the assay, the algal density in the wells was estimated through a previously established calibration curve relating absorbance at 440 nm (UV-1800 Spectrophotometer; Shimadzu) to microscopic cell counts. Growth rates, GR (day^{-1}), were calculated according to the expression $GR = \ln (X_f) - \ln (X_i) / \Delta t$, where X_f (cells mL^{-1}) stands for cell density at the end of the test, X_i (cells mL^{-1}) for the cell density used to start the test (10^4 cells mL^{-1}) and Δt (day) for the length of the test.

Immobilisation of *D. magna* was assessed following the OECD guideline 202. A static design was applied, using twenty neonates (< 24 h-old and born within the 3^{rd}-5^{th} brood in the cultures) per treatment, with five animals being randomly assigned to each of four replicates. The tests were carried out in glass vials filled with 25 mL of test solution or clean ASTM medium for the blank control. The daphnids were exposed to a

geometric dilution range of pre-filtration AMD-0, AMD-4, and AMD-10 (6.25, 12.5, 25, 50 and 100 %) and bio-filtered AMD-0 (6.25 to 100%; ratio = 2), AMD-4 (12 to 97.9%; ratio = 1.3) and AMD-10 (8 to 65.3%; ratio = 1.3). Assay conditions were kept as described above for culturing procedures. The test vessels were screened for immobilised daphnids after a 48-h exposure period.

6.3.5. Chemical analysis

The total concentration of Ag, Al, Ar, As, B, Ba, Be, Ca, Cd, Co, Cr, Cu, Fe, Hg, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, S, Sb, Se, Si, Sn, Sr, Ti, Tl, V and Zn were quantified in both water and biological samples, the latter consisting of soft tissues and shells of the clams used in the bio-filtration experiment. All analyses were performed externally by an accredited laboratory (according to DIN EN ISO/IEC 17025 notification under the DAkkS German Accreditation System for Testing). The quantification of all metals but Hg in water samples was carried out by inductively coupled plasma spectroscopy (ICP-OES) (ISO guideline 17294-2:2003 for Ag, Ar, Cd, Pb, Sb, Se, Ti, and Tl; ISO guideline 11885:2007 for all other metals). Atomic fluorescence spectrometry was used to quantify Hg concentration in water (ISO guideline 17852:2006). The metal concentrations in the solid matrices were determined by ICP-MS (ISO guideline 11885:2007 for Si and ISO guideline 17294-2:2005 for the other metals; due to methodological limitations, Ti was not determined in the clams' shells). Before quantification, the water samples were filtered (1.5 µm porosity); the soft tissues were cold dried, grinded and then subjected to microwave-assisted digestion with nitric acid PA; the shells were dried at 40 ºC, grinded and then digested with *aqua regia*.

6.3.6. Statistical analysis

Variation of metal concentrations in water was determined as a percent of the element initial contents, which in practice traduces clams' purification capacity. MANOVA was applied to identify significant differences in the variation of metal concentration for AMD-0, AMD-4 and AMD-10. The Pillai's Trace criterion was considered in the analyses (Huberty and Olejnik 2006). As significant differences between groups (effluent dilution levels) were found, one-way ANOVA, followed by Tukey *post-hoc* test, was performed in order to scrutinize the effect of effluent dilution level on each metal. The Bonferroni procedure was used to adjust the associate significance level of the family-wise type I error (Quinn and Keough 2002) hence a significance level of 0.017 was used in the analyses. A similar approach was followed to identify significant differences in metal concentrations in the clams' shells and soft tissues. Statistical analysis of the changes in metal concentrations in water as a result of filtration by the

clams was addressed by paired-sample t-tests. Pearson correlation was applied to assess the degree of association between metal removal from water and accumulation in the clams' soft tissues and shells. The difference between the metal concentrations in the pre-filtration and bio-filtered samples rather than the percent of the initial contents (which were previously used to prevent scale effects in the analyses) was considered here. Metal concentrations found below the detection limits were considered null for calculation purposes.

The analysis of the ecotoxicological data was based on the comparison between the EC_x values found for the pre-filtration bio-filtered effluents. *V. fischeri* bioluminescence and microalgae growth inhibition EC_{50} values and respective 95% confidence intervals were estimated by non-linear regression using the least-squares method to fit the logistic equation to the data (OECD guideline 201). *D. magna* immobilisation EC_{20} values and corresponding 95% confidence intervals were estimated by probit analysis (OECD guideline 202).

Unless stated otherwise, a significance level of 0.05 was considered in the analyses.

6.4. Results

As expected, clam mortality at the end of the bio-filtration test was very low, being null in the case of AMD-0 and 6.7 ± 11.5 and 16.7 ± 11.5 % (mean ± SD) for AMD-4 and AMD-10, respectively. Fig. 6.1 provides a detailed picture of the water quality during the bio-filtration test. Temperature and dissolved oxygen levels were similar in all treatments and kept generally constant throughout the test period. Lower pH levels and higher ammonia concentrations were recorded for AMD-10 as compared to the other treatments, with pH slightly increasing consistently throughout the experimental period. Conductivity and hardness increased with increasing effluent dilution while alkalinity had an opposite behavior, the three of them increasing across the test period.

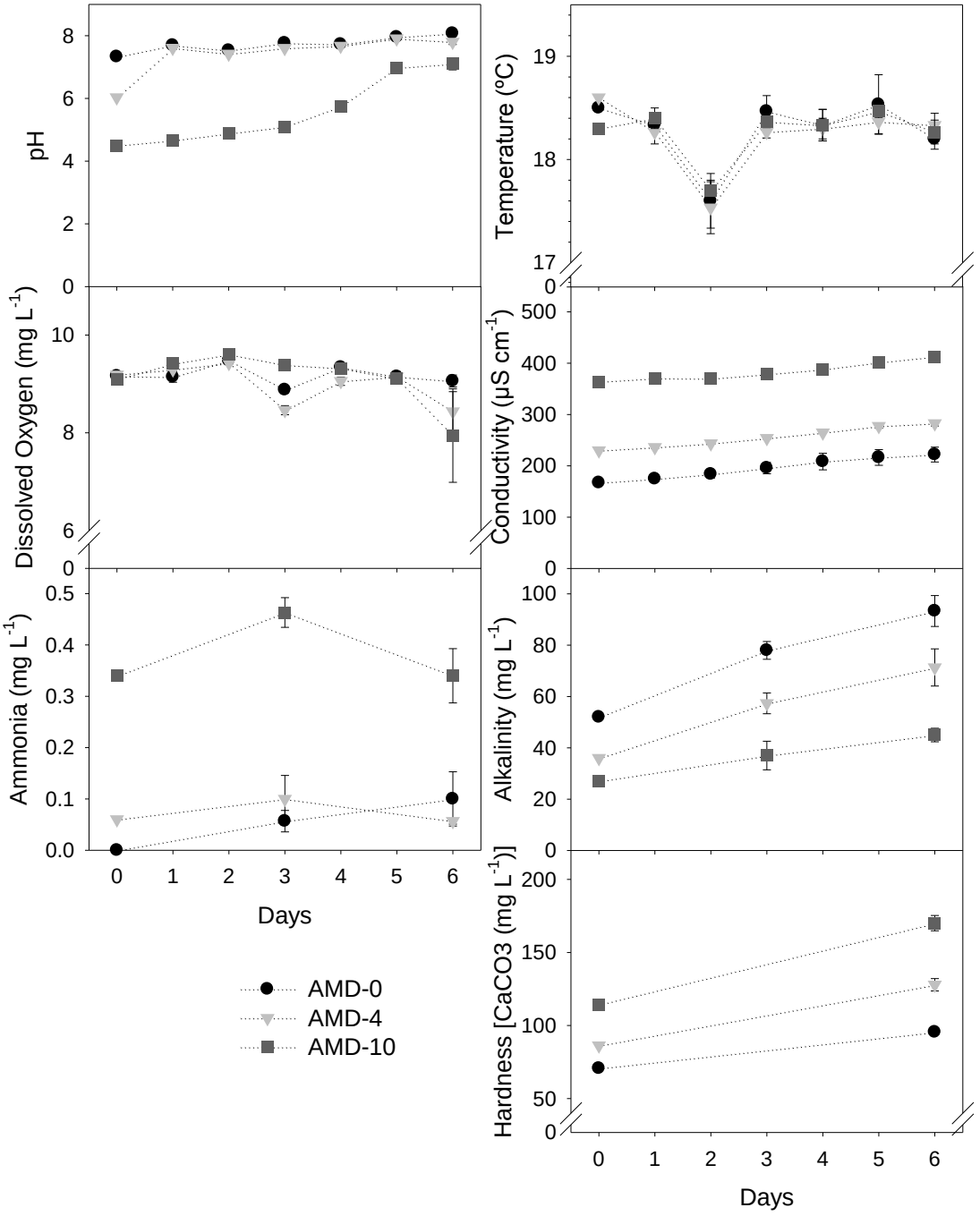

Fig. 6.1. Water quality parameters measured over the bio-filtration test. The points represent the mean of three experimental replicates and the error bars represent the corresponding standard deviation; the dotted lines represent no adjusted model and were used for clarity purposes only.

The variation of metal concentration in water due to bio-filtration was significantly affected by the AMD dilution level (one-way MANOVA: $F_{(12, 4)} = 466.011$; $p < 0.001$; Pillai's Trace = 1.999; partial $\varepsilon^2 = 0.999$), but no general dilution level-related trend was observed amongst the metals (Fig. 6.2; Table 6.1).

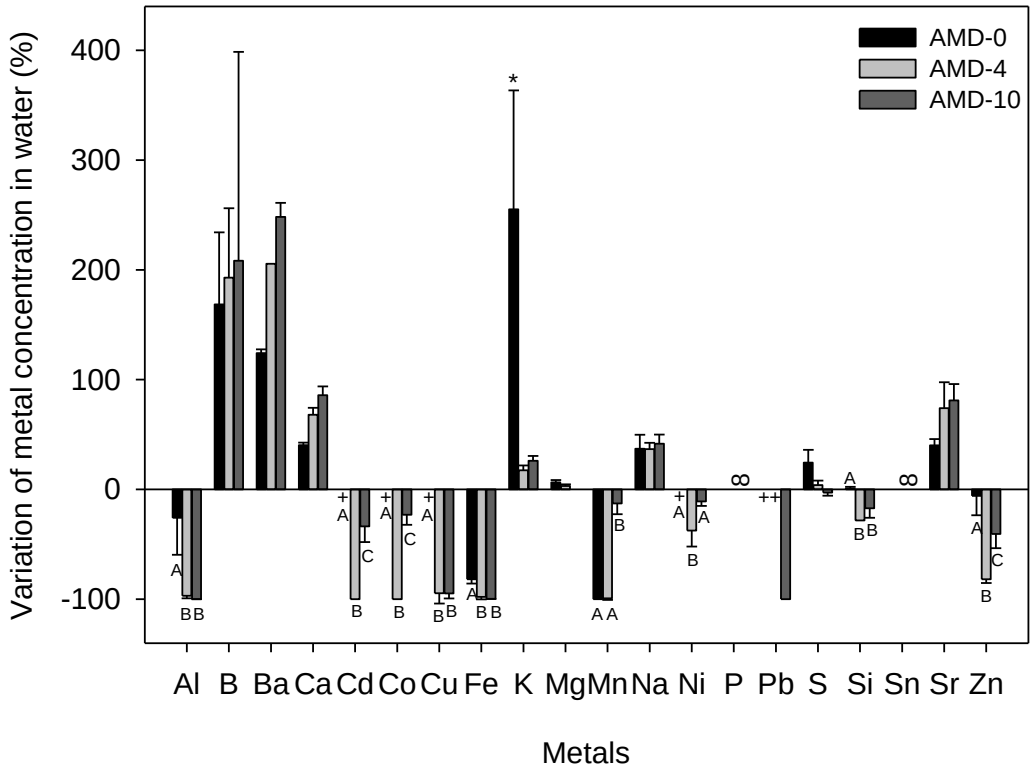

Fig. 6.2. Variation of metal concentrations in water (as a percent of the element initial contents) due to bio-filtration by *C. fluminea*. Only elements detected in at least one treatment replicate (see Table 6.1) were included in the graph; amongst these, + represents the treatments where the metal could not be detected at all and hence no concentration variation could be computed. Concentrations below the detection limits (see Table 6.1) were considered null for calculation purposes. ∞ stands for infinite variations obtained for the metals that were present in the bio-filtered medium but not in the pre-filtration form. Letters denote significant differences between treatments (Tukey *post-hoc* test).

Table 6.1. Metal concentrations in the original effluent from São Domingos mine and in samples taken at the beginning (pre-filtration) and end (bio-filtered) of the bio-filtration experiment. For bio-filtered samples, mean ± SD values (considering the three replicate treatments) are presented. bdl denotes the cases where the metals were present at levels below the respective detection limits.

Metal	Detection limit (mg L^{-1})	Original effluent (mg L^{-1})	Concentration in pre-fltration samples (mg L^{-1})			Concentration in bio-filtered samples (mg L^{-1})		
			AMD-0	AMD-4	AMD-10	AMD-0	AMD-4	AMD-10
Ag	0.001	0.001	bdl	bdl	bdl	bdl	bdl	bdl
Al	0.01	81.00	0.03	4.00	9.90	0.02 ± 0.01	0.13 ± 0.09	0.01 ± 0.01
Ar	0.001	0.001	bdl	bdl	bdl	bdl	bdl	bdl
As	0.001	0.001	bdl	bdl	bdl	bdl	bdl	bdl
B	0.005	0.210	0.019	0.033	0.048	0.050 ± 0.012	0.967 ± 0.021	0.148 ± 0.021
Ba	0.01	0.12	0.03	0.04	0.05	0.06 ± 1.00x10^{-3}	0.11	0.16 ± 0.01
Be	0.001	0.007	bdl	bdl	bdl	bdl	bdl	bdl
Ca	0.02	48.00	24.00	24.00	26.00	33.67 ± 0.58	40.33 ± 1.53	48.33 ± 2.08
Cd	0.001	0.065	bdl	0.002	0.008	bdl	bdl	0.005 ± 0.001
Co	0.01	0.86	bdl	0.04	0.11	bdl	bdl	0.09 ± 0.01
Cr	0.005	0.024	bdl	bdl	bdl	bdl	bdl	bdl
Cu	0.01	1.40	bdl	0.07	0.16	bdl	4.00x10^{-3} ± 0.01	8.00x10^{-3} ± 0.01
Fe	0.01	67.00	0.24	3.70	8.90	0.04 ± 0.01	0.08 ± 0.09	0.03 ± 0.01
Hg	0.00005	bdl	bdl	bdl	bdl	bdl	bdl	bdl
K	0.50	15.00	2.30	2.30	2.30	8.17 ± 2.50	2.70 ± 0.10	2.90 ± 0.10
Li	0.05	0.28	bdl	bdl	bdl	bdl	bdl	bdl
Mg	0.01	83.00	2.60	6.40	12.00	2.78 ± 0.06	6.60 ± 0.10	12.00
Mn	0.01	15.00	0.02	0.83	2.10	bdl	0.01 ± 0.01	1.83 ± 0.21
Mo	0.01	bdl	bdl	bdl	bdl	bdl	bdl	bdl
Na	0.05	35.00	9.00	10.00	12.00	12.33 ± 1.16	13.67 ± 0.58	17.00 ± 1.00
Ni	0.005	0.470	bdl	0.024	0.061	bdl	0.015 ± 0.003	0.054 ± 0.003
P	0.1	bdl	bdl	bdl	bdl	0.4 ± 0.1	0.2 ± 0.1	bdl
Pb	0.001	0.016	bdl	bdl	0.002	bdl	bdl	bdl
S	0.05	420.00	2.60	25.00	57.00	3.23 ± 0.31	26.00 ± 1.00	55.33 ± 1.53
Sb	0.001	bdl	bdl	bdl	bdl	bdl	bdl	bdl
Se	0.001	0.001	bdl	bdl	bdl	bdl	bdl	bdl
Si	0.01	0.34	4.00	3.90	3.70	4.03 ± 0.06	2.80	3.07 ± 0.32
Sn	0.005	0.009	bdl	bdl	bdl	0.013 ± 0.006	0.006 ± 0.005	0.012 ± 0.011
Sr	0.01	0.18	0.04	0.05	0.06	0.06 ± 3.00x10^{-3}	0.09 ± 0.01	0.11 ± 0.01
Ti	0.01	bdl	bdl	bdl	bdl	bdl	bdl	bdl
Tl	0.001	0.003	bdl	bdl	bdl	bdl	bdl	bdl
V	0.01	bdl	bdl	bdl	bdl	bdl	bdl	bdl
Zn	0.01	49.00	0.17	2.70	7.40	0.16 ± 0.03	0.49 ± 0.09	4.40 ± 0.01

Subsequent one-way ANOVA indicated that the concentration in water of all quantified metals, except B, Na, Sn and Sr, experienced changes during the bio-filtration period that significantly depended on the treatment (Table S6.1). A limited group of relevant metals (Al, Cd, Co, Cu, Fe, Mn, Ni, Pb, Si, and Zn) was selected for further analysis (Fig. 6.3). The quantified elements (i.e. present at levels above the respective detection limits) whose concentration either increased or did not change significantly (as assessed by the paired-sample t-test statistics) over the bio-filtration test (Fig. 6.2; Table 6.1) were excluded from this group because the focus of the study was on metal removal from water. The clams' capacity to remove metals from water depended on both the effluent dilution level and the metal itself (Fig. 6.2 and 6.3; Table 6.1). The concentration in AMD-4 and AMD-10 of almost all the selected metals underwent statistically significant reduction due to filtration by *C. fluminea* (Fig. 6.3). For these metals, effluent dilution influenced the clam purification capacity. In general, when the effluent dilution level increased, the purification degree provided by the clams

as measured by the relative decrease of the metals' initial contents decreased (Fig. 6.2). Such a dilution level-dependent behavior was observed for Cd, Co, Mn, Ni, Si and Zn, being Si the only metal where this trend was not statistically significant. The relative decrease of the initial contents of Al, Cu and Fe was similar for AMD-4 and AMD-10 (Fig. 6.2). It is worth noticing that when absolute metal removal (as the difference between the elements' concentrations in pre-filtration and bio-filtered samples) rather than relative purification degrees are considered, a different trend was observed for some metals, with clams exposed to increasing effluent dilution levels actually removing higher amounts of metal. This was the case for Al, Cd, Cu, Fe and Zn (Fig. 6.3.b). For example, for Zn the absolute removal from AMD-10 was 1.4 times higher than that from AMD-4 (Fig. 6.3.b). However, since the initial Zn concentration in AMD-10 was almost three times higher than that in AMD-4, actual metal removal corresponded to purification levels of 81% in AMD-4 but only 41% in AMD-10 (Fig. 6.2). In AMD-0, metal concentrations were below or just above the detection limits, and significant decrease resulting from clam filtration occurred only in the case of Fe and Mn (Fig. 6.2 and 6.3).

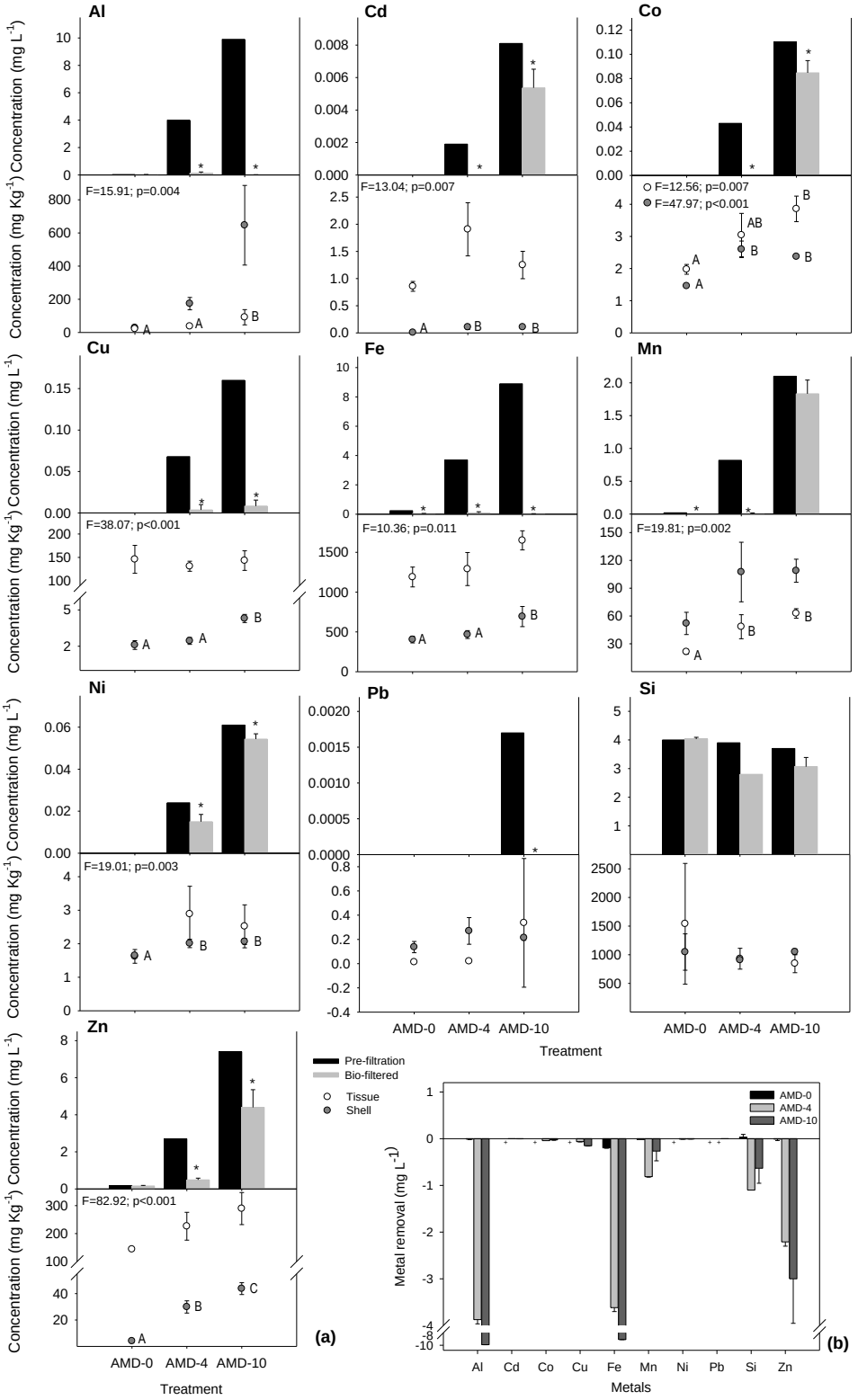

Fig. 6.3. (a) Concentration of selected metals in pre-filtration and bio-filtered media (upper bar graphs) and in the tissues and shells of the test clams (lower point graphs). Letters denote significant effects of the treatment (Tukey *post-hoc* test) on metal accumulation in the clams' tissues and shells. * denotes significant differences (paired-sample t-test) between metal concentrations in pre-filtration and biofiltered samples. **(b)** Absolute metal removal (as the difference between the concentrations in the pre-

filtration and bio-filtered samples) from water of selected metals due to bio-filtration by *C. fluminea*. + represents treatments where the metal could not be detected at all.

Along with the analysis of aqueous samples, metals were also quantified in *C. fluminea* soft tissues and shells (Table 6.2) in an attempt to establish a relationship between metal loss from water and accumulation in clams. In the search for such a relationship, particular attention was given to the sub-set (see above) of metals whose concentration in water decreased (Fig. 6.3.a). AMD dilution level significantly affected metal concentration in clams' tissues (one-way MANOVA: $F_{(12, 4)} = 12.130$; $p = 0.010$; Pillai's Trace = 1.947; partial $\varepsilon^2 = 0.973$) and shells (one-way MANOVA: $F_{(12, 4)} = 40.076$; $p = 0.001$; Pillai's Trace = 1.984; partial $\varepsilon^2 = 0.992$). Subsequent one-way ANOVA indicated that, for most metals, the concentration in the tissues did not significantly varied with the treatment (Fig. 6.3.a; Table S6.1), but Al, Co and Mn, which were also effectively removed from water (Fig. 6.2), accumulated significantly more in the soft tissues of clams exposed to AMD-4 and AMD-10 than in clams filtering AMD-0 (Fig. 6.3.a, Table S6.1). Cd, Co, Ni and Zn accumulated significantly more in the shells of clams exposed to AMD-4 and AMD-10 compared to untreated clams (AMD-0) (Fig. 6.3.a, Table S6.1) while Cu and Fe accumulated significantly more in clams exposed to AMD-10 compared to clams exposed to the other two treatments (Fig. 6.3.a, Table S6.1). For the metals that were actually removed from water (Fig. 6.3), the concentration decrease observed over the bio-filtration experiment was significantly correlated with metal concentration in the clams' shells (7 out of 10 metals) or both shells and tissues (4 out of 10 metals). Significant correlation between variation in metal water concentration and accumulation in clams (tissues or shell) was not found for Mn, Pb and Si (Table 6.3).

Table 6.2. Metal concentrations in soft tissues and shells of *C. fluminea* after the bio-filtration experiment. Mean ± SD values (considering the three replicate treatments) are presented. bdl denotes metals present at levels below the respective detection limits. ND denotes the cases where methodological shortcomings prevented metal quantification (see Materials and methods section for details).

Metal	Detection limit (mg Kg^{-1})	Concentration in the soft tissues (mg Kg^{-1})			Concentration in the shells (mg Kg^{-1})		
		AMD-0	AMD-4	AMD-10	AMD-0	AMD-4	AMD-10
Ag	0.01	0.74 ± 0.32	0.30 ± 0.02	0.25 ± 0.01	bdl	bdl	bdl
Al	0.3	28.7 ± 1.9	174.0 ± 37.2	647.0 ± 240.0	21.9 ± 1.40	37.2 ± 7.6	91.8 ± 45.9
Ar	0.02	7.71 ± 1.29	6.56 ± 0.17	6.79 ± 0.67	bdl	bdl	bdl
As	0.02	bdl	bdl	bdl	bdl	bdl	bdl
B	0.5	1.0 ± 0.2	1.1 ± 0.5	1.4 ± 0.1	bdl	bdl	bdl
Ba	0.09	20.12 ± 2.05	24.74 ± 4.08	27.35 ± 8.59	31.60 ± 5.48	34.00 ± 3.61	35.03 ± 3.26
Be	0.1	bdl	bdl	bdl	bdl	bdl	bdl
Ca	10	3200 ± 141	6000 ± 3969	5867 ± 252	$3.9x10^5$ ± 4725	$3.8x10^5$ ± 3512	$3.8x10^5$ ± 577
Cd	0.006	0.857 ± 0.090	1.910 ± 0.489	1.250 ± 0.252	0.007 ± 0.006	0.107 ± 0.046	0.107 ± 0.012
Co	0.01	1.98 ± 0.15	3.04 ± 0.68	3.86 ± 0.40	1.46 ± 0.03	2.60 ± 0.26	2.40 ± 0.04
Cr	0.01	bdl	bdl	bdl	0.07 ± 0.2	0.09 ± 0.02	0.12 ± 0.01
Cu	0.05	145.93 ± 29.72	131.17 ± 10.65	143.30 ± 20.92	2.10 ± 0.36	2.44 ± 0.29	4.29 ± 0.34
Fe	0.1	1190.0 ± 124.9	1290.0 ± 208.1	1650.0 ± 120.0	403.3 ± 41.6	466.6 ± 49.3	693.3 ± 126.6
Hg	0.006	1.113 ± 0.251	0.707 ± 0.075	0.627 ± 0.067	bdl	bdl	bdl
K	1.2	2706.7 ± 37.9	1950.0 ± 343.9	2096.7 ± 110.1	77.0 ± 64.3	27.6 ± 8.7	118.3 ± 78.2
Li	0.3	0.5 ± 0.5	0.3 ± 0.02	0.7 ± 0.06	bdl	bdl	bdl
Mg	0.2	1103.3 ± 61.1	1023.3 ± 40.4	1036.7 ± 46.2	22.5 ± 11.9	17.3 ± 8.7	41.0± 18.0
Mn	0.02	21.30 ± 2.65	48.43 ± 12.96	62.78 ± 5.14	51.97 ± 12.04	107.47 ± 32.15	108.83 ± 12.45
Mo	0.01	bdl	bdl	bdl	bdl	bdl	bdl
Na	0.5	4206.7 ± 818.2	2850.0 ± 490.0	2193.3 ± 158.9	2640.0 ± 120.0	2606.7 ± 75.7	2660.0 ± 96.4
Ni	0.1	1.6 ± 0.2	2.9 ± 0.8	2.5 ± 0.6	1.1 ± 0.02	2.0 ± 0.1	2.1 ± 0.1
P	3	9063 ± 356	8597 ± 439	8690 ± 157	83 ± 10	90± 6	1227 ± 12
Pb	0.01	0.01 ±0.01	0.02	0.34 ± 0.53	0.14 ± 0.05	0.27 ±0.11	0.21 ± 0.02
S	50	8873 ± 160	8726 ± 178	8597 ± 47	273 ± 49	373 ± 12	377 ± 32
Sb	0.01	0.03 ± 0.01	0.03 ± 0.02	0.04 ± 0.02	1.61 ± 0.15	1.73 ± 0.15	1.78 ± 0.18
Se	0.08	bdl	bdl	bdl	0.12 ± 0.03	0.21 ± 0.015	0.17 ± 0.035
Si	0.3	1540.0 ± 1054.3	930.0 ± 181.9	846.7 ± 160.7	1046.7 ± 317.9	906.7 ± 5.8	1050.0 ± 52
Sn	0.01	0.21 ± 0.07	0.20 ± 0.06	0.31 ± 0.04	0.03 ± 0.01	0.05 ± 0.02	0.07 ± 0.04
Sr	0.05	18.43 ± 17.99	15.43 ± 9.35	15.37 ± 1.24	451.40 ± 30.20	449.40 ± 21.57	457.83 ± 21.71
Ti	0.05	5.31 ± 0.36	0.52 ± 0.13	4.96 ± 0.23	ND	ND	ND
Tl	0.01	0.55 ± 0.51	0.59 ± 0.12	0.57 ± 0.48	bdl	bdl	bdl
V	0.01	bdl	bdl	bdl	0.15 ± 0.03	0.16 ± 0.02	0.22 ± 0.03
Zn	0.07	143.97 ± 4.95	226.27 ± 49.78	289.53 ± 57.38	4.06 ± 1.50	29.91 ± 4.64	43.76 ± 4.50

Metal	Decrease of concentration in water vs Concentration in soft tissues		Decrease of concentration in water vs Concentration in shells	
	r	p	*r*	p
Al	-0.903	**0.001**	-0.794	**0.011**
Cd	-0.477	0.194	-0.777	**0.014**
Co	-0.615	0.078	-0.921	**<0.001**
Cu	0.018	0.963	-0.922	**<0.001**
Fe	-0.827	**0.006**	-0.863	**0.003**
Mn	-0.436	0.241	-0.634	0.067
Ni	-0.842	**0.004**	-0.795	**0.011**
Pb	-0.516	0.155	-0.060	0.879
Si	0.379	0.315	0.310	0.417
Zn	-0.931	**<0.001**	-0.939	**<0.001**

Table 6.3. Pearson coefficient values (*r*) determined to identify significant correlation between the variation of metal concentration in water (as the difference between the concentrations in the pre-filtration and bio-filtered samples) and metal concentration quantified in biological samples. Significant effects (α = 0.05) were marked in bold. Only selected metals were considered (please see text for more details).

For a broader insight into the potential of *C. fluminea* as a bioremediator, the analytical characterization of pre-filtration and bio-filtered effluents was complemented with the analysis of their toxicity to standard organisms. Table 6.4 summarizes the results of the bioassays. AMD-0 was not particularly toxic to any of the test organisms, and, in general, the toxicity of the effluents tended to decrease after filtration by the Asian clam. *Vibrio fischeri* was negatively affected by pre-filtration AMD-4 and both pre-filtration and bio-filtered AMD-10, with bioluminescence EC_{50} increasing when the bacteria were exposed to bio-filtered AMD-10 as compared to the response observed in the exposure to the pre-filtration effluent. Decreased toxicity of the bio-filtered effluent was also observed for *P. subcapitata*, particularly for exposure to AMD-10, although the overlap of EC_{50} confidence intervals suggests that the trend is not statistically significant. The responses of *D. magna* were found to be the most consistent across treatments, with the toxicity of the effluents clearly decreasing after filtration by the Asian clam: when bio-filtered AMD-4 was tested, no adverse effects could be noticed, and the estimated AMD-10 EC_{20} increased after the bio-filtration process (average EC_{20} of 3.07 ± SD 1.154 % for the bio-filtered effluent as compared to an EC_{20} of 1.17 % for the pre-filtration medium; changes where statistically significant except for replicate 3).

Table 6.4 - Effect concentrations (EC_x) and respective 95 % confidence interval (within brackets) along with maximum responses observed and the dilution level eliciting such responses for the exposure of *V. fischeri*, *P. subcapitata* and *D. magna* to pre-filtration (1 replicate per treatment) and bio-filtered (three replicates per treatment) media from the bio-filtration experiment. Whenever EC_x values could not be determined (denoted by nd in the table), only information on the maximum effect observed is given. ne represents no observed effects.

		Pre-filtration medium	**Bio-filtered medium**		
			Replicate 1	Replicate 2	Replicate 3
***V. fischeri* luminescence**					
AMD-0	Max. effect (%) EC_{50} (%) (95% CI)	9.50 at 10.2 % nd	ne nd	Ne Nd	8.20 at 5.2 % Nd
AMD-4	Max. effect (%) EC_{50} (%) (95% CI)	38.1 at 81.9 % 4.57 (1.35-7.79)	5.1 at 0.6 % nd	Ne Nd	12.7 at 10.2 % Nd
AMD-10	Max. effect (%) EC_{50} (%) (95% CI)	75.7 at 81.9 % 2.47 (1.30-3.65)	51.1 at 40.9 % 3.87 (1.53-6.22)	53.0 at 20.5 % 3.05 (0-6.27)	65.7 at 81.9 % 3.28 (2.06-4.50
***P. subcapitata* growth inhibition**					
AMD-0	Max. effect (%) EC_{50} (%) (95% CI)	3.39 at 100 % nd	34.45 at 100 % nd	29.43 at 100 % Nd	38.21at 100 % Nd
AMD-4	Max. effect (%) EC_{50} (%) (95% CI)	5.64 at 75 % nd	14.10 at 12.5 % nd	7.11 at 12.5 % Nd	3.01 at 50 % Nd
AMD-10	Max. effect (%) EC_{50} (%) (95% CI)	52.68 at 75% 7.92 (5.45-10.39)	44.98 at 75% 9.18 (7.51-10.86)	46.60 at 75 % 8.87 (7.88-9.85)	46.60 at 50 % 8.96 (7.77-10.15)
***D. magna* immobilization**					
AMD-0	Max. effect (%) EC_{20} (%) (95% CI)	10 at 12.5 and 25 % nd	5 at 75 % nd	10 at 100% Nd	5 at 12.5 and 25 % Nd
AMD-4	Max. effect (%) EC_{20} (%) (95% CI)	100 at 50 % 1.17 (0.99-1.34)	5 at 12.0 % nd	5 at 26.4 and 34.3 % Nd	5 at 26.4 and 75.3 % Nd
AMD-10	Max. effect (%) EC_{20} (%) (95% CI)	100 at 25 % 1.17 (0.93-1.34)	60 at 50.2 % 3.99 (3.41-4.52)	55 at 65.3 % 3.46 (2.81-4.08)	85 at 50.2 % 1.78 (1.29-2.16)

6.5. Discussion

Many animals in aquatic ecosystems hyper-accumulate, stabilize or degrade pollutants (Oehlmann and Schulte-Oehlmann 2002, Ostroumov 2005). These characteristics provide these animals with the potential to act as bioremediators. However, biotic approaches involving bioremediator animals to treat polluted waters have not been consistently considered in the past, possibly due to ethical reasons or to human health concerns (Gifford et al. 2004). It seems that this picture has been changing recently, and there are already some bioremediation solutions that have been considered particularly for

microbial contaminants (Gifford S. et al. 2004), nutrient enrichment (Gulati et al. 2008, Nakamura and Kerciku 2000), persistent organic pollutants (Mackenzie et al. 2004), and metal-rich effluents (Jana and Das 1997). In fact, metals are environmental contaminants of great concern, because they are used or explored in a wide range of industrial applications and activities, and may easily accumulate to toxic levels in biological matrices being likely to biomagnify along the trophic chain (Rubio-Franchini et al. 2008). Accumulation in plankton, such as bacteria and phytoplankton (Rossi and Jamet 2008), and filter feeders, such as bivalves (Netpae and Phalaraksh 2009) constitutes a classic example of this hazardous environmental scenario. AMD was chosen as an experimental model in this study because it is one of the main metal-bearing effluents introducing metals into and promoting the acidification of different environmental compartments worldwide (Siegel 2002). The cost-effective mitigation of AMD impacts is thus of paramount interest. As most treatment solutions proposed for AMD so far involve economically prohibitive costs (Costa and Duarte 2005), the search for sustainable alternatives is worth continuing.

A suitable alternative for AMD treatment may rely on the use of tolerant bivalves, such as *C. fluminea*, that also have high filtering efficiency. This idea of using the Asian clam as bioremediator tool has been suggested as a theoretically interesting solution (Gifford et al. 2007) and has actually been tested by some authors (Cohen et al. 1984, Li et al. 2010). However, most of the studies conducted so far have focussed on the species' bioremediator potential to restore eutrophic habitats. As to our best knowledge, the present study is the first addressing and providing experimental evidence on the potential of *C. fluminea* as a bioremediator to assist the management and treatment of industrial metal-rich effluents. This study demonstrated that the chemical improvement in water quality (i.e. a decrease in metal concentrations) as a result of processing by the clams is likely to translate into an actual decrease of the effluent environmental toxicity.

Filtration by the Asian clam originated a significant decrease in the concentration of an important part of the AMD metal contents, in particular for Al, Cd, Co, Cu, Fe, Mn, Ni, Pb, Si and Zn. The present study indicates that, although absolute metal removal may increase as the clams are exposed to more concentrated effluents, there is a tendency for a reduction on the purification efficiency, i.e. the improvement of the overall water quality relative to the initial pollutant contents. Furthermore, after bio-filtration, less diluted effluent was observed to still be more toxic to aquatic biota than the more diluted effluent. The main practical implication of these results is that a potential bioremediation system based on filtration by the Asian clam is likely to be more effective if implemented as a sequential multi-stage assembly than as a single-step configuration. Other biologically-based wastewater treatment systems have been designed and tested that may inspire the

design of such a multi-stage system, e.g., the recirculating raceway system proposed by Riley (2008) integrating *Corbicula* to remove nutrients from wastewaters, eventually combined with cascade systems as used by Tanner et al. (2002) to test improvements in wetland-based wastewater treatment.However, further studies are necessary to assess this sequential multi-stage assembly once high levels of metal in water may cause a decrease in clams' filtration capacity by damaging the gill structure (Barrera-Escorcia et al. 2010) or by changing metals' physiological handling during accumulation, sequestration, distribution and elimination (Wang and Rainbow 2008).

Bivalves can uptake a significant portion of the water content into their soft tissues as well as incorporate them in the shell matrix during the process of biomineralisation or by passive adsorption (Gifford et al. 2004). For this reason, metal concentrations in the soft tissues and shell of the clams were determined in this study. In general, for the 10 elements that were effectively removed from water (Al, Cd, Co, Cu, Fe, Mn, Ni, Pb, Si and Zn) the respective concentration in the biological samples was higher in the clams that processed effluents than in those filtering uncontaminated water. In fact, removal of most metals from AMD significantly correlated to the clams' tissue and/or shells contents confirming that *C. fluminea* was indeed responsible for effluent purification. The literature indicates that toxic effects can be elicited by the metals that were accumulated by *C. fluminea*, including disruption of mechanisms involved in calcium uptake (e.g. Cd and Co; Machado and Lopes-Lima 2011, Vercauteren and Blust 1999). In this study, and although unmeasured toxic effects may have occurred, these metals did not reach lethal levels, with no significant mortality being observed during the bio-filtration experiment. The increase of metal concentration in *C. fluminea* soft tissues and shells as a consequence of exposure to increasing metal concentrations in water has been observed previously. Andres et al. (1999) and Porter and Nairn (2010) reported this pattern for soft tissues. Based on former published evidence, Doherty (1990) suggested that shells of *C. fluminea* can be a useful matrix for the monitoring of freshwater contamination by heavy metals. This is in agreement with the results obtained in the present study. However, other studies focusing on different bivalve species (e.g. Vercauteren and Blust 1999) concluded that metal uptake is not correlated with environmental concentration. Also in the present study, and considering the whole set of quantified metals, no significant correlation between uptake/accumulation and medium concentration could be found for the majority of the elements. These inconsistencies can be explained by the particular assessment conditions used in different studies, combined with the specificities of the focused metal(s). Clams accumulate an important fraction of the metals via filtration flow across the gills or ingestion (Porter and Nairn 2010) hence accumulation is likely to increase with augmented exposure periods. Another confounding factor is the ability of some bivalves,

including the Asian clam, to regulate their body metal contents as a short-time strategy, by closing the valves (Rainbow and Dallinger 1993). Additionally, the combined effect of abiotic and biotic factors on metal bioavailability and mechanisms involved in the uptake and detoxification of metal ions in organisms vary considerably with the environmental conditions, seasonality and species (Vercauteren and Blust 1999). It should also be noticed that continuous passive exchange of metals occurs between the shell and the surrounding medium (Rainbow and Dallinger 1993), which makes it difficult to draw sound conclusions on the concentrations in the exposure medium based on the metal content found in the shells of exposed organisms.

Although only a sub-set of metals were effectively removed from water by the clams, the improvement of the effluent quality through bio-filtration was clear, which reinforces the potential of *C. fluminea* as a bioremediator. In fact, a decrease in AMD toxicity to selected test species (*V. fischeri*, *P. subcapitata* and *D. magna*) was observed after filtration by the Asian clam. The use of biological indicators, such as the bioassays performed here, is essential for a final, robust conclusion on the water quality improvement after the evidence provided by chemical analysis (Porter and Nairn 2010). While the present and former studies indicate a high potential for *C. fluminea* to work as a tool for the treatment of contaminated water, the species' invasive character must be carefully taken into account before its integration in water treatment and remediation programmes. The use of invasive bivalves as bioremediators was already suggested by other authors (Diggins et al. 2002, Elliott et al. 2008, Gottlieb and Schweighofer 1996), all agreeing that caution is essential when introducing such species in non-invaded habitats. However, already invaded regions may benefit from the presence of the species to assist the treatment of the local effluents. Also, as part of transferring these preliminary results on the species' bioremediation potential to real treatment systems, it will be necessary to envisage and develop controlled configurations that can safely hold the organisms.

Further studies are still necessary to fully validate the use of the Asian clam as a bioremediation tool and the species' integration in water treatment programmes. Such studies include testing other metal-bearing effluents (e.g. smelting and metallurgic wastes) as well as distinct effluents, such as those processed in wastewater treatment plants. Maximising the species' bioremediation ability to increase the treatment efficiency should also be attempted; in this context, the combination of different treatment methods is an approach that is worth investigating. The concentration of the AMD to be processed is likely to be strongly restricted by the high metal content of the effluent and also by its very acidic nature, with concentrations higher than 10% producing high mortality of the clams. To promote the bio-filtration of more concentrated AMD, it may be useful to consider a preliminary step for pH adjustment. This adjustment may eventually be achieved by

combining AMD with an alkaline effluent, such as paper mill wastes, which has been proven by Perez-Lopez et al. (2011) to be suitable to treat and restore AMD. The benefits of using *C. fluminea* as a bioremediator in wastewater treatment fully justify the investment in further research efforts to understand the exact extent to which the species is efficient and the conditions in which its use is sustainable at a large scale.

6.6. Conclusions

This study follows previous suggestions on the use of bivalves for water bioremediation. The testing of the potential of the invasive bivalve *Corbicula fluminea* as a tool to bioremediate metal-rich effluents is its main original contribution. The Asian clam was proven to be able to efficiently remove an important part of the metal content of an acid mine drainage effluent, significantly improving water quality. The analytically quantified improvement (as the reduction of metal contents) in water quality was supported by a decrease in the effluent environmental toxicity. Research is still necessary to further develop a bioremediation system for effluents based on Asian clam filtration, being essential to refine its optimal configuration to maximize treatment efficiency and to fully account the invasive character of *C. fluminea*. The optimal treatment configuration will provide adequate purification levels ensuring absolute control of the species spread in neighboring ecosystems.

6.7. Aknowledgements

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6.8. References

Akpor OB, Muchie M. 2010. Bioremediation of polluted wastewater influent: Phosphorus and nitrogen removal. Scientific Research and Essays 5: 3222-3230.

Alakangas L, Ohlander B, Lundberg A. 2010. Estimation of temporal changes in oxidation rates of sulphides in copper mine tailings at Laver, Northern Sweden. Science of the Total Environment 408: 1386-1392.

Andres S, Baudrimont M, Lapaquellerie Y, Ribeyre F, Maillet N, Latouche C, Boudou A. 1999. Field transplantation of the freshwater bivalve *Corbicula fluminea* along a polymetallic contamination gradient (river Lot, France): I. Geochemical characteristics of the sampling sites and cadmium and zinc bioaccumulation kinetics. Environmental Toxicology and Chemistry 18: 2462-2471.

ASTM. 1980. Standard Pratice for conducting acute toxicity tests with fishes, macroinvertebrates and amphibians. Philadelphia: American Standards for Testing and Materials.

Baird D, Soares A, Girling A, Barber I, Bradley M, Calow P. 1989. The long-term maintenance of *Daphnia magna* Straus for use in ecotoxicity tests: Problems and prospects. Pages 144-148 in Lokke H, Tyle H, F B-R, eds. 1st European conference on ecotoxicology. Lyngby.

Barrera-Escorcia G, Vanegas-Perez C, Wong-Chang I. 2010. Filtration rate, assimilation and assimilation efficiency in *Crassostrea virginica* (Gmelin) fed with *Tetraselmis suecica* under cadmium exposure. Journal of Environmental Science and Health Part a-Toxic/Hazardous Substances & Environmental Engineering 45: 14-22.

Beltran KS, Pocsidio GN. 2009. Acetylcholinesterase activity in *Corbicula fluminea* Müller, as a biomarker of organophosphate pesticide pollution in Pinacanauan River, Philippines. Environmental Monitoring and Assessment 165: 331-340.

Blodau C. 2006. A review of acidity generation and consumption in acidic coal mine lakes and their watersheds. Science of the Total Environment 369: 307-332.

Cherry DS, Scheller JL, Cooper NL, Bidwell JR. 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.

Christensen B, Laake M, Lien T. 1996. Treatment of acid mine water by sulfate-reducing bacteria; Results from a bench scale experiment. Water Research 30: 1617-1624.

Cohen RRH, Dresler PV, Phillips EJP, Cory RL. 1984. The effect of the asiatic clam, *Corbicula fluminea*, on phytoplankton of the Potomac River, Maryland. Limnology and Oceanography 29: 170-180.

Colombo JC, Bilos C, Campanaho M, Presa MJR, Catoggio JA. 1995. Bioaccumulation of polychlorinatedbiphenyls and chlorinated pesticides by the asiatic clam *Corbicula fluminea*: Its use as sentinel organism in the Rio de La Plata estuary, Argentina. Environmental Science & Technology 29: 914-927.

Costa MC, Duarte JC. 2005. Bioremediation of acid mine drainage using acidic soil and organic wastes for promoting sulphate-reducing bacteria activity on a column reactor. Water Air and Soil Pollution 165: 325-345.

Diggins TP, Baier RE, Meyer AE, Forsberg RL. 2002. Potential for selective, controlled biofouling by *Dreissena* species to intercept pollutants from industrial effluents. Biofouling 18: 29-36.

Doherty FG. 1990. The asiatic clam, Corbicula spp, as a biological monitor in freshwater environments. Environmental Monitoring and Assessment 15: 143-181.

El Khalil H, El Hamiani O, Bitton G, Ouazzani N, Boularbah A. 2008. Heavy metal contamination from mining sites in South Morocco: Monitoring metal content and toxicity of soil runoff and groundwater. Environmental Monitoring and Assessment 136: 147-160.

Elendt B, Bias W. 1990. Trace nutrient deficiency in *Daphnia magna* cultured in standard medium for toxicity testing. Effects of the optimisation of culture conditions on life-history parameters of *D. magna*. Water Research 24: 1157-1167.

Elliott P, Aldridge DC, Mogyridge GD. 2008. Zebra mussel filtration and its potential uses in industrial water treatment. Water Research 42: 1664-1674.

Garcia C, Moreno DA, Ballester A, Blazquez ML, Gonzalez F. 2001. Bioremediation of an industrial acid mine water by metal-tolerant sulphate-reducing bacteria. Minerals Engineering 14: 997-1008.

Gifford S, Dunstan RH, O'Connor W, Roberts T, Toia R. 2004. Pearl aquaculture - profitable environmental remediation? Science of the Total Environment 319: 27-37.

Gifford S, Dunstan RH, O'Connor W, Koller CE, MacFarlane GR. 2007. Aquatic zooremediation: deploying animals to remediate contaminated aquatic environments. Trends in Biotechnology 25: 60-65.

Gottlieb SJ, Schweighofer ME. 1996. Oysters and the Chesapeake Bay ecosystem: A case for exotic species introduction to improve environmental quality? Estuaries 19: 639-650.

Gulati RD, Pires LMD, Van Donk E. 2008. Lake restoration studies: Failures, bottlenecks and prospects of new ecotechnological measures. Limnologica 38: 233-247.

Huberty CJ, Olejnik S. 2006. Applied MANOVA and discriminant analysis. Hoboken: Wiley. 488pp

Hudson-Edwards KA, Jamieson HE, Lottermoser BG. 2011. Mine Wastes: Past, Present, Future. Elements 7: 375-379.

Huertas JI, Huertas ME, Izquierdo S, Gonzalez ED. 2012. Air quality impact assessment of multiple open pit coal mines in northern Colombia. Journal of Environmental Management 93: 121-129.

Jana BB, Das S. 1997. Potential of freshwater mussel (*Lamellidens marginalis*) for cadmium clearance in a model system. Ecological Engineering 8: 179-193.

Li X-N, Song H-L, Li W, Lu X-W, Nishimura O. 2010. An integrated ecological floating-bed employing plant, freshwater clam and biofilm carrier for purification of eutrophic water. Ecological Engineering 36: 382-390.

Lopes I, Gonçalves F, Soares AMVM, Ribeiro R. 1999a. Ecotoxicological tools in the remediation of acid mine drainage. Toxicological and Environmental Chemistry 70: 441-460.

Lopes I, Goncalves F, Soares A, Ribeiro R. 1999b. Discriminating the ecotoxicity due to metals and to low pH in acid mine drainage. Ecotoxicology and Environmental Safety 44: 207-214.

Lottermoser BG. 2007. Mine Wastes - Characterization, Treatment, Environmental Impacts. New York: Springer. 304pp.

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

Mackenzie BR, Almesjo L, Hansson S. 2004. Fish, fishing, and pollutant reduction in the Baltic Sea. Environmental Science & Technology 38: 1970-1976.

Marie V, Baudrimont M, Boudou A. 2006. Cadmium and zinc bioaccumulation and metallothionein response in two freshwater bivalves (*Corbicula fluminea* and

Dreissena polymorpha) transplanted along a polymetallic gradient. Chemosphere 65: 609-617.

MINEO. 2000. Environmental State in the Portuguese Test Site - S. Domingos Mine: Past and Present. Mértola: Instituto Geológico e Mineiro.

Nakamura Y, Kerciku F. 2000. Effects of filter-feeding bivalves on the distribution of water quality and nutrient cycling in a eutrophic coastal lagoon. Journal of Marine Systems 26: 209-221.

Netpae T, Phalaraksh C. 2009. Water quality and heavy metal monitoring in water, sediments, and tissues of *Corbicula sp* from Bung Boraphet Reservoir, Thailand. Chiang Mai Journal of Science 36: 395-402.

Oehlmann J, Schulte-Oehlmann U. 2002. Molluscs as bioindicators. Pages 577-635 in B.A. Markert AMB, H.G. Zechmeister, ed. Bioindicators and biomonitors, Eselvier Science. 577-635 pp.

Ostroumov SA. 2005. Some aspects of water filtering activity of filter-feeders. Hydrobiologia 542: 275-286.

Page T, Neitzel D, Simmons M. 1986. Biofouling of power plant service systems by Corbicula in Britton JC, ed. Proceedings of the Second International *Corbicula* Symposium: American Malacological Bulletin - Special edition no.2.

Peplow D, Edmonds R. 2005. The effects of mine waste contamination at multiple levels of biological organization. Ecological Engineering 24: 101-119.

Pereira R, Pereira ML, Ribeiro R, Goncalves F. 2006. Tissues and hair residues and histopathology in wild rats (*Rattus rattus* L.) and Algerian mice (*Mus spretus* Lataste) from an abandoned mine area (Southeast Portugal). Environmental Pollution 139: 561-575.

Perez-Lopez R, Quispe D, Castillo J, Miguel Nieto J. 2011. Acid neutralization by dissolution of alkaline paper mill wastes and implications for treatment of sulfide-mine drainage. American Mineralogist 96: 781-791.

Porter CM, Nairn RW. 2010. Fluidized bed ash and passive treatment reduce the adverse effects of acid mine drainage on aquatic organisms. Science of the Total Environment 408: 5445-5451.

Quinn GP, Keough MJ, eds. 2002. Experimental design and data analysis for biologists New York: Cambridge University Press. 537 pp.

Rainbow PS, Dallinger R. 1993. Metal uptake, regulation, and excretion in freshwater invertebrates. Pages 461 in Dallinger R, Rainbow PS, eds. Ecotoxicology of metals in invertebrates, Lewis Publishers. 461 pp.

Riley L.W. 2008. Freshwater clams as a treatment mechanism for phosphorus in agricultural wastewater. University of Florida, Florida.Roetting TS, Thomas RC, Ayora C, Carrera J. 2008. Passive treatment of acid mine drainage with high metal concentrations using dispersed alkaline substrate. Journal of Environmental Quality 37: 1741-1751.

Rosa IC, Pereira JL, Gomes J, Saraiva PM, Goncalves 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.

Rossi N, Jamet JL. 2008. In situ heavy metals (copper, lead and cadmium) in different plankton compartments and suspended particulate matter in two coupled Mediterranean coastal ecosystems (Toulon Bay, France). Marine Pollution Bulletin 56: 1862-1870.

Rubio-Franchini I, Saavedra JM, Rico-Martinez R. 2008. Determination of lead in samples of zooplankton, water, and sediments in a Mexican reservoir: Evidence for lead biomagnification in lower/intermediate trophic levels? Environmental Toxicology 23: 459-465.

Siegel FR. 2002. Environmental geochemistry of potentially toxic metals. Berlin: Springer. 218 pp.

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

Stein J. 1973. Handbook of Phycological methods – Culture methods and growth measurements. Cambridge: Cambridge University Press. 448 pp.

Tanner CC, Kadlec RH, Gibbs MM, Sukias JPS, Nguyen ML. 2002. Nitrogen processing gradients in subsurface-flow treatment wetlands - Influence of wastewater characteristics. Ecological Engineering 18: 499-520.

Touze S, Battaglia-Brunet F, Ignatiadis I. 2008. Technical and economical assessment and extrapolation of a 200-dm^3 pilot bioreactor for reduction of sulphate and metals in acid mine waters. Water Air and Soil Pollution 187: 15-29.

Ulrich M. 1999. Non-metallic carbonous minerals in the passive treatment of mining wastewater in Slovenia. Chemical Engineering and Processing 38: 249-258.

Vercauteren K, Blust R. 1999. Uptake of cadmium and zinc by the mussel *Mytilus edulis* and inhibition by calcium channel and metabolic blockers. Marine Biology 135: 615-626.

Wang W-X, Rainbow PS. 2008. Comparative approaches to understand metal bioaccumulation in aquatic animals. Comparative Biochemistry and Physiology C-Toxicology & Pharmacology 148: 315-323.

Zhou Q, Zhang J, Fu J, Shi J, Jiang G. 2008. Biomonitoring: An appealing tool for assessment of metal pollution in the aquatic ecosystem. Analytica Chimica Acta 606: 135-150.

Suplementary material

Table S6.1. Summary of one-way ANOVA performed after MANOVA to assess the effect of effluent dilution level on the variation of metal concentration in water due to clams' filtration and on metal accumulation in the animals' soft tissues and shells. Significant effects (α = 0.017) were marked in bold.

		Variation of concentration in water			Accumulation in the soft tissues			Acumulation in the shells		
Metal	**df**	MS error	F	p	MS error	F	P	MS error	F	p
Ag	2, 6	-	-	-	$3.4x10^{-2}$	6.38	0.033	-	-	**-**
Al	2, 6	5274.9	13.89	**0.006**	$2.0x10^{4}$	15.91	**0.004**	4058.0	5.62	0.042
Ar	2, 6	-	-	**-**	1.1	1.57	0.282	$2.2x10^{-2}$	0.89	0.460
B	2, 6	1215.0	0.08	0.922	0.1	1.00	0.423	-	-	**-**
Ba	2, 6	11911.0	202.45	**<0.001**	31.5	1.28	0.346	9.3	0.52	0.619
Ca	2, 6	1585.5	43.09	**<0.001**	$3.8x10^{7}$	0.24	0.797	$1.5 x10^{7}$	1.27	0.348
Cd	2, 6	7764.2	115.48	**<0.001**	0.1	8.13	0.020	$1.0x10^{-2}$	13.04	**0.007**
Co	2, 6	8227.4	291.84	**<0.001**	0.2	12.56	**0.007**	1.1	47.97	**<0.001**
Cr	2, 6	-	-	**-**	-	-	-	$2.0 x10^{-3}$	10.06	**0.012**
Cu	2, 6	17936.1	8968.10	**<0.001**	478.2	0.39	0.694	4.2	38.07	**<0.001**
Fe	2, 6	288.7	42.79	**<0.001**	$2.4x10^{4}$	7.19	0.026	7.0	10.36	**0.011**
Hg	2, 6	-	-	-	$2.4x10^{-2}$	8.38	0.018	-	-	**-**
K	2, 6	54501.0	13.85	**0.006**	$4.4x10^{4}$	10.99	**0.010**	1428.0	0.14	0.872
Li	2, 6	-	-	**-**	0.1	3.30	0.122	-	-	**-**
Mg	2, 6	30.8	12.54	**0.007**	2500.0	2.20	0.192	469.9	2.61	0.153
Mn	2, 6	7554.7	226.53	**<0.001**	67.2	19.81	**0.002**	3158.0	7.10	0.026
Na	2, 6	23.29	0.26	0.778	$3.1x10^{5}$	10.15	**0.012**	2178.0	0.22	0.807
Ni	2, 6	1115.9	14.85	**0.005**	0.4	3.34	0.106	0.1	19.01	**0.003**
P	2, 6	-	-	-	$1.1x10^{5}$	1.59	0.278	1325.0	14.19	**0.005**
Pb	2,6	-	-	**-**	0.1	1.09	0.395	$1.3x10^{-2}$	2.75	0.142
S	2, 6	603.4	11.23	**0.009**	$2.0x10^{4}$	2.87	0.133	$1.0 x10^{5}$	8.62	**0.017**
Sb	2, 6	-	-	-	$2.0x10^{-4}$	1.61	0.289	-	-	**-**
Se	2, 6	-	-	**-**	-	-	**-**	$6.0x10^{-3}$	7.96	0.021
Si	2, 6	644.2	24.92	**0.001**	$3.9x10^{5}$	1.10	0.391	$2.0x10^{5}$	0.58	0.588
Sn	2, 6	1459.4	1.50	0.296	$3.0x10^{-3}$	3.83	0.085	$1.0x10^{-3}$	1.72	0.257
Sr	2, 6	1433.3	5.30	0.047	137.5	0.07	0.936	58.3	0.09	0.911
Ti	2, 6	-	-	**-**	$3.5x10^{-2}$	0.53	0.617	-	-	**-**
Tl	2, 6	-	-	**-**	$5.4x10^{-2}$	4.14	0.087	-	-	**-**
V	2, 6	-	-	-	-	-	-	$4.0x10^{-3}$	5.60	0.042
Zn	2, 6	4339.6	26.62	**0.001**	1932.0	8.27	0.019	1218.0	82.92	**<0.001**

CHAPTER 7

Final considerations

7.1. General conclusions and future research

Ecosystems have been facing constant intentional or accidental introduction of non-indigenous species (Davis 2003, Vitousek et al. 1996). After being introduced into a new habitat out of its native range, an invasive species is able to survive and establish itself there (Allendorf and Lundquist 2003). Such a colonization process tends to have several ecological and economic consequences, mainly negative (Sousa et al. 2012). Facing this problem, scientists and policy makers have been drawing potential solutions for the prevention, early detection and control of invasive species (e.g. European Comission 2008, Perrings 2005). However, many challenges remain unaddressed (see e.g Byers et al. 2002 for details). The present work contributes to the body of knowledge on one of the most relevant invasive freshwater bivalves, the Asian clam *Corbicula fluminea*, by covering topics related to dispersal, impacts on non-native habitats, pest control and as well as the possibility of taking profits from the species when accepting that invasion becomes unavoidable. Follow-ups that will allow consolidating and extending the research are referred below as the main conclusions on the studies compiled in this thesis are presented, and can be found outlined in Figure 7.1.

Asian clam dispersal is promoted by drifting in water, which is assisted by a mucilaginous drogue line produced by mucocytes packed along the inner demibranchs of the ctenidia of juveniles and small adult clams (Prezant and Chalermwat 1984). These processes remain, in many aspects, largely unknown. This thesis attempts to increase the understanding of this topic by providing fundamental information on factors that constrain the clams' drifting behavior. It was concluded that increasing water temperature triggers an acceleration of mucocytes production and stimulates flotation in the short-term, which suggests that increased Asian clam dispersal may constitute another threat linked to global warming (Vitousek et al. 1996, Wilby et al. 2010). The potential effects of genetic variability and seasonality on mucocyte production and flotation behavior were also assessed. The study indicated reduced flotation of the focused population (a Portuguese population was focused and compared to an American population presenting high flotation activity) and no season-driven changes in the mucous production, which was also insipient. A reasonable conclusion was that different populations experience local adaptation processes that translate environmental and/or ecological pressures, which can lead to physiological changes including in the production of the mucilaginous threads that assist flotation. The comparison between populations of different *Corbicula* species was also considered as a possible explanation to the inconsistency. This was not confirmed by the molecular assessment of the morphotypes involved, but there are still technical shortcomings preventing a definite conclusion. The data obtained thus suggest that

mucous production is not a necessary condition for successful clam reproduction to occur. Nevertheless, in populations where mucous is produced, mature parents, developing embryos and juvenile release from the parents' gills may actually benefit from it as suggested by some authors (Byrne et al. 2000, Morton 1977, Williams. and McMahon 1987). Further studies are still necessary to investigate if other factors affect clam drifting and mucocyte development as well as to clearly understand the influence of temperature in clam drift. In particular, it would be relevant to (i) assess temperature effects using sediment in the test system to clarify the extent of its protective role (seems to exist based on preliminary observations made during the present study), and (ii) to address the effects of low-range temperature changes. Other environmental factors, such as hypoxia and salinity, may affect *C. fluminea* flotation behavior, and it would be important to explore them, particularly in the context of global climate change (Whitworth et al. 2012, Wilby et al. 2010).

As the Asian clam entered Europe through Portugal in the 1980s (Mouthon 1981), understanding the pest dispersion and consequences here over time may assist official entities with the implementation of integrated pest management policies in other European countries at risk of invasion or where the pest has recently arrived. The ecological impacts of the Asian clam in some Portuguese watersheds have already been reported (Sousa et al. 2008a, Sousa et al. 2008b). However, this thesis provides the first systematic overview of the pest's biofouling impacts on national freshwater-dependent industries. Updated information on the species' distribution in national waterbodies is also provided, revealing that it can be found in most of the country's river basins. Nevertheless, only mild impacts on the drinking water treatment, thermal power, cement, pulp and paper and agricultural (irrigation) sectors were reported. Possible reasons for this apparent discrepancy between the wide distribution of the species and its economic consequences may be (i) the fact that *C. fluminea* invasive process in Portugal remains in the lag time phase which is supported by the documentation of this phenomenon in some Portuguese waterbodies (Sousa et al. 2008c) which translates into deficient recruitment in the industrial sets, and/or (ii) regular die-offs in the established populations, which are very characteristic in *C. fluminea* (e.g. Ilarri et al. 2011). Although the species has not been acting as a particularly aggressive enemy to industry for more than 30 years, taking into account previous experiences, especially in North America (Williams and McMahon 1986), it must be considered a latent threat to Europe rather. From now on it is recommended to repeat the documentation of the species' industrial impacts in Portugal at regular intervals to assess, and thus more effectively manage, the species' progression in such environment. Furthermore, in the context of pest management in the industrial sector, it is important to assist the national plants with the study of the *C. fluminea* population dynamics to support

the design of effective control measures as well as with the implementation of adequate systematic monitoring strategies.

As the methods currently available for controlling Asian clam infestations in the industrial environment are far from perfect and present several limitations (Doherty and Cherry 1988), it is important to keep searching for safer, more cost-effective, more selective and environmentally friendly alternative practices. In the present work, the potential of combining chemicals with different with oxygen depletion to control *C. fluminea* was examined. The possible effect of the biocide's mechanism of toxicity on the viability of such a control approach was also addressed in the study. While preliminary results prove that hypoxia increases the efficiency of the chemicals against the Asian clam, it is still essential to assess the toxicity of this method to non-target organisms, which is particularly relevant for the cases where application in open industrial sets is intended. The laboratory results reported here still need to be confirmed at a field-scale for a thorough evaluation of the control approach proposed.

It is extremely difficult, if not impossible, to completely eradicate *C. fluminea* populations established in natural waterbodies. The possibility of taking profit from such populations to, in some extent, offset their damaging effects was also addressed in this thesis. It was concluded that filtration by the Asian clam could promote significant removal of metals from a diluted acid mine drainage, which resulted in a general decrease of the effluents' environmental toxicity. Thus, *C. fluminea* has a great potential as a bioremediator of industrial metal-rich effluents, particularly if they are integrated in a multi-stage configuration system. In spite of the promising results presented here, many issues still need to be further explored to maximize the bioremediation abilities of the Asian clam. It would be interesting to test other effluents (e.g. smelting and metallurgical effluents) and assess the combination of different treatment methods (e.g. use alkaline paper mill wastes as a neutralization step, combined with a subsequent bio-filtration stage in cases where improving highly acidic effluents is the intent (Perez-Lopez et al. 2011)).

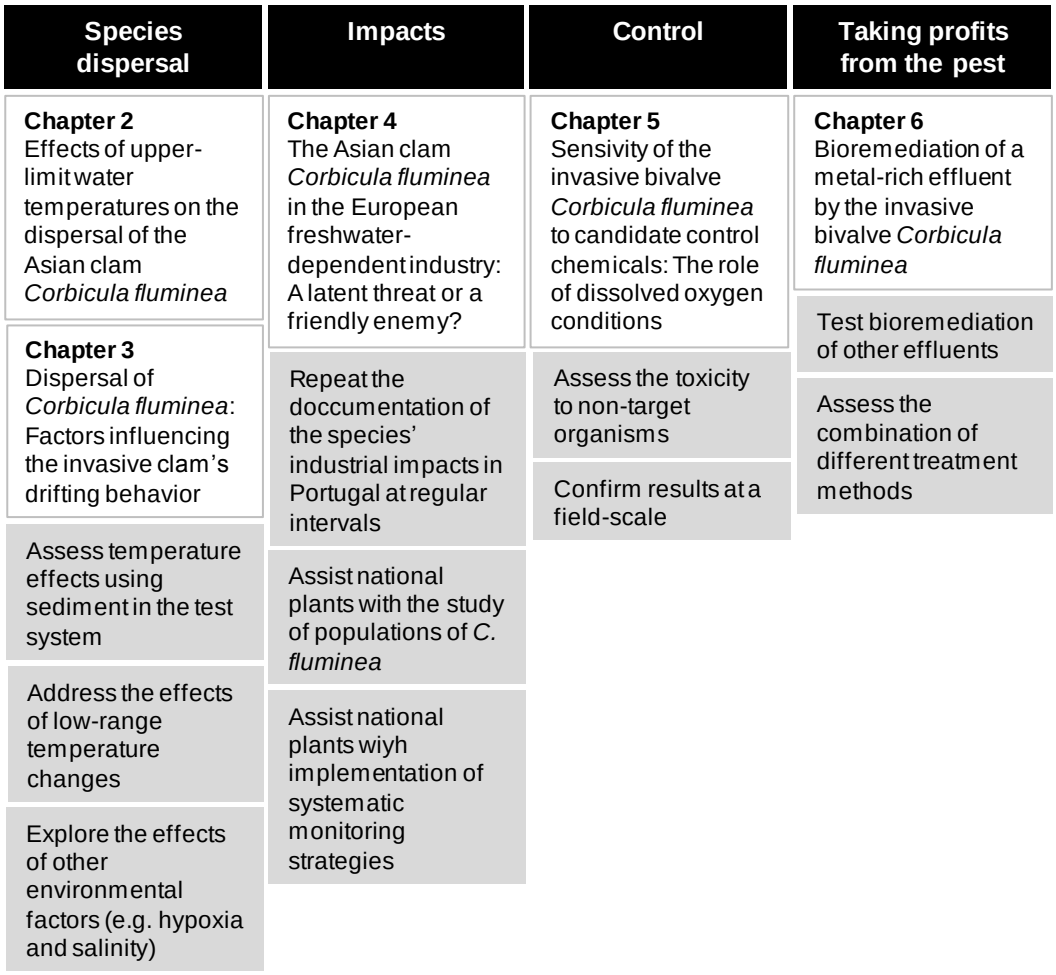

Figure 7.1: Roadmap for future research work.

7.2. References

Allendorf FW, Lundquist LL. 2003. Introduction: Population biology, evolution, and control of invasive species. Conservation Biology 17: 24-30.

Byers JE, et al. 2002. Directing research to reduce the impacts of nonindigenous species. Conservation Biology 16: 630-640.

Byrne M, Phelps H, Church T, Adair V, Selvakumaraswamy P, Potts J. 2000. Reproduction and development of the freshwater clam *Corbicula australis* in Southeast Australia. Hydrobiologia 418: 185-197.

Davis MA. 2003. Biotic globalization: Does competition from introduced species threaten biodiversity? Bioscience 53: 481-489.

Doherty FG, Cherry DS. 1988. Tolerance of the asiatic clam *Corbicula* spp to lethal levels of toxic stressors – A review. Environmental Pollution 51: 269-313.

European Comission. 2008. Comunicação da Comissão ao Conselho, ao Parlamento Europeu, ao Comité Económico e Social Europeu e ao Comité das Regiões - Por uma estratégia da UE em matéria de espécies invasivas. Brussels.

Ilarri MI, Antunes C, Guilhermino L, Sousa R. 2011. Massive mortality of the Asian clam *Corbicula fluminea* in a highly invaded area. Biological Invasions 13: 277-280.

Morton B. 1977. Freshwater fouling bivalves. Pages 1-14. First International *Corbicula* Symposium. Fort Worth, Texas, USA: Texas Christian University.

Mouthon J. 1981. Sur la présence en France et au Portugal de Corbicula (Bivalvia, Corbiculidae) originaire d' Asie. Basteria 45: 109-116.

Perez-Lopez R, Quispe D, Castillo J, Miguel Nieto J. 2011. Acid neutralization by dissolution of alkaline paper mill wastes and implications for treatment of sulfide-mine drainage. American Mineralogist 96: 781-791.

Perrings C. 2005. Mitigation and adaptation strategies for the control of biological invasions. Ecological Economics 52: 315-325.

Prezant RS, Chalermwat K. 1984. Flotation of the bivalve *Corbicula fluminea* as a means of dispersal. Science 225: 1491-1493.

Sousa R, Dias SC, Guilhermino L, Antunes C. 2008a. Minho River tidal freshwater wetlands: Threats to faunal biodiversity. Aquatic Biology 3: 237-250.

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

Sousa R, Rufino M, Gaspar M, Antunes C, Guilhermino L. 2008c. Abiotic impacts on spatial and temporal distribution ofCorbicula fluminea (Müller, 1774) in the River Minho estuary, Portugal. Aquatic Conservation: Marine and Freshwater Ecosystems 18: 98-110.

Sousa R, Novais A, Costa R, Strayer D. 2012. Invasive bivalves in fresh waters: Impacts from individuals to ecosystems and possible control strategies. Hydrobiologia.

Vitousek PM, Dantonio CM, Loope LL, R. W. 1996. Biological invasions as global environmental change. American Scientist 84: 468-478.

Whitworth KL, Baldwin DS, Kerr JL. 2012. Drought, floods and water quality: Drivers of a severe hypoxic blackwater event in a major river system (the Southern Murray–Darling Basin, Australia). Journal of Hydrology 450–451: 190-198.

Wilby RL, Orr H, Watts G, Battarbee RW, Berry PM, Chadd R, Dugdale SJ, Dunbar MJ, Elliott JA, Extence C. 2010. Evidence needed to manage freshwater ecosystems in a changing climate: Turning adaptation principles into practice. Science of the Total Environment 408: 4150-4164.

Williams CJ, McMahon RF. 1986. Power station entrainment of *Corbicula fluminea* (Müller) in relation to population dynamics, reproductive cycle and biotic and abiotic variables. Pages 99-111 in Britton JC, ed. Proceedings of the Second International *Corbicula* Symposium: American Malacological Bulletin - Special edition no.2.

Williams, C.J., McMahon, R.F., 1986. Power station entrainment of *Corbicula fluminea* (Müller) in relation to population dynamics, reproductive cycle and biotic and abiotic variables, in: Britton, J.C. (Ed.), Proceedings of the Second International *Corbicula* Symposium, 2nd ed. American Malacological Bulletin - Special edition no.2. 99-111.

Williams CJ, McMahon RF. 1987. Annual variation of tissue biomass and carbon nitrogen content in the freshwater bivalve *Corbicula fluminea* relative to downstream dispersal. Canadian Journal of Zoology 67: 82-90.