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Escola de Ciências

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## **Use and persistence of empty bivalve shells**

Master Thesis in Ecology

Work made under the orientation of

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January 2016

## **Acknowledgments**

To my supervisors Martina Ilarri and Ronaldo Sousa, thank you for all the support and guidance during this process, for always being available for me and making everything you could to help me. Thank you for giving me the opportunity to work with you both, and for being the most amazing supervisors I could possibly ask for.

To my family in special my parents and my brother for all the words of encouragement and help given not only throughout this process but my entire life, thank you for shaping me into what I am today.

To Paulo and Vera for their friendship and all the help, thank you Paulo for making every moment of work the most fun and enjoyable time.

To Adriana for her sympathy and all the theoretical and practical help, and to Francisco for helping me with the taxonomical identification of species.

To José and Elisabete Matos for lending me their computer during critical times and for all the support.

Finally to Joana, for believing and always being there for me, for not letting me lose focus and helping me in so many different levels. Thank you for being in my life.

## **Abstract**

Freshwater bivalves are remarkable organisms that can potentially influence biological communities through their engineering attributes, such as their hard shells. Bivalve shells can contribute to important physical modifications that can alter the structure of aquatic benthic communities. One of the most successful invasive species worldwide is the Asian clam *Corbicula fluminea*, it can cause severe ecological and economic impacts. Minho River is a highly invaded area by the species, currently representing more than 90% of the benthic biomass. Little is known about the effects that shells of different bivalve species, with different morphologies, and origins have on the associated fauna, and for how long they can persist in the freshwater systems. Given that, the objectives of the present study were, to assess the effect of shells of different bivalve species on the macrozoobenthic fauna, and to determine the shell decay rates of the different bivalve species, considering the influence of the size, river condition (lentic *versus* lotic) and season. For the experiments, shells of native (*Anodonta anatina*, *Potomida littoralis* and *Unio delphinus*) and one invasive (*Corbicula fluminea*) bivalve species were used. The study results suggest that substrate heterogeneity may play an important role in attracting the macrozoobenthic fauna, as the heterogeneous treatments (the ones that had more than one bivalve species) were associated with more abundant, rich and diverse macrozoobenthic community than the homogeneous treatments, although significant differences were only found for abundance. Furthermore, shell decays varied among species, season, river condition and shell size. *Anodonta anatina* was the species that exhibited the highest percentage of decay during the four seasons, followed by *U. delphinus*, *C. fluminea* and *P. littoralis*. Also, shells decayed around 50% faster under lotic than under lentic conditions, and the size of the shells also influenced the decay rates. The results of the present study suggest that the structures formed with two species of bivalve shells support significantly more abundant macrozoobenthic communities, and that the shell size, season and river condition associated to other factors (i.e. leaf deposition and agglomerations of shells) can have major influence on the shell decays.

## **Resumo**

Bivalves de água doce são organismos notáveis que potencialmente podem influenciar as comunidades biológicas através dos seus atributos de engenheiros do ecossistema, como as suas conchas. As conchas dos bivalves podem contribuir para alterações físicas importantes que podem modificar a estrutura das comunidades bentônicas. Uma das espécies invasoras mais bem-sucedidas em todo o mundo é a ameijoia Asiática *Corbicula fluminea*, esta pode causar impactos ecológicos e económicos graves. O Rio Minho constitui uma área altamente invadida por esta espécie, que representa atualmente mais de 90% da biomassa bentónica. Pouco se sabe sobre os efeitos que conchas de diferentes espécies de bivalves, com diferentes morfologias e origens, têm na fauna associada, e por quanto tempo estas podem persistir nos sistemas de água doce. Dito isto, os objetivos do presente estudo foram, avaliar o efeito de conchas de diferentes espécies de bivalve na fauna macrozoobentónica e determinar as taxas de decaimento das conchas destas espécies de bivalves, considerando, a influência do tamanho, condições do rio (lêntico *versus* lótico) e estações do ano. Para as experiências foram usadas conchas de espécies nativas (*Anodonta anatina*, *Potomida littoralis* e *Unio delphinus*) e da espécie invasora (*Corbicula fluminea*). Os resultados do presente estudo sugerem que a heterogeneidade do substrato pode ser importante para atrair a fauna macrozoobentónica, uma vez que tratamentos heterogêneos (que continham conchas de mais do que uma espécie de bivalve) estiveram associados a uma mais abundante, rica e diversificada comunidade macrozoobentónica do que os tratamentos homogêneos, porém diferenças significativas foram encontradas apenas para a abundância. Além disso, os decaimentos das conchas variaram entre espécies, estação do ano, condições do rio e tamanho. A espécie *A. anatina* foi a que exibiu o maior percentual de decaimento durante as estações do ano, seguida das espécies *U. delphinus*, *C. fluminea* e *P. littoralis*. Ainda, as conchas decaíram cerca de 50% mais rápido em condições lóticicas, e o tamanho das conchas também influenciou as taxas de decaimento. Os resultados do presente estudo sugerem que estruturas formadas por conchas de duas espécies de bivalve, suportam uma abundância significativamente maior de comunidades macrozoobentónicas, e que o tamanho das conchas, estação do ano, e condições do rio, associados a outros fatores (p.ex. deposição de folhas e aglomeração de conchas) podem ter grande influência no decaimento das conchas.

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# **1. Introduction**

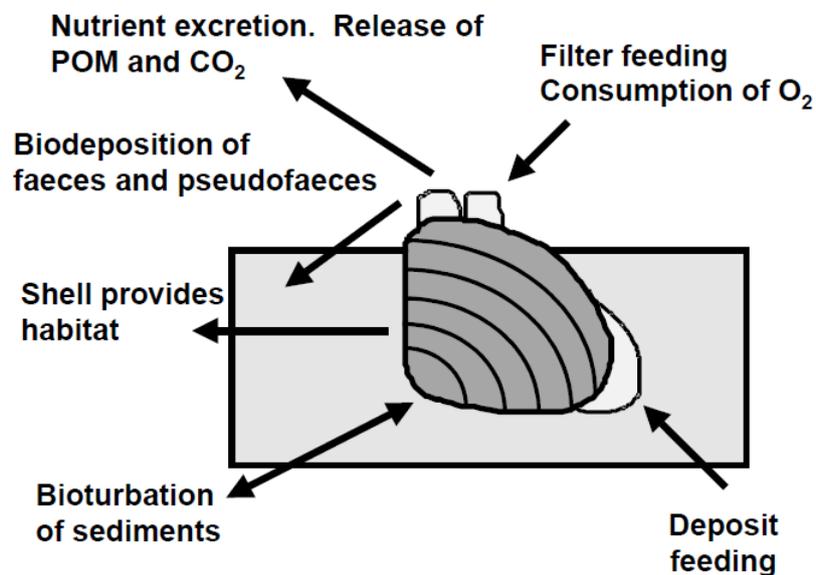
## 1.1 Freshwater bivalves as ecosystem engineers – ecological importance

Ecosystem engineer, a term introduced by Jones et al. (1994, 1997), is defined as an organism capable to modulate the availability of resources to other species by promoting physical state changes in biotic or abiotic materials. This concept has gained prominence in ecological research in the last two decades (Wright & Jones, 2006; Jones et al., 2010).

In essence, ecosystem engineering is the creation, maintenance or modification of habitats by organisms via two mechanisms: allogenic and autogenic engineering. Allogenic engineering consists in the ability of the organism to alter the structure of biotic or abiotic materials via their own activities (Jones et al., 1997). Beavers and their dam construction ability is a well-known example of this type of engineering mechanism (Wright et al., 2002). On the other hand, autogenic engineering consists in the ability of an organism to promote habitat modifications via their own physical structures (Jones et al., 1997). Coral reefs are an example of this engineering mechanism, since they provide habitat and improve abiotic (i.e. reduction in the stress-wave profile) conditions to many other species (Bruno et al., 2003).

Bivalves are remarkable organisms that can both act as allogenic and autogenic ecosystem engineers and have several attributes to promote modifications at various ecological levels (from individuals to ecosystems), thus representing an important group of ecosystem engineers in aquatic ecosystems (Crooks, 2002; Gutiérrez et al., 2003; Sousa et al., 2009, 2014). Bivalve species are distributed across the globe in all types of aquatic ecosystems (i.e., marine, estuarine and freshwater systems). One of the most well-known examples of ecosystem engineers in marine systems are oysters. For example, the Pacific oyster *Crassostrea gigas* (Thunberg, 1793) creates massive reefs (similar to coral reefs) with important consequences for biodiversity and ecosystem functioning (Ruesink et al., 2005). Being a commercial species, *C. gigas* was subjected to many studies addressing its ecology, genetics and toxicology (Boudry et al., 2003; Ruesink et al., 2005; Gagnaire et al., 2006). Despite the usual higher attention for the marine and/or estuarine bivalve species, freshwater bivalves of the Order Unionida, also known as freshwater mussels are an important faunal group that provides a wide range of ecosystem functions and services. Indeed, these freshwater bivalves transfer nutrients and energy between the water column and the benthos,

having strong effects on primary and secondary production, sedimentation rates, biogeochemical cycles and water clarity (Strayer et al., 1999). Their shells provide habitat to other organisms and because of their direct and indirect physical effects on freshwater systems these bivalves are described as ecosystem engineers (Gutiérrez *et al.*, 2003). Main engineering mechanisms by freshwater bivalves include: filtration that can lead to a decrease in phytoplankton and other particles in the water column and in this way increase clarity; bioturbation activity through bivalve movements that may increase sediment oxygen content and release of nutrients from the sediment to the water column; and shell production, which provides habitat to a myriad of organisms (Fig. 1) (Strayer et al., 1999; Vaughn & Hakenkamp, 2001).



**Fig. 1.** Ecosystem functions performed by burrowing bivalves in freshwater ecosystems being several of them related to ecosystem engineering activities. Adapted from Vaughn & Hakenkamp (2001).

Bivalve shells are an important ecosystem engineering attribute to freshwater systems. Despite being an often overlooked feature, shells can introduce complexity and heterogeneity into the benthic environment, creating colonisable substrate for several species, and increasing heterogeneity in provide more complex microhabitats (Gutierrez et al., 2003; Bódis et al., 2014). In doing so, large accumulation of bivalves (live and dead) can be responsible for changes at the individual, population, community and ecosystem levels (Gutiérrez & Iribarne, 1999; Palacios et al., 2000; Sousa et al., 2009; Ilarri et al., 2012, 2015a). In most cases on freshwater systems,

the bivalve shells are exploited by the associated organisms to avoid predators, as well as to reduce the physical and/or physiological stress as they can be responsible for changes in current velocity (Gutierrez et al., 2003; Erwin, 2008). After the death of bivalves, shells may persist for many years in the environment and this physical structure left behind by the living organism is particularly relevant from an ecosystem engineering perspective (Bódis et al., 2013, 2014; Ilarri et al., 2015a, 2015b). As an example, in Grays harbor (West coast of North America) extensive shell deposits of the soft-shell clam *Mya arenaria* (Linnaeus, 1758) cover parts of the intertidal flats in that estuarine ecosystem and have received much attention because of their role as nursery ground for Dugess crab *Cancer magister* (Dana, 1852). The most interesting part is that the majority of the shells were deposited after a mass-mortality event that took place around 1895 (Palacios et al., 2000). After more than 100 years shells still persist in that area constituting a dominant ecological and structural feature of the ecosystem (Palacios et al., 2000). The abundance, size, spatial distribution and persistence of empty bivalve shells are relevant factors that can act as an important biological legacy, given their long lasting effects to the ecosystem. Despite their possible high ecological relevance very few studies explore this perspective and there is a lack of quantitative assessments regarding this topic (but see Bódis et al., 2014; Ilarri et al., 2015a, 2015b; Novais et al., 2015a).

In addition to the above mentioned potential ecological importance, freshwater bivalve populations have been declining worldwide. Indeed, 224 (44%) of the 511 assessed freshwater mussel species are classified as Threatened or Near Threatened by the 2015 IUCN Red List of Threatened Species. In Europe, from the 16 currently recognized freshwater mussel species, 12 are also Threatened or Near Threatened. The major threats to these species include loss and fragmentation of habitat, pollution, overexploitation, climate change, and introduction of non-indigenous invasive species (NIS). Freshwater bivalves are vulnerable to all these threats, and resultant declines in their diversity and population structure may have important repercussions in ecosystem functions and services including those resulting from the loss of ecosystem engineering activities.

## 1.2. Non-Indigenous species – *Corbicula fluminea* (Müller, 1774)

Biological invasions are a process representing the arrival of a species into an area in which it did not exist in a historical time (Carlton, 1979). The dispersion of species was, until recently, mainly a natural process, with high temporal variability, wherein species sought to colonize new areas due to a number of possible reasons associated with a constant dynamic world and due to environmental and climatic changes (e.g. tectonic movements, glaciations, among others).

In the last decades the rate at which species are crossing biogeographic barriers is completely unprecedented. This situation is associated with anthropogenic activities (Sakai et al., 2001; Simberloff et al., 2013). Indeed, these biological invasions are mostly related to anthropogenic activities such as global trade, agriculture, construction of canals, aquaculture, recreational activities, transportation by transatlantic ships and consequent ballast water release, pet industry, among others, that either intentionally or unintentionally transport species beyond their limits of natural dispersal (Kolar & Lodge, 2001; Sousa et al., 2008b). In addition, some intrinsic features of some NIS make them more prone to be introduced, establish new populations and disperse to new habitats. In theory (and many exceptions exist in the literature), features that confer NIS their invasiveness include, a wide geographical distribution, genetic variability, rapid growth, rapid sexual maturity, short generation time, physiologic tolerance to abiotic changes and interaction with human activities that can promote their dispersal (Darrigran, 2002; Sousa et al., 2008b).

With the increasing concern over this problem, the introduction, establishment and further dispersion has become one of the greater topics of research in ecology mainly because of the high ecological and economic impacts generated by some invasive species (Facon et al., 2006; Sax et al., 2007; Simberloff et al., 2013). Given this situation it is of most importance to predict potential establishment and dispersal of NIS, as well as to quantitatively assess the possible effects in the invaded areas in order to prevent or reduce future ecological, economic and social impacts.

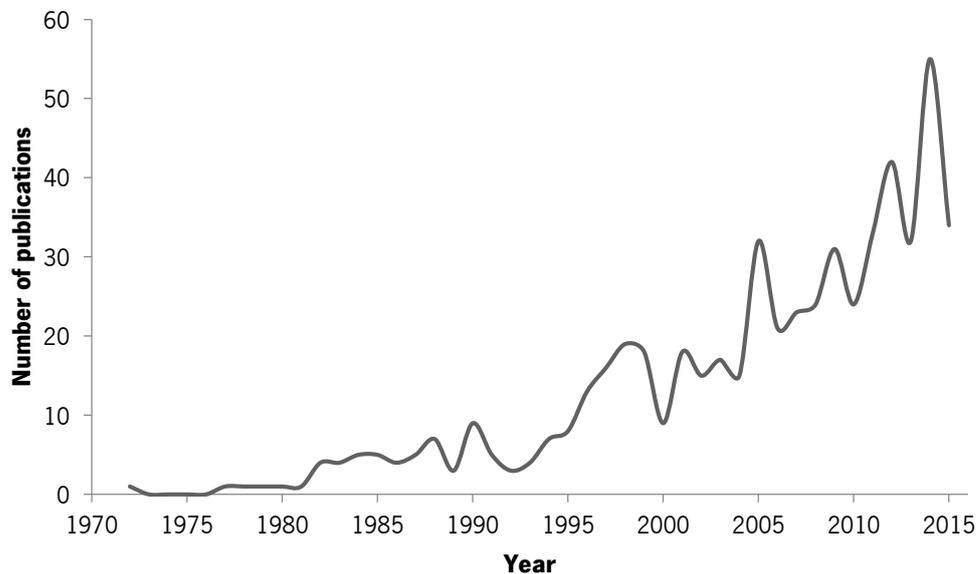
Any type of ecosystem (terrestrial, freshwater and marine) is immune to NIS introductions. However, freshwater ecosystems are especially vulnerable to these introductions and this thematic has gained a fundamental importance in aquatic ecology, mainly in the last two decades (Strayer, 2010). Carlton (1992), stated that an increasing number of future aquatic system invasions could be expected with confidence. In fact, this statement has been confirmed through the years, and it is also known that the number of introduced species will even accelerate in the future (Strayer, 2010; Sousa et al., 2014). Biological invasions in aquatic ecosystems can have important

environmental effects, including reduction of biodiversity and the disruption of ecological processes and functions (Byrnes et al., 2007; Sousa et al., 2011; Gutiérrez et al., 2014).

Theoretically, NIS with great density and biomass, the ones that can force new biological interactions (e.g. introduction of top predators, introduction of new parasites and diseases) or the ones that change biogeochemical cycles in the invaded ecosystem are those with more probability to generate higher negative impacts (Sousa et al., 2011; Strayer, 2012). In freshwater ecosystems, some bivalve species that can reach massive densities, such as the zebra mussel *Dreissena polymorpha* (Pallas, 1771), the quagga mussel *Dreissena bugensis* (Andrusov, 1897), the Asian clam *Corbicula fluminea* (Müller, 1774), and the Chinese pond mussel *Sinanodonta woodiana* (Lea, 1834) are well known invaders (Sousa et al., 2014). These invasive bivalves have been colonizing freshwater ecosystems worldwide and contributed to important disruptions of ecosystem processes and functions, representing a significant threat to biodiversity (Strayer & Dudgeon, 2010; Strayer, 2010; Sousa et al., 2009, 2014).

The Asian clam *Corbicula fluminea* (Müller, 1774) is one of the most successful NIS in freshwater ecosystems, and it is considered one of the most problematic invasive species in the world (McMahon, 2002; Pigneur et al., 2011). This species is described as a threat to various elements of the ecosystem, including phyto and zooplankton, macrozoobenthos and submerged vegetation and it can lead to important economic losses (Sousa et al., 2008a; Sousa et al., 2009). The original distribution of the *Corbicula* genus was confined to Asia, Africa and Australia in the beginning of the 20<sup>th</sup> century, and since then it has dispersed worldwide (Mouthon, 1981; Araujo et al., 1993; Ituarte, 1994; McMahon, 1999). *Corbicula fluminea* was first reported in USA during the first half of the twentieth century, first in the Pacific coast but rapidly spread to the Atlantic coast. Nowadays the species is widespread in North America, even colonizing some areas in the south of Canada (Crespo et al., 2015). In South America, the first specimens of the *Corbicula* genus were detected around the 1970s, in Rio de la Plata estuary (Ituarte, 1994). In Europe its presence was described for the first time in the early 1980's (Mouthon, 1981), occurring nowadays in almost all European countries from Portugal in the west to Romania in the east and Ireland in the North (Sousa et al., 2008a; Ilarri & Sousa, 2012; Crespo et al. 2015). *Corbicula fluminea* was firstly reported in Portugal in the 1980's in River Tejo (Mounthon, 1981) and presently occurs in most Portuguese hydrological basins (from the Minho in the North to Guadiana in the South), including many estuarine systems (Sousa et al., 2007a). With the worldwide dispersal of this

species and the negative ecological and economic described is not a surprise that the number of studies addressing the species greatly increased in the last two decades (Fig. 2).



**Fig. 2.** Number of *C. fluminea* publications per year assessed from Scopus until December 31, 2015.

The *C. fluminea* successful introduction, establishment and dispersal possibly relies in several biological traits, such as early sexual maturity and rapid growth, high fecundity and association with anthropogenic activities (i.e. juvenile's transportation through attachment on boats, ballast water release, utilization of individuals as fish bait, usage of specimens as food resource and transportation of sediments between different hydrological basins) (McMahon, 2002; Ilari & Sousa, 2012). Species from the *Corbicula* genus comprise different reproductive modes; however, *C. fluminea* is generally described as a hermaphroditic species, with a single organism theoretically being enough to generate a population.

When considering *C. fluminea* physiological tolerance, it appears to be lower than other freshwater bivalve species. Environmental fluctuations in oxygen, temperature, pH and low calcium concentrations are some of the abiotic conditions in which *C. fluminea* reveals less resistance compared to other freshwater bivalve species (McMahon, 1999, 2002; Sousa et al., 2008b). Despite *C. fluminea* lower resistance to some abiotic variations the species ability to rapidly recover due to its high growth and fecundity, makes this NIS much more resilient than the native bivalve species.

Ecosystem engineering actions performed by bivalves can have huge impacts in the structure and functions of aquatic systems if the species reaches a high density and biomass, such as the ones already observed for some systems invaded by the NIS *C. fluminea*. For instance, in the River Minho (NW of the Iberian Peninsula) some sites may reach more than 4000 ind.m<sup>-2</sup> (Sousa et al., 2008a). When present in such high density, *C. fluminea* high filtration rates influence the flow of organic matter in the water column, increasing light penetration and consequently the water clarity. In one hand, these conditions (i.e. more nutrients and higher clarity) influence the submerged vegetation growth. On the other hand, make the species an overall better competitor, given that it can reduce the food and energy resources to native bivalve species, even because this NIS can also rely in organic matter available in the sediments due to pedal feeding (Sousa et al., 2008b; Novais et al., 2016). Also, movements in the sediments via bioturbation activity can lead to changes in the abiotic conditions, as well as reduction of available habitat, and/or creating unsuitable abiotic conditions for native bivalves' recruitment (Sousa et al., 2008b, 2009; Caffrey et al., 2011; Ilarri et al., 2012). However, in some invaded areas this NIS appears to positively influence the macrozoobenthos community, as there is evidence of an increased density, biomass and diversity in areas where the density of *C. fluminea* is higher (Ilarri et al., 2012). The reasons behind this positive correlation are still not totally clear, but may be highly associated with the increase of biodeposits and the modification of substrate mediated by the presence of their shells (Novais et al., 2015a).

Massive rapid die-off events of *C. fluminea* have been reported, caused by changes in abiotic conditions such as increased temperature in summer, associated with low dissolved oxygen levels and alterations in the water flow (Cooper et al., 2005; Ilarri et al., 2011; Ilarri & Sousa, 2012). These mortalities can be an important trophic subsidy to the adjacent terrestrial ecosystem and to some aquatic species such as detritivores and scavengers, given during these events the amount of nutrients and energy released to the ecosystem can reach massive levels (Sousa et al., 2007c., 2008a; Bódis et al., 2013; Novais et al., 2015b). On the other hand, these tremendous mortalities create a chain of events that may result in catastrophic conditions to the entire benthic fauna. *Corbicula fluminea* die-offs may increase eutrophication levels, leading to increased decomposition rates and subsequent higher ammonia concentrations in the aquatic system, resulting in levels that are not tolerated by the native unionoid mussels, particularly juveniles (Cherry et al., 2005). These conditions can be tolerated by *C. fluminea*, as it was recently found that this species presents lower sensibility to ammonia (Costa & Guilhermino, 2015), with this

feature being another factor in favour of *C. fluminea* competitive advantage over native species and contributing to their invasive success. However, an unstudied consequence of these massive die-offs is the legacy generated by the huge accumulation of empty shells and given the potential ecosystem engineering effects as described above this subject deserves attention.

In economic terms, *C. fluminea* can be responsible for great financial losses if the invaded system is responsible for important ecosystem services, such as provision of cooling water for a power plant, drinking and irrigation water, supports aquaculture, recreational activities and commercial or sport fishing. Despite *C. fluminea* inability to physically attach to solid structures like other invasive bivalves (i.e. zebra mussel *Dreissena polymorpha*), the accumulation of shells can translate into economic losses by creating detrimental conditions for fisheries and tourism in fluvial beaches, as well as serious biofouling problems in small diameter pipelines, heat exchanger tubing and fire protection lines in power plants and irrigation pipes. These biofouling problems may result in system failures and reduction of water quality by filter damage in water treatment plants (Rosa et al., 2011). Frequent pipe replacement or mechanical cleaning can become financially costly over time, and chemical cleaning may involve environmental impacts like heavy metal pollution in the water systems (Cooper et al., 2005; Caffrey et al., 2011; Ilarri & Sousa, 2012). As eradication is virtually impossible in systems where *C. fluminea* reach high density and is widespread, more scientific knowledge about the species is required in order to understand their ecological (and economic) impacts, aiming to mitigate the overall negative effects of the species on the invaded systems. Since, *C. fluminea* is categorized as one of 100 worst invasive species in Europe, and in addition to the possible effects that this species may have on native species, studies addressing important ecological mechanisms such as ecosystem engineering are essential and highly timely.

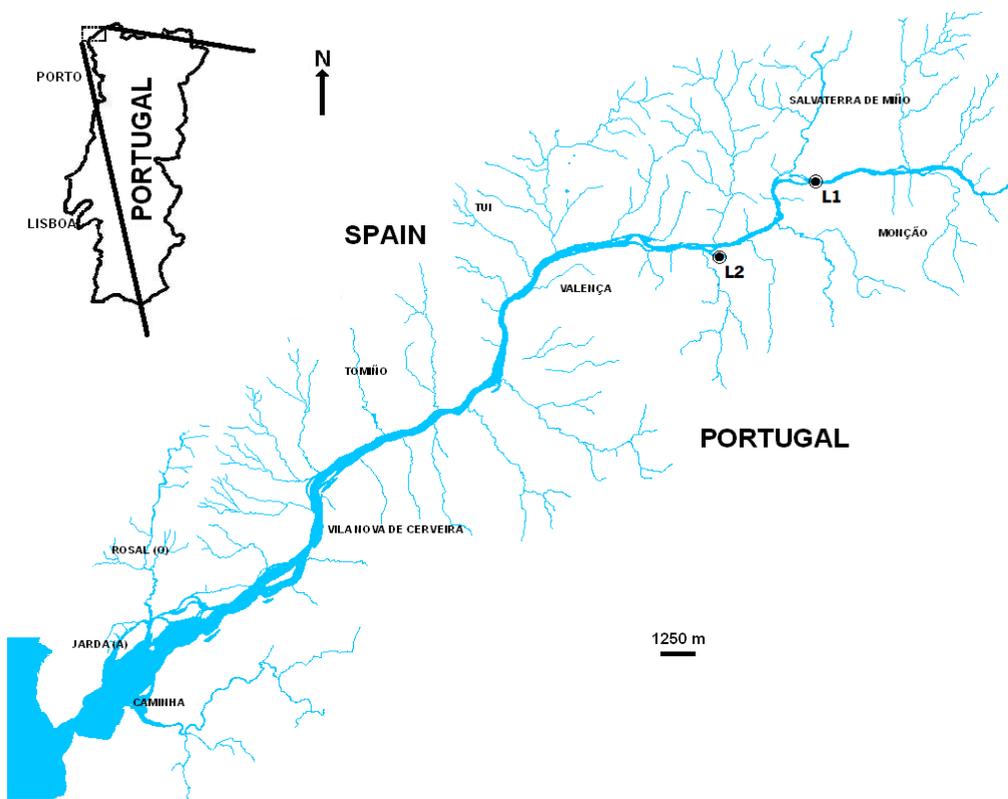
### 1.3. Objectives of the study

Since the introduction of *C. fluminea* in the River Minho in 1989 (Araujo et al., 1993), the density of native bivalve species (i.e. *Anodonta anatina* (Linnaeus, 1785), *Potomida littoralis* (Cuvier, 1798) and *Unio delphinus* (Spengler, 1793)) drastically declined (Sousa et al., 2008d). Currently, *C. fluminea* is the dominant species in the last 70km of the river, in terms of density, biomass and secondary production (Sousa et al., 2005, 2007b, 2008a,b,e; Ilarri et al., 2012). The species is also responsible for the majority of empty shells deposited in the river bottom, with some areas reaching empty shell densities of more than 2000 ind.m<sup>2</sup> (Ilarri et al., 2015b). These empty shells associated with the huge densities of living organisms lead to a homogenization of the substrate due to the massive accumulation of *C. fluminea* shells. Very few studies addressed the theoretical importance of empty bivalve shells as an ecosystem engineering process with relevant impacts in the freshwater benthic environment. Indeed, little is known about the faunal groups that may utilize the empty shells as substrata for attachment or refuge. Having this premise in mind, and in the context of this study, it is fundamental to understand if shells of different bivalve species (native and NIS) support distinct associated fauna, aiming to evaluate the effect of substrate homogenization mediated by the presence of a successful and dominant non-indigenous bivalve species. Assuming that shells with distinct morphologies and origins (native and NIS) can influence differently the macrozoobenthic community, and given the huge amount of *C. fluminea* shells deposited in the invaded areas, it is of most importance to determine also the shell persistence and decay rates of the different bivalve species, and how much time they can persist in freshwater ecosystems subjected to different environmental conditions.

## 2. Methods

### 2.1. Study Area

The study was performed in the River Minho (NW of the Iberian Peninsula). The River Minho drains a hydrological basin with a total area of 17080 km<sup>2</sup>, of which 95% is located in Spain and 5% in Portugal. This river originates in Serra da Meira, in the province of Lugo, Spain, and has an extent of approximately 300 km. The last 70 km are located in the Portuguese/Spanish Border, draining NNE-SSW into the Atlantic Ocean (Sousa et al., 2005, 2007e). The study was conducted in two sites located in the international section of the River Minho. The first location (L1) in the village of Cortes, Monção (42°04'36.90''N; 8°30'54.42''W) and the second location (L2) in the village of Friestas, Valença (42°02'59.60''N; 8°33'29.75''W) (Fig. 3). Both sites were shallow, with permanent freshwater conditions and with similar substratum composition mainly consisting of pebbles, cobbles and coarse and medium sand, macrophytes and *C. fluminea* shells. The main difference between sites was the water current: L1 is characterized by fast-flowing (lotic) water conditions and L2 by a slow-flowing (lentic) water regime throughout the entire year.



**Fig. 3.** Map of the River Minho showing the location of the lotic (L1) and lentic (L2) sites chosen for the experiments.

## 2.2. Experimental design and laboratory procedures

### 2.2.1. Field assay 1 - Empty bivalve shells as hard substrata for the associated macrozoobenthic fauna

To study the effects of the bivalve shells on the associated macrozoobenthic community, empty shells of two native (*P. littoralis* and *U. delphinus*) and one non-indigenous (*C. fluminea*) species were used. In order to simulate different substrates, seven treatments were used, three of them consisting in homogeneous substrates, with shells of only one species (*C. fluminea*; *P.littoralis*; *U. delphinus*) and four of them consisting in heterogeneous substrates, with shells of more than one species, for these it was considered all the possible combinations using two and three types of shells of the different species (i.e. combinations of shells of two and three species) (Table1). Shells of recently dead organisms that were intact were selected and manually cleaned to remove any traces of soft tissues, dried at 50 C° for 48 hours, weighted, and their diameter measured to nearest 0.1 mm. In order to standardize the treatments, an identical sum of shell 's outer surface area (1000cm<sup>2</sup>) was considered (i.e. homogeneous: 1000cm<sup>2</sup>; heterogeneous with two species: 500cm<sup>2</sup>+500cm<sup>2</sup>; and heterogeneous with three species: 333.3cm<sup>2</sup> x3) (Table 1). Surface areas were estimated by covering the outer surface of the different bivalve shells with aluminum foil. The foil was subsequently weighted and converted to area using a standard area-mass ratio (following Ricciardi et al., 1995 and Sousa et al., 2010), and linear regressions were established correlating area and diameter of the different bivalve shells. Ten replicates of each treatment were prepared, totalizing seventy samples.

**Table 1.** Experimental design of the seven different treatments, considering the total outer shell area and type of substratum.

<b>Treatment</b>	<b>Bivalve Shells</b>	<b>Shell Area</b>	<b>Substratum</b>
1 ('Corbicula')	<i>C. fluminea</i>	1000cm <sup>2</sup>	Homogeneous
2 ('Potomida')	<i>P. littoralis</i>		
3 ('Unio')	<i>U. delphinus</i>		
4 ('Corbicula+Potomida')	<i>C. fluminea</i> + <i>P. littoralis</i>	500cm <sup>2</sup> +500cm <sup>2</sup>	Heterogeneous (2 species)
5 ('Corbicula+Unio')	<i>C. fluminea</i> + <i>U. delphinus</i>		
6 ('Unio+Potomida')	<i>P. littoralis</i> + <i>U. delphinus</i>		
7('Corbicula+Unio+Potomida')	<i>C. fluminea</i> + <i>P. littoralis</i> + <i>U. delphinus</i>	333.3cm <sup>2</sup> + 333.3cm <sup>2</sup> + 333.3cm <sup>2</sup>	Heterogeneous (3 species)

Shells of each treatment were placed in sealed nylon net bags with 10mm of mesh size in order to facilitate the colonization of the macrozoobenthic community and exclude large animals. The seven treatments were tied together into one iron stake (ten replicates were used in the experiment), and placed underwater (around 70cm depth) and near the river bank (around 10m away from the left bank). Shells remained underwater for two months (July and August 2014). This period of time was sufficient for substantial colonization because these months of the year are associated with the highest rates of recruitment by many invertebrate species in the River Minho (Sousa et al., 2007b, 2007c, 2008c). The experiment was conducted in site L1. At the end of the experiment, the bag contents were sieved using a 500µm, and the macrozoobenthos associated with the empty shells were sorted and fixed in 70% ethanol. During the following weeks, organisms were identified to the lowest practical taxonomic level using Tachet et al. (2003).

### 2.2.2. Field assay 2- Empty bivalve shell decays

To study the empty shell decay rates in the aquatic system, shells of four bivalve species were used, namely *A. anatina*, *C. fluminea*, *P. littoralis* and *U. delphinus* (Fig. 4). The study considered the shell decays of individual shells of the four species and also of three different sizes (i.e. small, medium and large). In this last case, only shells of *C. fluminea* and *U. delphinus* were used. Following the same procedure as in the field assay 1 (described above), intact shells were selected and manually cleaned. Shells were placed in sealed nylon net bags with 10mm mesh size. Six treatments were performed, four of them consisting in one individual shell of each species (i.e. *A. anatina*, *C. fluminea*, *P. littoralis* and *U. delphinus*) per sealed nylon bag, and two treatments consisting in the three shells of different sizes of the same specie (*C. fluminea* and *U. delphinus*) per individual nylon bag. Each treatment was replicated twelve times totalizing seventy two samples. Each of the six treatments were tied together with a string into one iron stake and placed underwater (around 70cm deep) and near the river bank (around 10m away of the left bank). Shells remained underwater for one month during each season (spring, summer, autumn, winter) in order to assess possible seasonal variations in the shell decay rates. After each month of experiment, the shells were cleaned, dried, measured and weighted following the same procedure as described above. The experiment was conducted simultaneously in the two study areas (L1 and L2) in order to determine also if the different water flow regimes (lentic and lotic) affect the shell decay rates.



**Fig. 4.** Bivalve species used in the shell decay experiment conducted in both selected areas (L1 and L2) in the River Minho: *Anodonta anatina* (a), *Potomida littoralis* (b), *Unio delphinus* (c), and *Corbicula fluminea* (d).

### 2.3. Data analysis

Permutational multivariate analysis of variances (PERMANOVA) were performed to compare possible differences in the associated macrozoobenthic fauna between different treatments and also to assess possible spatial and temporal differences in shell decay rates between bivalve species. In the experiment “empty bivalve shells as hard substrata for the associated macrozoobenthic fauna” a one-way PERMANOVA (type-III) was performed, with treatment (seven levels: Corbicula, Potomida, Unio, Corbicula + Potomida, Corbicula + Unio, Unio + Potomida, Corbicula + Unio + Potomida) as a fixed factor. The ecological indexes, species richness (S), the Shannon-Wiener index ( $H'$ ) and Pielou's evenness index ( $J'$ ) of the associated macrozoobenthic community were calculated using DIVERSE analysis. In the experiment “Empty bivalve shell decays” for the comparison between the four species a three-way PERMANOVA (type-III) was performed, with treatment (four levels: Corbicula, Potomida, Unio and Anodonta), season (four levels: spring, summer, autumn and winter) and river condition (two levels: lentic and lotic) as fixed factors. In the comparison between the different size classes, a four-way PERMANOVA (type-III) was used, with treatment (two levels: Corbicula and Unio), size (three levels: small, medium and large), season (four levels: spring, summer, autumn and winter) and river condition (two levels: lentic and lotic) as fixed factors.

Before each PERMANOVA analysis the variables were always normalized without data transformation and a resemblance matrix based on the Euclidean distances were calculated. In all PERMANOVA tests was considered a statistical significance of variance of ( $\alpha=0.05$ ) with 9999 permutations of residuals within a reduced model. When the number of permutations were lower than 150, the Monte Carlo  $p$ value was considered. For the PERMANOVA significant results, pairwise comparisons were also performed.

The contribution of the shell size in the shell decays was accessed through a distance-based linear modelling (DistLM). In the DistLM, was selected the AIC (Akaike Information Criterion) based on the analyses on the Euclidean distance resemblance after normalisation of the data.

PRIMER software (vers. 6.1.6, PRIMER-E Ltd, Plymouth, U.K.) with PERMANOVA + 1.0.1 add-on (Anderson et al., 2008) was used for all statistical tests and analyses.

The estimative of the number of years necessary for the total shell disintegration of the bivalve's species was calculated considering the mean percent of shell decay loss per month of each species.

### 3. Results

#### 3.1 Field Assay 1- Empty bivalve shells as hard substrata for the associated macrozoobenthic fauna

The treatment containing the largest size of shells corresponded to 'Potomida' (69.6±2.7mm) and the smallest corresponded to 'Corbicula' (26.7±0.7mm). As *C. fluminea* presented the smallest shells, the treatment 'Corbicula' contained the highest number of shells per bag (53.6±1.3). On the other hand, 'Potomida' contained the lowest amount of shells per bag (13.6±0.5) (Table 2).

**Table 2.** Mean (±sd) size (mm) and number of shells used in each of the seven treatments.

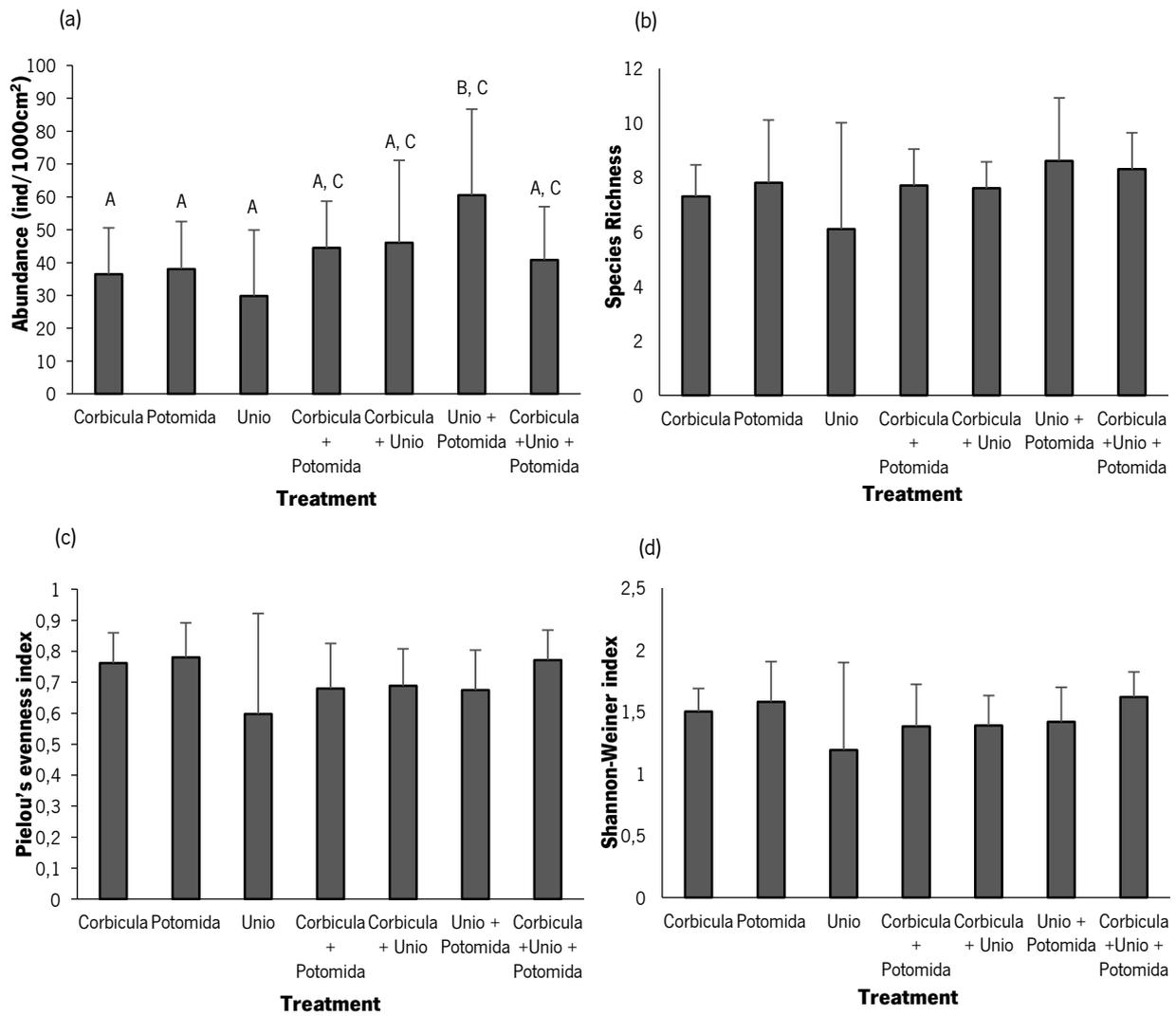
	Treatment						
	'Corbicula'	'Potomida'	'Unio'	'Corbicula + Potomida'	'Corbicula + Unio'	'Unio + Potomida'	'Corbicula + Unio + Potomida'
<b>Shell size (mm)</b>	26.7±0.7	69.6±2.7	50.7±26	33.8±0.9	33.4±1.5	56.5±3.5	38.3±1.3
<b>Number of shells</b>	53.6±1.3	13.6±0.5	26.5±1.4	42.7±1.2	54.7±2.8	28.0±1.5	33.6±1.1

A total of 2961 individuals corresponding to 30 macrozoobenthic taxa, representing four phyla (Annelidia, Arthropoda, Mollusca, Platyhelminthes), were associated with the empty bivalve shells. The two most diverse groups were the insects (15 taxa) and molluscs (10 taxa), being responsible for 83.3% of the taxa observed. The four most abundant taxa were the gastropod *Bithynia tentaculata* (46.1%), the planarians of the Tricladida order (11.2%), the water hoglouse *Asellus aquaticus* (9.8%) and the Asian clam *C. fluminea* (9.4%). The remaining 26 taxa made up only 23.5%. The ten most abundant species accounted for 96.4% of all the taxa recorded in this study (Table 3).

**Table 3.** Mean ( $\pm$ sd) abundance (ind/1000cm<sup>3</sup>) of the top ten dominant taxa recorded associated to the different treatments used in the River Minho.

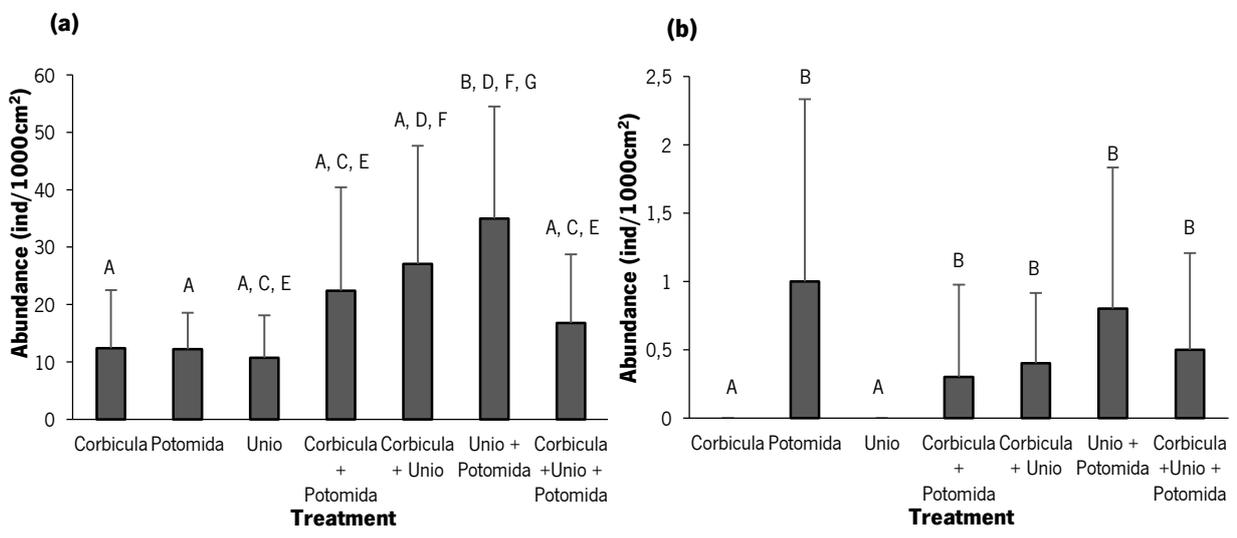
Taxonomic group	Treatment							Total mean abundance	Abundance Rank
	'Corbicula'	'Potomida'	'Unio'	'Corbicula + Potomida'	'Corbicula + Unio'	'Unio + Potomida'	'Corbicula + Unio + Potomida'		
Mollusca									
<i>Bithynia tentaculata</i>	12.4 $\pm$ 10.1	12.2 $\pm$ 6.3	10.7 $\pm$ 7.4	22.4 $\pm$ 18.0	27.1 $\pm$ 20.5	35 $\pm$ 19.5	16.8 $\pm$ 12.0	19.5 $\pm$ 13.4	1
<i>Corbicula fluminea</i>	2.5 $\pm$ 2.3	4.7 $\pm$ 3.6	2.4 $\pm$ 3.2	5.7 $\pm$ 6.5	2.2 $\pm$ 1.8	5.7 $\pm$ 4.7	4.7 $\pm$ 4.7	4.0 $\pm$ 3.9	4
<i>Physella acuta</i>	0	1.0 $\pm$ 1.3	0	0.3 $\pm$ 0.7	0.4 $\pm$ 0.5	0.8 $\pm$ 1.0	0.5 $\pm$ 0.7	0.4 $\pm$ 0.6	10
Annelida									
Hirudinea									
sp1	2.1 $\pm$ 2.2	1.6 $\pm$ 1.2	1.0 $\pm$ 1.5	1.0 $\pm$ 0.9	0.7 $\pm$ 1.3	1.9 $\pm$ 1.0	2.1 $\pm$ 2.0	1.5 $\pm$ 1.4	7
sp2	2.2 $\pm$ 1.3	2.1 $\pm$ 1.5	0.7 $\pm$ 1.3	1.7 $\pm$ 1.6	1.2 $\pm$ 1.4	2.5 $\pm$ 2.2	2.1 $\pm$ 1.6	1.8 $\pm$ 1.6	6
Oligochaeta									
Lumbriculidae	0.7 $\pm$ 1.1	1.1 $\pm$ 2.0	0.6 $\pm$ 0.9	1.0 $\pm$ 0.9	0.9 $\pm$ 1.0	0.6 $\pm$ 1.6	1.5 $\pm$ 2.2	0.9 $\pm$ 1.4	8
Naididae	5.3 $\pm$ 9.1	6.9 $\pm$ 14.6	3.2 $\pm$ 9.1	1.7 $\pm$ 2.7	0.6 $\pm$ 0.8	1.2 $\pm$ 1.6	2.0 $\pm$ 3.5	3.0 $\pm$ 6.0	5
Arthropoda									
Crustacea									
<i>Aselus aquaticus</i>	2.8 $\pm$ 2.5	2.7 $\pm$ 2.2	3.2 $\pm$ 3.6	3.8 $\pm$ 2.6	5.6 $\pm$ 4.1	6.5 $\pm$ 4.4	4.4 $\pm$ 4.1	4.1 $\pm$ 3.4	3
Insecta									
Leuctridae	0.8 $\pm$ 1.0	0.2 $\pm$ 0.6	1.0 $\pm$ 1.6	0.6 $\pm$ 1.1	1.2 $\pm$ 2.1	1.2 $\pm$ 1.6	0.5 $\pm$ 0.9	0.8 $\pm$ 1.3	9
Platyhelminthes									
Tricladida	6.6 $\pm$ 8.3	3.9 $\pm$ 4.4	5.0 $\pm$ 4.9	5.0 $\pm$ 6.9	4.8 $\pm$ 2.3	3.3 $\pm$ 2.3	4.7 $\pm$ 3.9	4.8 $\pm$ 4.8	2

Significant differences in the abundance of the associated macrozoobenthic organisms were observed in the comparison between treatments (Pseudo-F=2.52,  $p < 0.05$ ). The treatment that had the highest abundance (mean $\pm$ sd) of organisms was 'Unio + Potomida' (60.50 $\pm$ 26.24 individuals/1000cm<sup>2</sup>) followed by 'Corbicula + Unio' (46.0 $\pm$ 25.2 individuals/1000cm<sup>2</sup>), and the lowest was obtained in 'Unio' (29.80 $\pm$ 20.12 individuals/1000cm<sup>2</sup>) Fig. 5a). The highest species richness (mean $\pm$ sd) was also observed for the treatment 'Unio+Potomida' (8.60 $\pm$ 2.32) followed by 'Corbicula+Unio+Potomida' (8.30 $\pm$ 1.34), and the lowest was obtained in 'Unio' (6.10 $\pm$ 3.90); no significant differences regarding the species richness were observed in the comparison between treatments (Fig. 5b). The highest evenness (Pielou's index) (mean $\pm$ sd) was observed for 'Potomida' (0.78 $\pm$ 0.11) followed by 'Corbicula' (0.76 $\pm$ 0.10), and the lowest was obtained in 'Unio' (0.60 $\pm$ 0.32); no significant differences regarding evenness were observed in the comparison between treatments (Fig. 5c). The highest diversity values (Shannon-Weiner index) (mean $\pm$ sd) were observed for the treatment containing the mix of three types of bivalve shell, namely 'Corbicula + Unio + Potomida' (1.62 $\pm$ 0.20) followed by 'Potomida' treatment (1.58 $\pm$ 0.33), being the treatment with the lowest diversity 'Unio' (1.19 $\pm$ 0.71); no significant differences regarding the diversity were observed in the comparison between treatments (Fig. 5d).



**Fig. 5.** Mean ( $\pm$ sd) abundance (ind/1000cm<sup>2</sup>) (a), species richness (b), Pielou's evenness index (c) and Shannon-Weiner index (d) of the macrozoobenthic associated communities that colonized the empty shells of the seven treatments. Distinct letters indicate significant differences between treatments (one-way PERMANOVA pairwise tests;  $P < 0.05$ ).

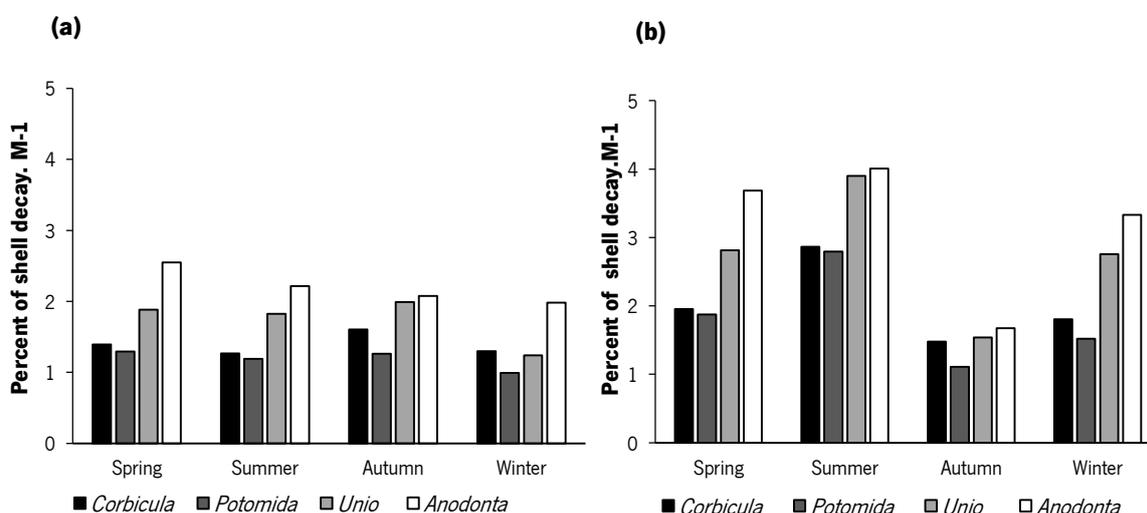
The abundance of 2 taxa were significantly different between treatments. Significant differences were observed for the gastropod *Bithynia tentaculata* (Pseudo-F=3.94,  $p<0.05$ ). This species was the most abundant species colonizing the empty shells, with the highest abundance values (mean $\pm$ sd) observed in the heterogeneous substrates composed by two types of shells, namely 'Unio+Potomida' ( $35\pm 19.5$ ), 'Corbicula+Unio' ( $27.1\pm 20.5$ ) and 'Corbicula+Potomida' ( $22.4\pm 18.0$ ) (Fig. 6a). Significant differences were also observed for the gastropod *Physella acuta* (Pseudo-F=2.45,  $p<0.05$ ), with the treatments containing *P. littoralis* shells showing the highest abundance values, namely 'Potomida' ( $1.0\pm 1.3$ ), 'Unio + Potomida' ( $0.8\pm 1.0$ ) and 'Corbicula + Unio + Potomida' ( $0.5\pm 0.7$ ) (Fig. 6b). Beyond these two species, three other also had higher abundances; however, no significant differences were observed. Indeed, the water hoglouse *Asellus aquaticus* also had high abundance values in the heterogeneous treatments of two and three types of shells. *Corbicula fluminea* and the planarians from the Tricladida order were also very abundant; however, both species did not reveal any pattern of preference between homogeneous and heterogeneous substrates.



**Fig. 6.** Mean ( $\pm$ sd) abundance (ind/1000cm<sup>2</sup>) of the two species that presented significant differences in the comparison between the different treatments: *Bithynia tentaculata* (a) and *Physella acuta* (b). Distinct letters indicate significant differences between treatments (one-way PERMANOVA pairwise tests;  $P<0.05$ ).

### 3.2. Field assay 2- Empty bivalve shell decays

*Anodonta anatina* was the species that exhibited the highest percentage of decay, followed by *U. delphinus*, *C. fluminea* and *P. littoralis* (Fig. 7). This pattern repeated during all seasons and independently of the river condition (Fig. 7). The highest percentage of decay was observed for the shells submitted to lotic condition, with the highest values during the summer, whereas under lentic conditions the highest values were observed during spring (for *A. anatina* and *P. littoralis*) and autumn (for *C. fluminea* and *U. delphinus*) (Fig. 7).



**Fig. 7.** Mean values of the percent of shell decay per month of the different bivalve species submitted to lentic (a) and lotic (b) conditions in the River Minho.

The shell decay differed significantly between species, season and river condition (Pseudo- $F=2.89$ ,  $p<0.05$ ) (Table 4). Pairwise tests indicated that the differences were associated to different shell decays observed for the four species. Also under lentic conditions the differences were associated to the lower decays observed during the winter, whereas under lotic conditions summer and autumn contributed to the differences observed. Shell decays for each species also differed when comparing the river condition, the only exception was during autumn. *Anodonta anatina* was the species that presented the largest shells, followed by *P. littoralis*, *U. delphinus* and *C. fluminea* (Table 4). In fact, the size of the shells have influenced the shell decays, explaining 61.51% of the variance (AIC=-359.77, SS=233.11, Pseudo- $F=603.96$ ,  $p<0.05$ ).

**Table 4.** Mean ( $\pm$ sd) shell size and decay (g) per season (spring, summer, autumn and winter) and river condition (lentic and lotic) and three-way PERMANOVA test results of the shell decay comparison between species, season and river condition. Different letters indicate significant differences between them (three-way PERMANOVA pairwise tests;  $p < 0.05$ ). Lower case letters refers to comparisons among season, while upper case letters refers to comparisons among river conditions.

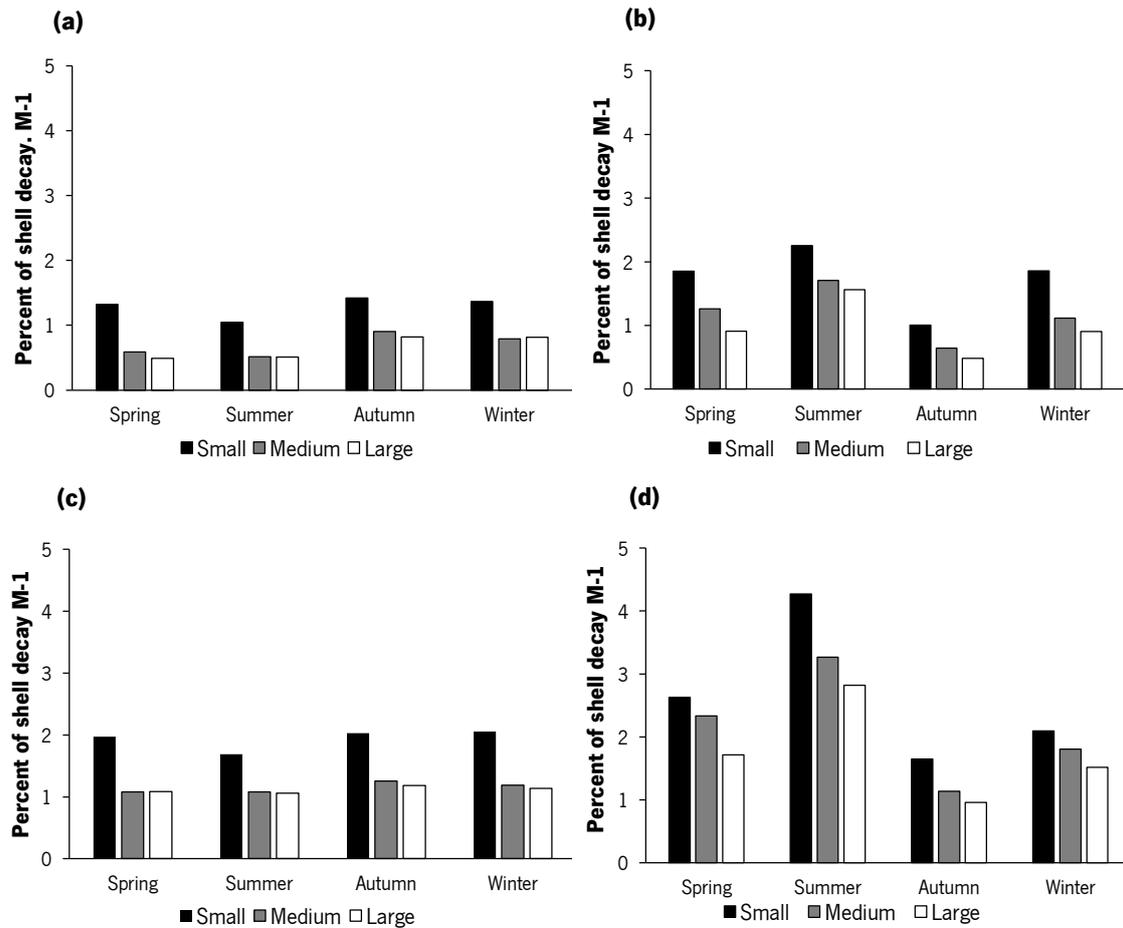
Source	Df	SS	MS	Pseudo-F	p-value	
Species x Season x River condition	9	7.48	0.83	2.89	<0.05	
Residual	348	99.88	0.29			
Total	379	379				
Pairwise results						
Species	Shell size	River condition	Season			
			Spring	Summer	Autumn	Winter
<i>Corbicula</i>	31.28 $\pm$ 1.82	lentic	0.04 $\pm$ 0.009 <sup>a;A</sup>	0.03 $\pm$ 0.008 <sup>a,b;A</sup>	0.04 $\pm$ 0.01 <sup>a,b,c;A</sup>	0.05 $\pm$ 0.009 <sup>a,c;A</sup>
<i>fluminea</i>	31.11 $\pm$ 3.01	lotic	0.06 $\pm$ 0.02 <sup>a;B</sup>	0.09 $\pm$ 0.02 <sup>b;B</sup>	0.04 $\pm$ 0.01 <sup>c;A</sup>	0.06 $\pm$ 0.009 <sup>a;B</sup>
<i>Potomida</i>	69.41 $\pm$ 6.05	lentic	0.17 $\pm$ 0.03 <sup>a;A</sup>	0.16 $\pm$ 0.06 <sup>a,c;A</sup>	0.19 $\pm$ 0.04 <sup>a,c,d;A</sup>	0.14 $\pm$ 0.03 <sup>b,c;A</sup>
<i>littoralis</i>	71.56 $\pm$ 9.12	lotic	0.30 $\pm$ 0.16 <sup>a;B</sup>	0.40 $\pm$ 0.15 <sup>a,c;B</sup>	0.18 $\pm$ 0.05 <sup>b;A</sup>	0.26 $\pm$ 0.09 <sup>a;B</sup>
<i>Unio</i>	60.48 $\pm$ 5.72	lentic	0.10 $\pm$ 0.04 <sup>a;A</sup>	0.08 $\pm$ 0.02 <sup>a,b;A</sup>	0.11 $\pm$ 0.02 <sup>a,c;A</sup>	0.09 $\pm$ 0.02 <sup>a,b;A</sup>
<i>delphinus</i>	60.88 $\pm$ 5.39	lotic	0.16 $\pm$ 0.05 <sup>a;B</sup>	0.22 $\pm$ 0.06 <sup>b;B</sup>	0.08 $\pm$ 0.02 <sup>c;B</sup>	0.16 $\pm$ 0.02 <sup>a;B</sup>
<i>Anodonta</i>	105.19 $\pm$ 21.14	lentic	0.37 $\pm$ 0.18 <sup>a;A</sup>	0.29 $\pm$ 0.13 <sup>a;A</sup>	0.30 $\pm$ 0.13 <sup>a;A</sup>	0.26 $\pm$ 0.10 <sup>a;A</sup>
<i>anatina</i>	106.37 $\pm$ 24.35	lotic	0.60 $\pm$ 0.33 <sup>a;B</sup>	0.69 $\pm$ 0.23 <sup>a;B</sup>	0.26 $\pm$ 0.10 <sup>b;A</sup>	0.56 $\pm$ 0.16 <sup>a;B</sup>

Differences in the total time for the disintegration was also observed, when comparing the species and river condition. The species that presented the most resistant shells was *P. littoralis*, followed by *C. fluminea*, *U. delphinus* and *A. anatina* (Table 5). The same pattern was observed for the species when comparing the river conditions; nonetheless, shells under lentic conditions have a higher persistence in the system (Table 5).

**Table 5.** Estimative of the number of years necessary for the total shell disintegration of four bivalve species of the River Minho when submitted to lentic and lotic conditions.

Species	Total decay time (years)	
	Lentic	Lotic
<i>Corbicula fluminea</i>	6.0	4.1
<i>Potomida littoralis</i>	7.0	4.6
<i>Unio delphinus</i>	4.8	3.0
<i>Anodonta anatina</i>	3.8	2.6

Regarding shell decay with size variation we observed that *U. delphinus* had higher percentage of shell decays compared to *C. fluminea* (Fig. 8). The small size class had the highest decay percentage, followed by the medium and large size classes (Fig. 8). The highest decays were observed for the shells submitted to lotic conditions (Fig. 8). The highest percentage values were observed for *C. fluminea* (2.3%) and *U. delphinus* (4.3%) during the summer and for the small size class. Under lentic conditions the highest values were also observed for the small size class; however, for *C. fluminea* was during autumn (1.4%) whereas for *U. delphinus* was during winter (2.1%) (Fig. 8).



**Fig. 8.** Mean values of the percent of shell decay per month of *Corbicula fluminea* and *Unio delphinus* shells of different size classes when submitted to lentic and lotic conditions. *Corbicula fluminea* (lentic) (a); *Corbicula fluminea* (lotic) (b); *Unio delphinus* (lentic) (c); *Unio delphinus* (lotic) (d).

The shell decay differed significantly between species, season, river condition and size (Pseudo- $F=3.55$ ,  $p \leq 0.05$ ) (Table 6). Pairwise tests indicated that the differences were associated to different shell decays observed for the two species. Furthermore, under lentic conditions the shell decays of both species for the small size class were very similar and did not present any differences. For the other size classes this pattern was different. There was not a clear pattern for each species during the seasons. When comparing the river conditions, in most of the cases, the lotic was very different from the lentic being the only exception *U. delphinus* during autumn for the small and medium size classes (Table 6).

**Table 6.** Mean ( $\pm$ sd) shell size and decay (g) per season (spring, summer, autumn and winter) river condition (lentic and lotic) and size (small, medium and large) and four-way PERMANOVA test results of the shell decay comparison between species, size, season and river condition. Different letters indicate significant differences between them (four-way PERMANOVA pairwise tests;  $P < 0.05$ ). Lower case letters refers to comparisons among season, while upper case letters refers to comparisons among shell size.

Source		df	SS	MS	Pseudo-F	p-value	
Species x Size x Season x River condition		6	2.64	0.44	3.55	<0.05	
Residual		521	64.63	0.12			
Total		568					
Pairwise results							
Species	Size	Shell size	River condition	Season			
				Spring	Summer	Autumn	Winter
<i>Corbicula fluminea</i>	small	21.57 $\pm$ 1.57	lentic	0.02 $\pm$ 0.004 <sup>aA</sup>	0.01 $\pm$ 0.002 <sup>bA</sup>	0.01 $\pm$ 0.003 <sup>cA</sup>	0.02 $\pm$ 0.003 <sup>a,c;A</sup>
	medium	28.07 $\pm$ 1.49	lentic	0.02 $\pm$ 0.006 <sup>aA</sup>	0.01 $\pm$ 0.004 <sup>aA</sup>	0.02 $\pm$ 0.005 <sup>b;B</sup>	0.02 $\pm$ 0.002 <sup>b,c;B</sup>
	large	36.79 $\pm$ 2.47	lentic	0.03 $\pm$ 0.009 <sup>a;B</sup>	0.03 $\pm$ 0.007 <sup>a;B</sup>	0.04 $\pm$ 0.007 <sup>b;C</sup>	0.04 $\pm$ 0.009 <sup>b,c;C</sup>
<i>Unio delphinus</i>	small	30.39 $\pm$ 3.77	lentic	0.02 $\pm$ 0.006 <sup>aA</sup>	0.01 $\pm$ 0.005 <sup>bA</sup>	0.02 $\pm$ 0.005 <sup>a,b;d;A</sup>	0.01 $\pm$ 0.003 <sup>b,c;e;A</sup>
	medium	45.38 $\pm$ 2.55	lentic	0.03 $\pm$ 0.007 <sup>a;B</sup>	0.03 $\pm$ 0.005 <sup>a;B</sup>	0.03 $\pm$ 0.008 <sup>a,b;B</sup>	0.03 $\pm$ 0.004 <sup>a,c;B</sup>
	large	65.99 $\pm$ 4.25	lentic	0.08 $\pm$ 0.01 <sup>a;C</sup>	0.08 $\pm$ 0.01 <sup>a;C</sup>	0.09 $\pm$ 0.02 <sup>a,b;C</sup>	0.07 $\pm$ 0.02 <sup>a,c;C</sup>
<i>Corbicula fluminea</i>	small	21.89 $\pm$ 1.90	lotic	0.03 $\pm$ 0.006 <sup>aA</sup>	0.02 $\pm$ 0.008 <sup>aA</sup>	0.01 $\pm$ 0.002 <sup>bA</sup>	0.03 $\pm$ 0.007 <sup>a;A</sup>
	medium	28.42 $\pm$ 4.36	lotic	0.03 $\pm$ 0.01 <sup>a;B</sup>	0.04 $\pm$ 0.02 <sup>a,c;B</sup>	0.02 $\pm$ 0.02 <sup>b;B</sup>	0.03 $\pm$ 0.008 <sup>a,d;A</sup>
	large	37.73 $\pm$ 2.20	lotic	0.05 $\pm$ 0.01 <sup>a;C</sup>	0.09 $\pm$ 0.04 <sup>b;C</sup>	0.03 $\pm$ 0.005 <sup>c;C</sup>	0.06 $\pm$ 0.02 <sup>a;B</sup>
<i>Unio delphinus</i>	small	31.44 $\pm$ 5.00	lotic	0.02 $\pm$ 0.006 <sup>aA</sup>	0.04 $\pm$ 0.009 <sup>bA</sup>	0.01 $\pm$ 0.004 <sup>cA</sup>	0.02 $\pm$ 0.005 <sup>d;A</sup>
	medium	44.09 $\pm$ 9.06	lotic	0.06 $\pm$ 0.02 <sup>a;B</sup>	0.08 $\pm$ 0.03 <sup>b;B</sup>	0.03 $\pm$ 0.006 <sup>c;B</sup>	0.05 $\pm$ 0.02 <sup>d;B</sup>
	large	64.59 $\pm$ 10.63	lotic	0.14 $\pm$ 0.04 <sup>a;C</sup>	0.2 $\pm$ 0.05 <sup>b;C</sup>	0.07 $\pm$ 0.02 <sup>c;C</sup>	0.11 $\pm$ 0.03 <sup>d;C</sup>

The size of the shells have influenced the shell decays, explaining 54.27% of the variance (AIC=-442.22, SS=308.27, Pseudo- $F$ =672.95,  $p < 0.05$ ). The total time for the disintegration was also different when comparing the species, size and river condition (Table 7). *Corbicula fluminea* presented the most resistant shell compared to *U. delphinus*. The large shells persist more in the system than the shells of the other size classes (Table 7). Under lentic conditions the shells persist more in the system (Table 7).

**Table 7.** Estimative of the number of years necessary for the total shell disintegration of three different size (small, medium and large) *Corbicula fluminea* and *Unio delphinus* shells in the River Minho, when submitted to lentic and lotic conditions.

		Total decay time (years)		
Species	River condition	small	medium	large
<i>C. fluminea</i>	lentic	6.46	11.90	12.65
	lotic	4.79	7.05	8.64
<i>U. delphinus</i>	lentic	4.30	7.24	7.47
	lotic	3.13	3.90	4.75

## **4. Discussion**

### 4.1. Empty bivalve shells as hard substrata for the associated macrozoobenthic fauna

The results obtained in this study suggest that the empty bivalve shells create suitable habitat for many benthic organisms, especially for molluscs, platyhelminthes, crustaceans and insects. Overall, differences in the abundance of macrozoobenthic fauna colonizing homogeneous and heterogeneous shell substrates were found since treatments containing a mix of two types of bivalve shells had higher values. Indeed, those treatments had a significantly higher abundance of associated macrofauna, suggesting that substrate heterogeneity plays an important role in the macrozoobenthos colonization. The observed differences might be due to structural and morphological differences between the bivalve shells, as the three bivalve species used in this experiment have different morphological features. Shell hardness is different between species, with *P. littoralis* presenting a thick and hard shell, *C. fluminea* with a slightly thinner shell, and *U. delphinus* with a thin and somewhat fragile shell. Outer surface area is also different between the invasive and the native species, *C. fluminea* shells have a rough sculpture with raised concentric ribs, and both native species have an almost flat and smooth surface. Data suggest that the more complex physical structures formed with different bivalve species may support a more abundant and diverse macrozoobenthic community than treatments comprising a single species. For example, *U. delphinus* shells had the lowest abundance, species richness, diversity and evenness index values. However, in treatments when associated with *P. littoralis* shells the physical structure formed by these two types of shells revealed the highest abundance and species richness between all treatments. In fact, between the heterogeneous treatments, the one containing only native bivalve species shells, namely 'Potomida + Unio' was associated with the highest abundance values compared to the heterogeneous treatments containing the invasive species *C. fluminea*. The highest values of diversity were recorded for the treatment containing the mix of all three bivalve species shells ('Corbicula+Unio+Potomida'), this was expected as more diverse and physically complex substrate may harbor several species with different substratum preferences, thus increasing species diversity (Medeiros et al. 2011). Results suggest that the substrate homogenization occurring in the River Minho due to the great densities of *C. fluminea*, and to the decline of the native species is not the ideal to an abundant, rich and diverse associated fauna. It

is important to state that previous studies have already observed a positive effect of *C. fluminea* in the associated fauna, with the macrozoobenthic density and diversity positively responding to the increasing density of *C. fluminea* (Ilarri et al., 2012). However, this study assessed the influence of *C. fluminea* live specimens and not empty shells. Anyway, it seems that the availability of a higher diversity of shells is more beneficial to the associated macrozoobenthic fauna. As species richness was mostly even through all treatments used, it is reasonable to assume that *C. fluminea* shells can partially mimic the ecological role played by native bivalves in terms of associated macrozoobenthic community, mainly diversity. The loss of bivalve diversity occurring in the River Minho could lead to decreased macrozoobenthos abundance; however, due to the largely higher densities of *C. fluminea* compared to native bivalve species (see Sousa et al., 2008b, 2008d), the amount of shells deposited provides a larger amount of colonisable hard substrata. Although *C. fluminea* shells appear to have the same positive effects on most macrozoobenthic species (Ilarri et al., 2012), the ecological implications of the huge amounts of deposited shells (e.g. physicochemical properties of the ecosystem) are still to be identified and future detailed studies should be performed in order to investigate this situation. To date few studies compared the associated fauna of native and the invasive bivalve species such as *C. fluminea* (but see Bódis et al., 2014, Ilarri et al., 2015a), with this study being one of the few to investigate the influence of different levels of substrate heterogeneity in the associated fauna. Both Bódis et al., (2014) and Ilarri et al. (2015a) studies revealed higher species richness and abundance in treatments containing the native bivalve species versus the treatments containing *C. fluminea*. The results of this study corroborates the results of both authors, and suggest that an optimal level of abundance and species richness are associated with, not only native bivalve shells, but also with substrate heterogeneity. Therefore, the disappearance of native bivalve species such as *P. littoralis* and *U. delphinus* (and other species) in the River Minho (and in many other aquatic ecosystems), may have impacts on the macrozoobenthic fauna. Also, the continuous accumulation of *C. fluminea* shells may influence several freshwater faunal groups.

#### 4.2. Empty bivalve shell decay

Shell decay rates varied among bivalve species, seasons of the year, water flow condition, and size of the shell. Differences in shell decay rates between bivalve species appear to be mostly related to the structural and morphological dissimilarities between them. The four selected species present remarkable differences in size and shell thickness. As described above, *P. littoralis* presents the thickest and most robust shell, followed by *C. fluminea*, and by *U. delphinus* with a thin and delicate shell. *Anodonta anatina* presents the largest, thinnest and fragile shell in comparison with the other three species. Interestingly, *A. anatina* presented the highest percentage of shell decay during the four seasons, followed by *U. delphinus*, *C. fluminea* and *P. littoralis*, respectively. Though chemical composition, crystal size and mineralogy of the shell might influence the decay rates in bivalves our results suggested that the robustness/thickness of shells may be one important factor in explaining the overall interspecific differences (see also Ilarri et al., 2015b). Shell size also plays an important role in the bivalve shell decay rates, with larger shells of a given species dissolving slower than smaller shells. This is explained by the difference in the surface area to mass ratio between different size classes, with larger shells presenting a smaller surface area to mass ratio, thus losing mass more slowly than smaller shells (with a high surface area to mass ratio). Significant differences in shell decay rates between the lentic and lotic sites and between seasons were found. In the lotic system shells decayed around 50% faster than on the lentic system. The only exception was during autumn, which revealed, for most of the species, no significant differences between lentic and lotic systems. This situation was due to the fact that during this season an unexpected leaf layer with around 30cm deposited above the treatments in the lotic site possibly shielding the shells from the abrasive agents and decreasing the decay rates in this season. Despite the lack of studies regarding shell decays, results were concordant with those of Strayer & Malcom (2007), in which the authors observed that shell decay rates were also faster at sites with higher current velocity.

Although the estimative of the number of years necessary for the total shell disintegration only take into account species, size, season and river condition many other unexpected abiotic factors can influence the decays. The aggregations of shells is also an important factor to be considered. In fact, in the present study this effect was also observed, with the shells of both, *C. fluminea* and *U. delphinus* showing a proportional lower percent of decay rates in the treatments

containing three shell sizes than when compared to the individual shell treatments. Therefore, the agglomerations of shells may somehow protect the inner shells from abrasion, increasing shells persistence in the environment. However, more studies addressing shell decay rates are necessary to better understand these patterns. According to the present study, *C. fluminea* shells were the second most persistent shells between the bivalve species used in the experiment. Due to global climate change, the frequency and severity of extreme climatic events are predicted to increase in the future (IPCC, 2007). Therefore massive bivalve die-offs will probably occur more frequently, leading to decreased water quality due to the extensive decomposition of bivalve biomass, and increasing accumulation of shells (Ilarri et al., 2011, Bódis et al., 2013). Consequently the continue increase of *C. fluminea* shells in the river bed might lead to significant modifications in the community structure of invaded areas. Detailed studies regarding the shell decays, emphasizing on the impacts of the accumulation of bivalve shells may have in the aquatic environments and macrozoobenthic fauna associated with different bivalve species are necessary in order to understand the importance of bivalve shells as a biological legacy.

## **5. Conclusion**

The results of the present study suggest that empty bivalve shells create suitable habitat for most macrozoobenthic organisms and that *C. fluminea* seems to be functionally equivalent to the native species in the River Minho. In general the physical structures formed with different bivalve shells, especially the ones containing two or more species, appeared to support more abundant, rich and diverse macrozoobenthic communities than bivalve assemblages containing a single species (however only in abundance values significant differences were noticed). Furthermore, shell decay rates varied among species, river condition, seasons and shell sizes. From this study we were able to conclude that other factors (i.e. leaf deposition and agglomerations of shells), beyond the species, size, season and river condition could have major influence on the shell decays.

Given the invasive species broad geographic dispersal and their high densities reached, associated to the decline of the native bivalve species, it is important that further studies addressing the engineering characteristics of different invasive (i.e. *D. polymorpha* and *S. woodiana*) and native bivalve species be performed in order to understand if the results of the present study were a pattern or an exception in the context of the macrozoobenthic colonization and shell decay rates.

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