Universidade do Minho
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Adriana Araújo Novais

Invasive species as resource subsidies: functional importance

April 2017

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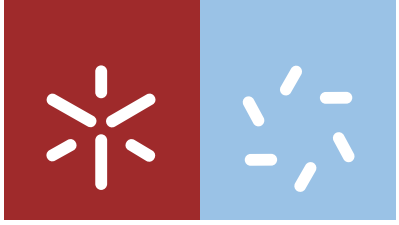

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Invasive species as resource subsidies: functional importance

PhD Thesis in Molecular and Environmental Biology
Specialization in Evolution, Biodiversity and Ecology

Work Supervised by
Professor Doutor Ronaldo Gomes de Sousa
Professora Doutora Cláudia Pascoal

April 2017

DECLARAÇÃO DE INTEGRIDADE

Eu, Adriana Araújo Novais, com o número de identificação 13764450, declaro ter atuado com integridade na elaboração da presente tese. Confirmo que em todo o trabalho conducente à sua elaboração não recorri à prática de plágio ou a qualquer forma de falsificação de resultados.

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Universidade do Minho, 28 / 04 / 2017

Adriana Araújo Novais

(Adriana Araújo Novais)

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Abstract

Biological invasions are one of the main threats to global biodiversity, causing profound changes in ecosystems structure and functioning. So, understanding the functional impact of invasive alien species (IAS) is a major goal in ecology and conservation biology. Recent advances have shown that assimilatory-dissimilatory and physical ecosystem engineering are the two main mechanisms by which IAS can affect entire ecosystems. Although the number of studies has increased over the last years, a considerable lack of knowledge still exists about the possible effects of IAS on ecosystems. The Asian clam *Corbicula fluminea* (Müller, 1774) is well recognized by its invasive behaviour and high ecological impacts, being classified as one of 100 worst IAS in Europe. Despite the negative impacts, the presence of *C. fluminea* was lately associated with positive changes in some estuarine faunal groups and so far the mechanisms responsible for these effects remain unidentified. This thesis intended to understand and disentangle the main mechanisms explaining possible changes in an estuarine benthic community (Chapter 2), and in estuarine sediments biochemistry and microbial communities (Chapter 3). For that, a manipulative experiment under natural conditions was performed using five clam treatments (control, rock, closed shells, live clams, open shells), which were placed in a low sandy intertidal soft bottom area in the Minho River estuary (NW Iberian Peninsula) for 2 months. The presence of live and open shells of *C. fluminea* increased the density, biomass and species richness of the benthic community, mainly of species belonging to Annelida, Mollusca and Crustacea. These results may be explained by two mechanisms: (1) production of feces and pseudofeces by *C. fluminea*, which increases organic matter content and food resources for some benthic species; (2) ecosystem engineering activities by *C. fluminea*, which can create conditions for the establishment of other species via shell production and bioturbation in the sediments (Chapter 2). With the exception of potassium (K) that had higher concentration values in the open treatment, no differences were detected between treatments regarding the concentration values of carbon (C), nitrite (NO^{-}_{2}), ammonium (NH^{+}_{4}), phosphate (PO^{3-}_{4}) and calcium (Ca) in the sediments. Furthermore, the presence of live *C. fluminea* stimulated fungal biomass and bacterial diversity. Bioturbation activities by *C. fluminea* are possibly the main mechanism explaining these results; although factors such as the presence of other macroinvertebrate species and/or production of feces and pseudofeces by *C. fluminea* cannot be excluded (Chapter 3). Recent studies have also reported massive die-offs of *C. fluminea* in response to extreme climatic events (e.g.

floods and droughts), which can have a significant importance on the invaded ecosystem acting as a resource pulse. In fact, during major floods the biomass transported to river banks can enter to the terrestrial food web, but also act as a carrion to the aquatic food web during major droughts. Despite the possible importance of resource pulses, studies addressing the transport of resources by IAS from aquatic to terrestrial areas are rare. Thus, this thesis also aimed to assess the possible importance of massive die-offs of *C. fluminea* as a resource pulse to terrestrial invertebrate (Chapter 4) and microbial communities and soil chemistry (Chapter 5). In addition, we also assess the importance of these mortalities as an additional resource to aquatic invertebrate and microbial communities and leaf litter decomposition (Chapter 6). In Chapters 4 and 5, a manipulative experiment using five levels of *C. fluminea* density (0, 100, 500, 1000 and 2000 ind. m^{-2}) were placed in the left bank of the Minho River, and samples were collected 7, 30 and 90 days after *C. fluminea* addition. Clear differences were detected in abundance, biomass, richness and diversity of terrestrial invertebrates depending on the *C. fluminea* density, time and position. Interestingly, the highest abundance of adult Diptera was observed 7 days after *C. fluminea* addition, whereas that of the other terrestrial invertebrates was on day 30, both with *C. fluminea* densities higher than 500 ind. m^{-2} located on the edge of the experimental design (Chapter 4). Furthermore, *C. fluminea* carrion have significant effects on nutrients content [mainly NH^{+}_{4}, NO_{2}, nitrate (NO_{3}) and PO^{3-}_{4}], fungal biomass and fungal and bacterial diversity (Chapter 5). Lastly in Chapter 6, a manipulative experiment under natural conditions was performed in an arm of the Minho River for 33 days. Results showed that *C. fluminea* die-offs did not affect the structure of microbial and invertebrate communities neither leaf decomposition. However, the presence of live *C. fluminea* stimulated fungal biomass and leaf mass loss, probably due to an increased availability of nutrients via production of feces and pseudofeces. Overall, the results confirmed that the presence of *C. fluminea* could have strong effects on native communities highlighting the main mechanisms underlying these effects (ecosystem engineering and increase in organic matter via production of feces and pseudofeces). Results also revealed that massive die-offs of *C. fluminea*, contributing with remarkable amounts of carrion for adjacent terrestrial ecosystems, may function as a resource pulse. Given the high density, biomass and widespread distribution of *C. fluminea* and the predicted increase and intensification of extreme climatic events, the ecological importance of these phenomena cannot be ignored and should be further investigated.

Espécies invasoras como subsídios de recursos: importância funcional

Resumo

As invasões biológicas são uma das principais ameaças à biodiversidade global, causando alterações profundas na estrutura e funcionamento dos ecossistemas. Assim, perceber a importância funcional das espécies invasoras é um dos principais objetivos em ecologia e conservação. Avanços recentes têm mostrado que a assimilação-dissimilação e engenharia de ecossistemas são os dois mecanismos principais através dos quais as espécies invasoras podem afetar os ecossistemas. Embora o número de estudos tenham aumentado nos últimos anos, há uma considerável falta de conhecimento em torno dos possíveis efeitos dessas espécies nos ecossistemas. A ameijoa Asiática *Corbicula fluminea* (Müller, 1774) é bem reconhecida pelo seu comportamento invasor e pelos graves impactos ecológicos que pode causar, sendo mesmo classificada como uma das 100 piores espécies invasoras na Europa. Apesar destes impactos negativos, a presença de *C. fluminea* tem sido recentemente associada a efeitos positivos em alguns grupos faunísticos estuarinos mas, até ao momento, os mecanismos responsáveis por esses efeitos não foram esclarecidos. Esta tese teve por objetivo perceber quais os principais mecanismos subjacentes às possíveis alterações nas comunidades bentónicas estuarinas (Capítulo 2), e avaliar possíveis efeitos na bioquímica do sedimento e nas comunidades microbianas estuarinas (Capítulo 3). Para isso, foi realizada uma experiência manipulativa em condições naturais usando cinco tratamentos com a amêijoa Asiática (controlo, pedras, conchas fechadas, amêijoas vivas, conchas abertas), os quais foram colocados numa zona intertidal do estuário do Rio Minho (NO Península Ibérica) durante dois meses. A presença de amêijoas vivas e de conchas abertas de *C. fluminea* aumentou a densidade, a biomassa e a riqueza de espécies da comunidade bentónica, principalmente de espécies pertencentes aos Annelida, Mollusca e Crustacea. Estes resultados podem ser explicados por dois mecanismos: (1) produção de fezes por *C. fluminea*, o que aumenta o conteúdo de matéria orgânica, funcionando como um recurso trófico para algumas espécies bentónicas; (2) atividades de engenharia de ecossistemas por parte de *C. fluminea*, o que pode criar condições para o estabelecimento de outras espécies através das conchas e bioturbação do sedimento (Capítulo 2). Com a exceção do potássio (K), que apresentou valores de concentração elevados no tratamento com conchas abertas, não foram detetadas diferenças entre os tratamentos nos valores de concentração de carbono (C), nitrito (NO^{-}_{2}), amónia (NH^{+}_{4}), fosfato (PO^{3-}_{4}) e cálcio (Ca) no sedimento. Para além disso, a presença de indivíduos vivos de *C. fluminea* estimulou a biomassa dos fungos e a diversidade das bactérias associadas ao sedimento. Atividades de bioturbação realizadas por *C. fluminea* são possivelmente o

mecanismo principal que explica estes resultados; embora fatores como a presença de outras espécies de macroinvertebrados e/ou produção de fezes por *C. fluminea* não possam ser excluídos (Capítulo 3). Estudos recentes também têm reportado mortalidades massivas de *C. fluminea* como resultado de eventos climáticos extremos (e.g. cheias e secas), os quais podem ter uma importância significativa no ecossistema invadido atuando como um pulso de recurso. De facto, durante eventos de cheias, a biomassa transportada para as margens do rio pode entrar na cadeia trófica terrestre, mas também pode atuar como um recurso para a cadeia trófica aquática durante eventos de seca. Apesar da possível importância dos pulsos de recursos, estudos que abordem o transporte de recursos por espécies invasoras de ecossistemas aquáticos para terrestres são raros. Assim, esta tese também teve por objetivo avaliar o possível efeito das mortalidades massivas de *C. fluminea* como um pulso de recurso para as comunidades de invertebrados (Capítulo 4) e de microrganismos terrestres e para a química do solo (Capítulo 5). Adicionalmente, foi estimado também o seu efeito como um recurso adicional para as comunidades de invertebrados e de microrganismos aquáticos e na decomposição da folhada (Capítulo 6). Nos Capítulos 4 e 5, foi realizada uma experiência manipulativa usando cinco níveis de densidade de *C. fluminea* (0, 100, 500, 1000 e 2000 ind. m^{-2}) os quais foram colocados na margem esquerda do Rio Minho, e recolhidas após 7, 30 e 90. Foram detetadas diferenças claras na abundância, biomassa, riqueza em espécies e na diversidade de invertebrados terrestres dependendo da densidade de *C. fluminea*, do tempo e da posição (centro ou periferia) em que foram colocadas as amostras. Interessante, a abundância mais elevada de Diptera foi observada 7 dias depois da adição de *C. fluminea*, enquanto que a de outros invertebrados terrestres foi no dia 30, ambos para densidades de *C. fluminea* superiores a 500 ind. m^{-2} localizadas na periferia da área de estudo (Capítulo 4). Para além disso, foram observados efeitos significativos no conteúdo de nutrientes (principalmente em NH^{+}_{4}, NO^{-}_{2}, NO^{-}_{3} e PO^{3-}_{4}), na biomassa de fungos e na diversidade de fungos e bactérias (Capítulo 5). Por fim, no Capítulo 6, foi realizada uma experiência manipulativa em condições naturais num braço do Rio Minho durante 33 dias. Os resultados mostraram que as mortalidades de *C. fluminea* não afetaram a estrutura das comunidades de invertebrados e de microrganismos, nem a decomposição da folhada. No entanto, a presença de indivíduos vivos de *C. fluminea* estimulou a biomassa dos fungos e o processo de decomposição de folhada, provavelmente devido ao aumento da disponibilidade de nutrientes via a produção de fezes. Em termos gerais, os resultados confirmaram que a presença de *C. fluminea* pode ter fortes efeitos nas comunidades nativas, e nos processos ecológicos pela atividade que desempenham como engenheiro de ecossistemas e por contribuírem para o aumento de matéria orgânica via produção de fezes. Além disso, revelaram que as mortalidades massivas de *C. fluminea* podem funcionar como um pulso de recurso. Dada a grande densidade, biomassa e distribuição de *C. fluminea* e o previsível aumento e intensificação de eventos climáticos extremos, a importância ecológica destes fenómenos não pode ser ignorada e deverá ser investigada.

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Chapter 1

General introduction

Biological invasions are one of the main threats to global biodiversity, considered as important as climate change, habitat loss and fragmentation, overexploitation and pollution (Simberloff et al. 2013). Over the years, the study of biological invasions has demonstrated that invasive alien species (IAS) contribute to biotic homogenization (i.e. replacement of endemic/specialist by common/generalist species increasing the similarity between communities) and global biodiversity decline due to species extinctions (although it may be responsible for local biodiversity increases) (Olden 2006; Strayer 2010). These changes may be so profound that some researchers suggest that we are entering a new era, where all continents are interlinked and biogeographic barriers have been eliminated due to human activities, allowing the movement of organisms worldwide (Olden 2006).

Although the understanding of the impacts of biological invasions are growing rapidly, only recently, ecologists have started to recognize that IAS can affect entire ecosystems, causing profound changes in their structure and functioning (Ehrenfeld 2010; Strayer 2012; Simberloff et al. 2013). According to Gutiérrez et al. (2014) assimilatory-dissimilatory (uptake and release of materials, nutrients and energy) and physical ecosystem engineering (physical environmental modifications by organisms) are the two main mechanisms by which IAS can affect ecosystems structure and functioning. Indeed, assimilation-dissimilation can affect key ecosystem processes such as biogeochemical cycles and the fluxes and transformation of materials, nutrients and energy as well as the abundance of other species by consumption and/or the provision of resources in the form of living or dead tissues and waste products (Ehrenfeld 2010; Gutiérrez et al. 2014). On the other hand, physical ecosystem engineering can affect the inputs and outputs of materials, nutrients and energy to ecosystems together with the abundance and activity of other species via changes in abiotic resources and conditions (Gutiérrez et al. 2014).

IAS can significantly alter ecosystem structure and functioning (Ehrenfeld 2010) and so, the understanding of their functional importance is a major goal in ecology and conservation biology (Simberloff 2011). Although the number of studies on the impacts of IAS has increased over the last years, a considerable lack of knowledge still exists about the possible effects of these species on ecosystems (Sousa et al. 2011a).

1.1. Invasive alien species in aquatic ecosystems with emphasis on *Corbicula fluminea* (Müller, 1774)

Aquatic ecosystems are subject to hundreds of species introductions (Strayer 2010) and some of those are responsible for important ecological, economic and evolutionary impacts (Pimentel et al. 2000; Grosholz 2002). Aquatic IAS have been deliberately or accidentally transported by vectors that result from human activities (e.g. aquaculture, construction of canals, shipping, ballast water, sport fisheries, recreational activities, tourism) which has led to a dramatic increase in the rates of introduction, establishment and dispersion of these species (Strayer 2010). At the same time, freshwater ecosystems are being disturbed by habitat loss and fragmentation, pollution, flow regulation, resource exploitation and climate change, resulting in a decline in native species, which may benefit the spread of IAS due to lower biotic resistance of recipient ecosystems (Ricciardi and Rasmussen 1999; Holeck et al. 2004).

Loss or addiction of species can severely affect the functioning of aquatic ecosystems (Strayer 2010). Indeed, many studies have demonstrated that impacts generated by the introduction of IAS in aquatic ecosystems can often affect the entire food web and may trigger trophic cascades (Strayer 2010; Gallardo et al. 2016). Trophic interactions resulting from the introduction of herbivores, decomposers and predators can change community composition and the properties of the invaded area by modifying the material, nutrient and energy fluxes and changing the abundance of species that control these same fluxes (Gallardo et al. 2016). However, these changes are highly context dependent because they also depend on the abundance, spatial distribution, functional distinction, characteristics of the invaded area and time after the introduction of the IAS (Sousa et al. 2014). Some examples of IAS in aquatic ecosystems that have changed entire communities include: “top-down” control, where an invasive predator affects a basal trophic level through the direct consumption of an intermediate trophic level; for example, the introduction of the Nile perch *Lates niloticus* (Linnaeus, 1758) in the great African lakes that decimated a great diversity of cichlid fish (Kaufman 1992; Goldschmidt et al. 1993); and “bottom-up” control, when a basal trophic level indirectly affects a higher trophic level, as was the case of the shrimp *Mysis relicta* (Lovén, 1862) in Flathead Lake, North America, which affected zooplankton with important impacts on the upper trophic levels, including several species of fish, birds and mammals (Spencer et al. 1991).

Impacts mediated by IAS that can affect abiotic conditions (e.g. water clarity, nutrients levels, habitat complexity and physical transport of materials) are also able to significantly alter structure and functioning of aquatic ecosystems (Sousa et al. 2009). For example, reefs and artificial shell beds made by the invasive oyster *Crassostrea gigas* (Thunberg, 1793) increased the habitat available for infaunal polychaetes (Escapa et al. 2004) and protected juvenile Dungeness crab *Cancer magister* (Dana, 1852) from adult cannibalism and fish predation (Fernández et al. 1993). Invasive common carp *Cyprinus carpio* (Linnaeus, 1758) and red swamp crayfish *Procambarus clarkii* (Girard, 1852) can lead to abrupt shifts from macrophyte-dominated clear water state to a phytoplankton-dominated turbid state in lakes due to increasing sediment resuspension (Matsuzaki et al. 2009).

Bivalves are among the most invasive faunal groups in aquatic ecosystems mainly because they can develop massive populations in the invaded areas, eventually becoming the major portion of the total benthic biomass in many aquatic ecosystems (Sousa et al. 2009, 2014; Strayer 2010). There are several mechanisms through which invasive bivalve species can cause a variety of changes in the invaded area, including filter-feeding behaviour, ecosystem engineering, production of feces and pseudofeces, numerous biotic interactions, and bioamplification of pollutants (Sousa et al. 2014).

The Asian clam *Corbicula fluminea* (Müller, 1774) is one of the most recognized invasive bivalve species in aquatic ecosystems mainly due to its widespread distribution and high ecological impacts (Sousa et al. 2008a, 2014; Crespo et al. 2015). Originally, species belonging to the genus *Corbicula* were confined to aquatic ecosystems of Asia, Australia and Africa but during the twentieth century expanded their distribution worldwide (Ilarri and Sousa 2012; Crespo et al. 2015). Primarily, *C. fluminea* established in North America, possible introduced by Chinese immigrants as a food resource, and then expanded to South America (Ilarri and Sousa 2012; Crespo et al. 2015). During the 1980s, *C. fluminea* was introduced to Europe reaching nowadays a considerable distribution and being listed as one of 100 worst IAS in Europe (Ilarri and Sousa 2012; Crespo et al. 2015). Recently, its presence was also described in many Moroccan rivers of North Africa (Clavero et al. 2012; Ronaldo Sousa, personal observation).

When *C. fluminea* is present in high densities it can cause a wide range of impacts, including alteration of biogeochemical cycles and changes to submerged vegetation, phytoplankton, zooplankton and higher trophic levels (Vaughn and Hakenkamp 2001; Sousa et al. 2008a; Ilarri and Sousa 2012; Ilarri et al. 2012, 2014; Lopes-Lima et al. 2016). This IAS is also capable of bioturbate the sediments by pedal feeding (Sousa et al. 2008b) and excrete large amounts of nutrients in the form of feces and pseudofeces

(Strayer et al. 1999). Several studies have also shown that this IAS may have a negative impact on the abundance and diversity of native bivalves belonging to the Margaritiferidae, Unionidae and Sphaeriidae families through reduction of available habitat and food resources, as well as through a reduction in recruitment and in the number of native juveniles that survive (McMahon 1991; Hakenkamp and Palmer 1999; Strayer et al. 1999; Vaughn and Hakenkamp 2001). This species can also be a vector of introduction of parasites and diseases in the invaded ecosystems (Strayer et al. 1999).

Corbicula fluminea can also have high potential for ecosystem engineering since possess key attributes such as production of durable shells, bioturbating and filter-feeding behaviour, relatively large size, high density, and widespread distribution (Sousa et al. 2009). In fact, the structure provided by shells might serve to ameliorate environmental extremes, change abiotic factors (e.g. habitat complexity, sediment granulometry, dissolved oxygen, flow rate) and provide substrate for colonization. Its feeding behaviour (filter and pedal feeding) also strongly changes the benthic environment and the water column, specifically by changing sedimentation rates and increasing water clarity and light penetration (Phelps 1994; Strayer et al. 1999). These changes can, in turn, influence primary producers (Strayer et al. 1999; Sousa et al. 2009).

Although the majority of studies addressed the negative impacts of IAS on native biodiversity in terrestrial and aquatic ecosystems, some of these species can interact positively with native species, including *C. fluminea* (Ilarri et al. 2012). For example, Ilarri et al. (2012) reported that the presence of *C. fluminea* could have a positive effect on the density, biomass and diversity of some faunal groups such as Gastropoda, Crustacea and Insecta in estuarine environments. The mechanisms responsible for these positive effects still need to be established although the structure provided by the shells via engineering activities and the release of large amounts of nutrients in the form of feces and pseudofeces may play essential roles (Ilarri et al. 2012).

Earlier studies have reported that massive die-offs of *C. fluminea* as a result of extreme climatic events (e.g. intensive droughts and floods) can also have a significant importance on the invaded ecosystem (Ilarri et al. 2011; Sousa et al. 2012; Bódis et al. 2014). In fact, these mortalities together with the frequent high biomass attained by *C. fluminea* in invaded ecosystems and its consequent decomposition can lead to mortalities in other benthic species (Sousa et al. 2007a, 2011b). Events of this type occurred in the summers of 2005 and 2009 in the Minho River (Portugal). In these years, the flow rate was particularly low, which combined with high temperatures and decreased dissolved oxygen, resulted in

the death not only of *C. fluminea* specimens, but also of other species living in sympatry, including native bivalves (Ilarri et al. 2011). A similar event, but resulting from an increase in the flow during the winters of 2000/2001 and 2009/2010, caused the movement of freshwater bivalve species [*Anodonta anatina* (Linnaeus, 1758), *Potomida littoralis* (Cuvier, 1798), *Unio delphinus* (Spengler, 1793) and *C. fluminea*] from the river bed to the river banks (Sousa et al. 2012). When the river flow returned to normal in the spring/summer bivalves got stranded on the banks and died, reaching up to 2280 ind. m^{-2} and 10 225 g wet weight. m^{-2}, where *C. fluminea* represented more than 99% of the total bivalve biomass (Sousa et al. 2012). Since *C. fluminea* has an opportunistic life cycle, it can quickly recover to previous density levels, contrasting with native species that present a much lower resilience (Sousa et al. 2012). These massive mortalities may also have high importance in food web dynamics acting as a resource pulse (Yang et al. 2008; Bódis et al. 2014; Ilarri et al. 2015a). Actually, the biomass resulting from major droughts can act as a carrion to the aquatic food web, but also can enter to the adjacent terrestrial food web. However, this type of phenomenon remains almost ignored and their ecological importance not properly evaluated (but see below).

1.2. Pulsed subsidies mediated by invasive alien species

The movement of resources (e.g. materials, nutrients and energy) across spatial boundaries is termed spatial subsidies and, in theory, can have profound consequences for the structure and composition of the recipient community and food web (Polis et al. 1997). These trophic subsidies may arrive continuously, following a time trend with regular intervals, or in certain circumstances, can result from sporadic events of superabundance, named resource pulses (Polis et al. 1997; WB Anderson et al. 2008; Yang et al. 2008). Resource pulses are defined as episodes of low frequency (rarity), large magnitude (intensity) and short duration (brevity) that result in increased resource availability in space and time (Ostfeld and Keesing 2000; Yang et al. 2008). This resource superabundance may have originated and consumed in the same habitat or, in some cases, originated in one habitat but moved into another (Polis et al. 1997; WB Anderson et al. 2008; Yang et al. 2008). Although resource pulses are considered occasional or rare events, they are possibly much more frequent than previously thought (Yang et al.

2008).

Possible causes of resource pulses include: climatic or environmentally driven events, for example El Niño Southern Oscillation episodes (e.g. Jaksic 2001; Letnic et al. 2005), unusual precipitation events (e.g. Vanni et al. 2001) and extreme flooding events (Nakamura et al. 2005); processes of temporal resource accumulation and release, such as the gradual storage and rapid availability of resources in a particular component of the ecosystem such as those resulting from reproductive events (e.g. periodical cicadas emergence events, Yang 2004); processes of spatial resource accumulation and release, in this case, resource pulses can provide materials, nutrients and energy that have been accumulated and transported over space, being released suddenly to consumers in the recipient community, and include hurricanes or major storms that can transport materials, nutrients and energy, as well as storm driven runoffs from terrestrial to aquatic ecosystems (e.g. Novak et al. 2003; Kim et al. 2006); outbreak of population dynamics, for example, birds consumed outbreaking insects (e.g. Cooper and Smith 1995; Hoi et al. 2004; Hogstad 2005); or a combination of these events (Yang et al. 2008, 2010).

Resource pulses can directly affect the life cycle and behaviour at the individual level, for example producing an opportunistic response in consumers (Curran and Leighton 2000; Ostfeld and Keesing 2000; Lithner and Jonsson 2002; Meserve et al. 2003); trigger numerical responses at the population level, resulting from behavioural aggregative responses, increases in reproduction, or a combination of both (Ostfeld and Keesing 2000; Yang et al. 2008); and indirectly affect the communities and ecosystems, for example creating a succession of direct and indirect bottom-up effects that can be followed by delayed top-down effects or by significant changes in nutrient cycling (Ostfeld and Keesing 2000; Yang et al. 2008). Although resource pulses are defined as short duration events, sometimes the ecological effects persist over time (Yang et al. 2008; Armstrong and Bond 2013).

In recent years, an increasing number of studies have investigated the influence of pulsed subsidies on the structure and dynamics of recipient communities and food webs (WB Anderson et al. 2008; Yang et al. 2008, 2010). Interestingly, studies addressing pulsed subsidies mediated by IAS are rare, and the lack of knowledge contrasts with the potential ecological relevance of this topic. Therefore, and trying to call attention to this neglected ecological issue, paradigmatic examples of pulsed subsidies mediated by IAS between the main ecosystems (marine, freshwater and terrestrial) are summarized in Table 1.1.

Pulsed subsidies mediated by IAS between marine ecosystems occur through water movements and are related to the transport of detritus and nutrients. These movements may be vertical and the transport

occurs when pelagic resources fallout to benthos, or horizontal, when the transport occurs due to the action of currents, waves and/or tidal movements (Polis et al. 1997). Some examples of these pulsed subsidies include two species of invasive algae, *Codium fragile* ssp. *fragile* [(Suringar) Hariot, 1889] and *Caulerpa taxifolia* [(M. Vahl) C. Agardh, 1817], and one species of invasive bryozoan, *Membranipora membranacea* (Linnaeus, 1767) (Krumhansl and Scheibling 2011, 2012; Bishop and Kelaher 2013). Krumhansl and Scheibling (2012) studied the export of *C. fragile* detritus from subtidal algal beds to adjacent shallow areas during winter storms in Nova Scotia, Canada, and found that *C. fragile* acted as a trophic subsidy to the macrofaunal assemblages in areas linked to shallow algal beds via the transfer of detritus. In the case of *C. taxifolia*, Bishop and Kelaher (2013) assessed how detritus of this invasive seaweed influences invertebrate communities in estuarine mudflats of Quibray Bay, Australia, and found that plots with detritus of *C. taxifolia* contained higher abundance and diversity of invertebrates than control plots. In the Atlantic coast of Nova Scotia, Krumhansl and Scheibling (2011) observed that the erosion of the two dominant species of kelp, *Laminaria digitata* [(Huds.) Lamouroux, 1813] and *Saccharina longicruris* [(Bachelot de la Pylaie) Kuntze, 1891] increased with the presence of the invasive bryozoan *M. membranacea*, which consequently increased the amount of detritus exported from subtidal kelp beds, contributing to food webs in communities inhabiting deeper waters offshore.

IAS also mediate pulsed subsidies in the direction from marine to freshwater ecosystems and the examples found comprise the movement of consumers as a result of long distance migrations. Species that migrate from the sea up into freshwater to spawn (anadromous species) are the most compelling example regarding this type of trophic subsidy (Polis et al. 1997). An example was the case of the invasive Pacific salmon [*Oncorhynchus tshawytscha* (Walbaum, 1792)] in rivers of Chile and Argentina, South America (Soto et al. 2007; Correa and Gross 2008). This anadromous salmon species migrates into these rivers to spawn, depositing large amounts of marine nutrients and energy through excretion, reproductive products (e.g. sperm and eggs), and carcasses (Sarica et al. 2004; Soto et al. 2007; Correa and Gross 2008). These inputs of marine-derived nutrients increased basal food web production which may cascade to higher trophic levels (Bilby et al. 1998; Zhang et al. 2003).

Pulsed subsidies from marine to terrestrial ecosystems can also be mediated by IAS and examples include the movement of detritus and consumers. Rossi et al. (2010) studied the trophic significance of the invasive brown seaweed *Sargassum muticum* [(Yendo) Fensholt, 1955] on sandy beaches of Northwest coast of Spain, and found that this Japanese macroalgae seemed to be one of the main food

resources for amphipods and isopods. According to Branch and Steffani (2004) the invasive Mediterranean mussel *Mytilus galloprovincialis* (Lamarck, 1819) provided an additional resource for higher terrestrial predators, including *Haematopus moquini* (Bonaparte, 1856) in shores of South Africa. Also the invasive Pacific oyster *Crassostrea gigas* (Thunberg, 1793) represented an extra food resource to the herring gulls [*Larus argentatus* (Pontoppidan, 1763)] in coastal areas of Ducth Wadden Sea (Cadée 2001).

Between terrestrial ecosystems pulsed subsidies mediated by IAS include the movement of nutrients and energy across the roots of invasive plant species to belowground food webs. Bradford et al. (2012) studied the root carbon flow from the invasive grass *Microstegium vimineum* (Trin.) A. Camus to the belowground food web, and found that through the root-carbon exudation the invasive grass subsidized and affected the structure and function of the recipient microbial community. The movement of native predators in response to dispersal of invasive preys can also occur, as was the case of the invasive gypsy moth [*Lymantria dispar* (Linnaeus, 1758)] in North America (Barber et al. 2008). Barber et al. (2008) examined how invasive gypsy moth outbreaks affect the abundance and distribution of two native cuckoos, *Coccyzus erythropthalmus* (Wilson, 1811) and *C. americanus* (Linnaeus, 1758), and found that the gypsy moth shifted the annual distribution of cuckoos. A similar example, was the invasive earthworm *Dendrobaena octaedra* (Savigny, 1826) in boreal forests of Alberta, Canada (Cameron and Bayne 2012). According to Cameron and Bayne (2012), which examined the effects of *D. octaedra* on the diet, abundance and distribution of the American robin *Turdus migratorius* (Linnaeus, 1766), the invasive earthworm influenced robins' distribution as a result of spatial variability in *D. octaedra* abundance since robins use invasive earthworms as a prey.

Table 1.1. Description of spatial subsidies mediated by IAS between different ecosystems (marine, freshwater and terrestrial).

Species	Donor habitat	Recipient habitat	Description	References
	Marine	Marine		
Codium fragile			Productive macroalgae can subsidize dense detritivore populations in the supralittoral, littoral or intertidal and deep benthic zones.	Krumhansl and Scheibling 2012
Caulerpa taxifolia			Detritus from invasive algae subsidize invertebrate communities on an intertidal mudflat in Quibray Bay, New South Wales, Australia.	Bishop and Kelaher 2013
Membranipora membranacea			Invasive bryozoan may increase the amount of material eroded from subtidal kelp beds (*Saccharina longicruris* and *Laminaria digitata*) to food webs in communities inhabiting deeper waters offshore.	Krumhansl and Scheibling 2011
	Marine	Freshwater		
Oncorhynchus tshawytscha			Anadromous invasive Pacific salmonids deposit great amounts of energy and nutrients of marine origin to rivers and lakes via reproductive products, excretion, and carcasses.	Schuldt and Hershey 1995 Sarica et al. 2004 Soto et al. 2007 Correa and Gross 2008
	Marine	Terrestrial		
Sargassum muticum			Productive macroalgae subsidize beaches and adjacent terrestrial areas.	Rossi et al. 2010
Mytilus galloprovincialis			Marine invasive bivalves in intertidal areas of South Africa are an important source of nutrients to terrestrial species, including birds (*Haematous moquini*) and mammals (*Nucella cingulata*) during low tides.	Hockey and van Erkom Schurink 1992 Branch and Steffani 2004
Crassostrea gigas			Invasive Pacific oyster subsidize herring gulls (*Larus argentatus*) in the Wadden Sea.	Cadée 2001

Table 1.1. Continued

Species	Donor habitat	Recipient habitat	Description	References
	Terrestrial	Terrestrial		
Microstegium vimineum			Invasive grass by root carbon-exudation subsidize belowground foob webs.	Bradford et al. 2012
Lymantria dispar			The invasive gypsy moth undergoes periodic outbreaks and represent a super-abundant food resource for predators, particularly native cuckoos (*Coccyzus erythropthalmus* and *C. americanus*).	Barber et al. 2008
Dendrobaena octaedra			Invasive earthworm as a prey can strongly influence native predators such as the Americam robin (*Turdus migratorius*), with shifts in robin distributions occurings as a result of spatial variability in earthworm abundance.	Cameron and Bayne 2012
	Terrestrial	Freshwater		
Silurus glanis			*Silurus* preying on terrestrial birds move nutrients and energy from terrestrial to freshwater aquatic ecosystems.	Cucherousset et al. 2012
Elaeagnus angustifolia			Leaf litter by invasive Russian olive subsidize freshwater ecosystems.	Mineau et al. 2011
Tamarix sp. *Elaeagnus angustifolia*			Leaf litter by invasive saltcedar and Russian olive subsidize the aquatic crane fly *Tipula* (Diptera: Tipulidae).	Moline and Poff 2008
Lythrum salicaria			High floral density of invasive purple loosestrife increased adult dragonfly oviopsition and subsquently high larval dragonfly abundance in the aquatic ecosystems.	Burkle et al. 2012
Castor canadensis			Beavers transport trees from terrestrial habitats to freshwater ecosystems adding nutrients and organic matter.	Anderson and Rosemond 2007, 2010
	Terrestrial	Marine		
Spartina alterniflora			Invasive salt marsh subsidize four nekton species (*Chelon haematocheilus*, *Synechogobius ommaturus*, *Lateolabrax maculatus* and *Exopalaemon carinicauda*) in the Yangtze River estuary, China.	Quan et al. 2007

Table 1.1. Continued

Species	Donor habitat	Recipient habitat	Description	References
Silurus glanis	Freshwater	Freshwater	Invasive fish feeding in one area and aggregating in a different area may originate important biogeochemical hotspots via excretation.	Boulêtreau et al. 2011
Pacifastacus leniusculus			Invasive signal crayfish creat a new link in energy tranfer from litoral to profundal areas in large boreal lakes.	Ruokonen et al. 2012
Corbicula fluminea *Sinanodonta woodiana*	Freshwater	Terrestrial	Massive die-offs by invasive bivalves resulting from floods or droughts function as an important source of nutrients to plants, invertebrates and vertebrates.	Sousa et al. 2012 Bódis et al. 2014
Castor canadensis			Invasive Canadian beavers in southern Finland subsidize two bat species (*Eptesicus nilssoni* and *Myotis daubentoni*) by increased insect emergence from beaver ponds.	Nummi et al. 2011

Pulsed subsidies by IAS in the terrestrial to freshwater direction are often related to the movement of leaf detritus. Invasion of riparian trees can alter the quantity and quality of leaf litter inputs to streams and thus have the potential to alter stream food web dynamics (Moline and Poff 2008). According to Mineau et al. (2011) the invasive riparian Russian olive *Elaeagnus angustifolia* L. subsidized freshwater ecosystems in western North America, with high potential to alter biogeochemical cycling due to its dinitrogen (N_2)-fixing ability. An analogous example was given by Moline and Poff (2008), which studied the impacts of two invasive riparian trees, saltcedar (*Tamarix* sp. L.) and Russian olive, on the growth of the aquatic crane fly *Tipula* (Diptera: Tipulidae) in streams of western North America. In this study, *Tipula* showed high growth on leaf litter of *Tamarix* followed by *E. angustifolia* and the native cottonwood (*Populus*), respectively. Other examples of this type of pulsed subsidy includes an unusual predation behaviour by the invasive catfish *Silurus glanis* (Linnaeus, 1758) in Tarn River, Southwestern France, where this IAS developed a new behavioural strategy, similar to beaching behaviour used by killer whales, to capture birds [i.e. pigeons, *Columbia livia* (Gmelin, 1789)] on terrestrial ecosystems (Cucherousset et al. 2012); ecosystem engineering activities, for example, the introduction of the North American beavers *Castor canadensis* (Kuhl, 1820) in the Cape Horn Biosphere Reserve, Chile, which subsidized aquatic food web with terrestrially derived organic matter (amorphous detritus, leaves and wood) through

the construction of ponds (Anderson and Rosemond 2007, 2010); and bottom-up and top-down processes, as was the case of the invasive plant purple loosestrife (*Lythrum salicaria* L.) in North America, which attracted high levels of visiting insects pollinators and, subsequently, adult dragonflies (bottom-up effect), resulting in increased dragonfly oviposition and high abundance of predaceous larval dragonfly that change the structure and composition of zooplankton communities (top-down effect) on aquatic ecosystems (Burkle et al. 2012).

The invasive plant *Spartina alterniflora* Loisel. mediated pulsed subsidies from terrestrial to marine ecosystems (Quan et al. 2007). According to Quan et al. (2007), which examined the contribution from different food resources to commercial nektonic species [*Chelon haematocheilus* (Temminck and Schlegel, 1854), *Synechogobius ommaturus* (Richardson, 1845), *Lateolabrax maculatus* (McClelland, 1844) and *Exopalaemon carinicauda* (Holthuis, 1950)] in the tidal marshes of the Yangtze River estuary, China, *S. alterniflora* present in tidal marshes provided important food resources for some dominant estuarine nektonic species.

In freshwater ecosystems, pulsed subsidies mediated by IAS can comprise the movement of consumers. An example was the case of the natural aggregations of the invasive catfish *S. glanis* in Rhône River, Lyon, France (Boulêtreau et al. 2011). These aggregations, sometimes composed by dozens of adults, occurred within the same location and represented important biogeochemical hotspots through the excretion of large quantities of nutrients (Boulêtreau et al. 2011). Also, the invasive signal crayfish [*Pacifastacus leniusculus* (Dana, 1852)] in Lake Päijänne, Finland, increased connectivity between spatially distinct habitats, transferring organic matter, nutrients and energy from littoral to deep areas due to its great mobility (Ruokonen et al. 2012).

Pulsed subsidies mediated by IAS from freshwater to terrestrial ecosystems are also possible and comprise, for example, the massive die-offs of invasive bivalves *C. fluminea* and *Sinanodonta woodiana* (Lea, 1834), which subsidized adjacent terrestrial communities during extreme climatic events, such as intensive droughts and floods (Sousa et al. 2012; Bódis et al 2014). Another example was the case of *C. canadensis* in south of Finland (Nummi et al. 2011). According to Nummi et al. (2011) beaver ponds increased the production of aquatic invertebrates and, subsequently, the number of insects emerging, which benefited the population of two native bats, *Eptesicus nilssoni* (Keyserling and Blasis, 1839) and *Myotis daubentonii* (Kuhl, 1817).

The above mentioned examples suggest the important role of IAS in the establishment of spatial

subsidies between different ecosystems. The movement of detritus, nutrients, preys and consumers mediated by IAS is characteristic of all ecosystems and plays a determinant role in the flow of food resources between different habitats with potential impacts on the recipient community dynamics and food webs structure. However, the assessment of these effects remained mostly ignored and the number of quantitative studies on the subject remain very low.

1.3. Statement of the problem

Corbicula fluminea invaded almost all major rivers in Portugal, assuming a prominent role in the Minho River due to its high invasive success in this ecosystem (Sousa et al. 2008b,c,d). Since its introduction in the Minho estuary in 1989 (Araujo et al. 1993), *C. fluminea* suffered a rapid dispersion and nowadays is present in 150 km of the river length, and in many stretches (international section) represents more than 95% of the total benthic faunal biomass (Sousa et al. 2008c,d,e; Ferreira-Rodríguez and Pardo 2016). Over the years, the Minho River has functioned as model for the study of numerous ecological aspects inherent to the *C. fluminea* invasion. Some results have shown that after this species introduction a significant decline in the abundance, biomass and diversity of native species has been observed (Sousa et al. 2008c,d,e). However, recent studies in the Minho estuary reported that the presence of *C. fluminea* could also have a positive influence on some faunal groups (Ilarri et al. 2012). Yet nothing is known about the mechanisms responsible for these effects of *C. fluminea* on estuarine macrozoobenthos. This thesis intended to clarify the main mechanisms explaining the changes in the macrozoobenthic estuarine community (Chapter 2), and to assess possible effects on estuarine sediments biochemistry and microbial communities induced by the presence of *C. fluminea* (Chapter 3). Furthermore, periodic massive die-offs have also been reported in this river and earlier studies advance with some quantitative data about fluctuations on density and biomass of *C. fluminea* as a result of extreme events (Ilarri et al. 2011; Sousa et al. 2012). This last aspect needs further attention and this thesis aimed to assess the possible importance of the transference of materials, nutrients and energy from aquatic to adjacent terrestrial areas (the few ecological studies with resource pulses in aquatic ecosystems addressed the transference of energy and nutrients in the opposite direction), a possible important but neglected

pathway (Chapters 4 and 5), and also the importance of these mortalities as an additional resource to aquatic communities (Chapter 6).

1.4. Aims

The central aim of this study was to assess how *C. fluminea* (including changes resulting from massive mortalities) change above and belowground processes in aquatic and adjacent terrestrial areas. To that end, a multidisciplinary integrative approach combining ecology, microbiology and sediment chemistry was chosen in order to increase our knowledge about the potential ecological changes mediated by *C. fluminea*, giving special attention to effects on biological communities and ecosystem functions. Given that *C. fluminea* can influence ecosystem proprieties and biological communities in various ways, studies comprising distinct hypotheses were carried out, having the follow specific objectives:

- To understand and disentangle the main mechanisms that justify the higher biomass, density and diversity of estuarine benthic macroinvertebrates in the presence of *C. fluminea* (Chapter 2);
- To identify possible effects of *C. fluminea* on sediments biochemistry and on an estuarine microbial (both fungi and bacteria) community, and disentangle the main mechanisms explaining these results (Chapter 3);
- To estimate the importance of *C. fluminea* massive die-offs as a resource subsidy to adjacent terrestrial areas, assessing possible differences in the structure of the terrestrial invertebrate community (Chapter 4);
- To estimate the relevance of *C. fluminea* massive die-offs as a resource subsidy to adjacent terrestrial areas, evaluating the possible effects on the terrestrial soil chemistry and the structure of microbial (both fungi and bacteria) community (Chapter 5);
- To understand the importance of *C. fluminea* massive die-offs as additional resource to aquatic communities, evaluating the structure of microbial (both fungi and bacteria) and invertebrate communities and a key ecosystem process, the decomposition of leaf litter (Chapter 6).

Chapter 2

Facilitation in the low intertidal: effects of an invasive species on the structure of an estuarine macrozoobenthic assemblage

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Abstract

The Asian clam *Corbicula fluminea* (Müller, 1774) has been recognized as one of the most important IAS in aquatic ecosystems and may have significant ecological and economic impacts. Recently, the presence of *C. fluminea* was associated with changes in benthic and epibenthic fauna. In this study, we aimed to understand the mechanisms underlying the effects of *C. fluminea* on an estuarine macrozoobenthic assemblage using a manipulative experiment. We used five different treatments (control, rock, closed, live, open), which were placed in a low sandy intertidal soft bottom area in the Minho estuary (NW Iberian Peninsula) for 2 months. We found that the presence of live and open empty shells of *C. fluminea* had positive effects on the density, biomass and species richness of macrozoobenthos, specifically on species belonging to Annelida, Mollusca and Crustacea. Our results may be explained by two main mechanisms: (1) the production of feces and pseudofeces by *C. fluminea*, which increases organic matter content and food resources for some macrozoobenthic species; and (2) ecosystem engineering activities by *C. fluminea*, which can create conditions for the establishment of other species via shell production and bioturbation in the sediments.

2.1. Introduction

The introduction of IAS is one of the main threats to global biodiversity, causing significant changes in ecosystem structure and functioning (Grosholz 2002; Cox 2004; Davis 2009; Simberloff et al. 2013). Impacts generated by these introductions have contributed to biotic homogenization, reduction in global biodiversity and extinctions of native species (Olden 2006; Strayer 2010).

Many studies have addressed the negative impacts of IAS on native species biodiversity in terrestrial and aquatic ecosystems (Mack et al. 2000; Pimentel et al. 2000; Byers 2009). In most cases, the negative influences were due to new biotic interactions (predation, competition, allelopathy, introduction of diseases and parasitism) or by changes in biogeochemical cycles or physical structures (Ehrenfeld 2010; Sousa et al. 2011a). However, some IAS can interact positively with native species by providing food resources and habitat to rare species (see for example Schlaepfer et al. 2011). In some circumstances, IAS may behave as a foundation species because they have the ability to create habitats and/or modify environmental conditions, as well as change species interactions and resource availability in invaded ecosystems (Bruno and Bertness 2001; Crooks 2002; Altieri and van de Koppel 2014). There has been growing evidence in recent years that ecosystem engineering activities are one of the most important mechanisms underlying these facilitative interactions (Jones et al. 1994, 1997; Altieri and van de Koppel 2014).

Bivalves are one of the most invasive faunal groups in aquatic ecosystems and can significantly influence biological communities and alter ecosystem structure and functioning through several mechanisms, including ecosystem engineering (Gutiérrez et al. 2003; Sousa et al. 2009, 2014). Several invasive bivalve species in freshwater, estuarine and marine ecosystems have high potential for ecosystem engineering since they possess key attributes such as the production of durable shells, bioturbating and filter-feeding behaviour, relatively large size, high densities and widespread distribution (reviewed in Sousa et al. 2009). In fact, the structure provided by their shells might serve as a refuge from biotic and abiotic stress, predation and competition, ameliorate environmental extremes, change abiotic factors and provide a substrate for colonization (Gutiérrez et al. 2003; Sousa et al. 2009).

The Asian clam *C. fluminea* is well recognized for its invasive behaviour (e.g. it is listed as one of 100 worst invasive species; DAISIE 2009) and therefore the number of published articles using this species as a model organism has increased in recent years (Sousa et al. 2008a, 2014). In the 20th century,

species belonging to the genus *Corbicula* have expanded their distributions to North and South America, Europe and North Africa (reviewed in Ilarri and Sousa 2012). When this IAS is present in high densities, it can cause a wide range of abiotic and biotic impacts, including changes to submerged vegetation, phytoplankton and zooplankton communities and decreases in abundance and diversity of native bivalve species (Vaughn and Hakenkamp 2001; Darrigran 2002; McMahon 2002; Sousa et al. 2008a; Ilarri and Sousa 2012). However, recent studies have shown that the presence of *C. fluminea* could also have a positive influence on the density, biomass and diversity of some faunal groups such as Gastropoda, Crustacea and Insecta in estuarine environments (Ilarri et al. 2012). Yet nothing is known about the mechanisms responsible for the positive effects of *C. fluminea* on estuarine macrozoobenthos. It is expected that the high excretion rates of *C. fluminea*, which result in the release of large amounts of nutrients in the form of feces and pseudofeces, along with the structure provided by the species' shells via ecosystem engineering activities can be relevant to estuarine macrozoobenthos. Building on the results of an earlier study in the Minho estuary (Ilarri et al. 2012), we performed a manipulative experiment to understand and disentangle the main mechanisms explaining changes in the density, biomass and diversity of a macrozoobenthic estuarine assemblage induced by the presence of *C. fluminea* under natural environmental conditions.

2.2. Materials and Methods

2.2.1. Study area and sampling design

The experiment was carried out in the Minho River estuary (NW Iberian Peninsula) (Fig. 2.1), which has a maximum width of 2 km and is approximately 35 km long. Several studies have been performed in this estuary in the last two decades, and a detailed description of the macrozoobenthic and epibenthic communities are available in Sousa et al. (2008c) and Costa-Dias et al. (2010), respectively. Although controversy exists regarding the taxonomic status in the *Corbicula* genus, mainly due to high phenotypic variation in shell shape, an earlier genetic study performed by Sousa et al. (2007b) in five different sites along the Minho estuary identified the species found in this estuary as *C. fluminea*. This IAS was first reported in the Minho estuary in 1989, and now represents more than 95% of the total

benthic biomass in this estuarine ecosystem and has had several ecological and economic impacts (see for example Sousa et al. 2008c,d).

The lower intertidal portion of a sandflat in the lower estuary, approximately 8 km upstream of the mouth of the estuary (41° 54' 37'' N, 08° 47' 22'' W; Fig. 2.1) was used to determine the main mechanisms responsible for the effects of *C. fluminea* on the estuarine macrozoobenthos (following Ilarri et al. 2012). The studied area was selected due to its low density of *C. fluminea* (<50 ind. m^{-2}) when compared to adjacent areas (see Sousa et al. 2008c for comparison) in order to minimize the perturbation caused by the presence of this IAS. Throughout the experiment, abiotic variables were measured at two weeks intervals during high tide. Temperature, redox potential, salinity, dissolved oxygen and pH were measured 20 cm above the bottom with a multiparameter sea gage (YSI 6820). In addition, three random samples of sediment were collected to characterize the granulometry of the sediment in the study area. In the laboratory, sediment samples were oven-dried for 72 h at 60°C, then sieved with Ro-Tap agitation using columns of sieves of different mesh sizes (>2 mm: gravel; 1–2 mm: very coarse sand; 0.5–1 mm: coarse sand; 0.25–0.5 mm: medium sand; 0.125–0.25 mm: fine sand; 0.063–0.125 mm: very fine sand; <0.063 mm: silt + clay). The frequency of each size class was expressed as a percentage of total weight following Sousa et al. (2006).

All adult *C. fluminea* individuals (i.e. clams with shell length >10 mm) were removed from the sandflat one week before the start of the experiment. The experiment was conducted in a complete randomized block design with six blocks. Each block contained five 400 cm^2 boxes (with open tops and laterally lined with a net with a mesh size of 10 mm) corresponding to five different treatments: (1) bare sediment (hereafter, control treatment); (2) inanimate substrate consisting of small rocks with a similar oval shape as *C. fluminea* (hereafter, rock treatment); (3) dead *C. fluminea* shells filled with sand and glued together (hereafter, closed treatment); (4) live *C. fluminea* individuals (hereafter, live treatment); and (5) open empty *C. fluminea* shells (hereafter, open treatment). The control treatment was used to recreate a site without *C. fluminea* influence, while the rock treatment functioned as a control for the effect of a physically inert substrate. The closed treatment was used to detect only the effect of colonization on the outside of the shells while clams were alive, and the live treatment was used to detect the total effect of the presence of living *C. fluminea* (shell as a substrate, production of feces and pseudofeces and bioturbation activities). Finally, the open treatment was used to detect the effect of open empty shells after the death of individual clams. All treatments, except the control (no clams),

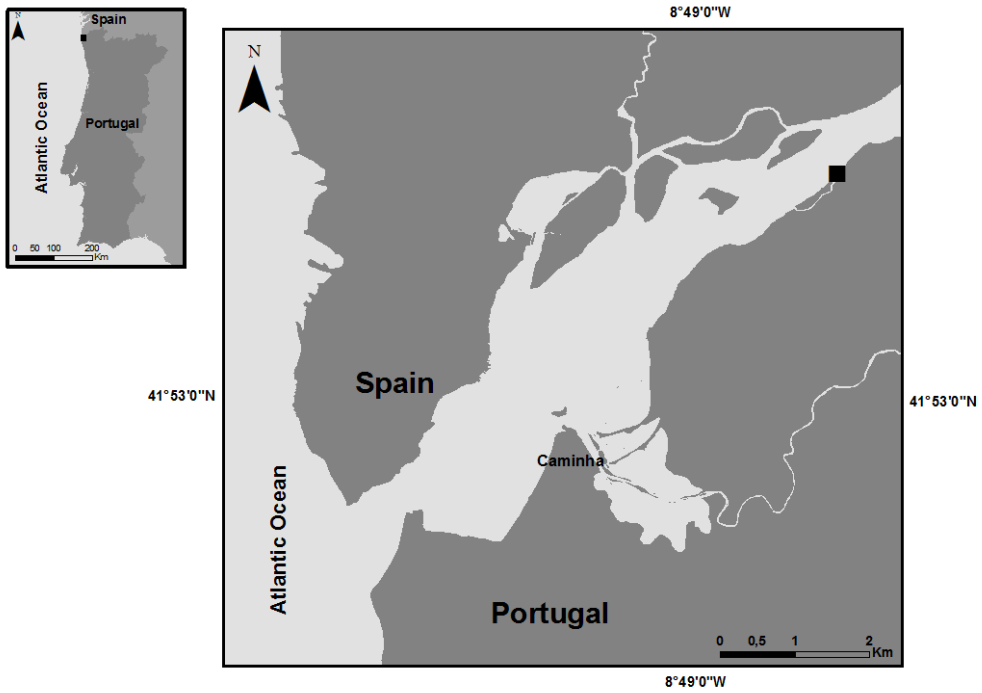

Figure 2.1. Study area showing the selected site in the lower Minho River estuary, NW Iberian Peninsula.

had a density of 1200 ind. m^{-2}, which reflects mean values in the Minho estuary (Sousa et al. 2008d). All *C. fluminea* individuals and rocks used were measured to minimize possible differences in size and surface area available for colonization between treatments. Boxes were distributed within a grid of ca. 1 m intervals, chosen to minimize habitat variability and inter-plot interactions. The experiment lasted 2 months (July and August). After that, sediment samples were collected for organic matter determination and macrozoobenthos characterization using cores with an area of 10 and 45 cm^2, respectively. The organic matter in the sediment was determined by combustion for 24 h at 550°C in a muffle furnace, and was estimated as the weight loss on ignition, expressed as a percentage of the dry weight (DW) of the whole sample, following Sousa et al. (2006). Samples containing biological material were sieved through a 500 µm mesh, and the macrozoobenthos was preserved in 70% ethanol. Organisms were counted and identified to species level whenever possible. To determine biomass, organisms were oven-dried for 72 h at 60°C.

2.2.2. Data analysis

All statistical tests were conducted using the PRIMER analytical software (v.6.1.6, PRIMER-E) with the permutational multivariate analysis of variance (PERMANOVA) + 1.0.1 add-on (MJ Anderson et al. 2008). PERMANOVA tests the simultaneous response of one or more variables to one or more factors in an ANOVA experimental design on the basis of any distance measure, using permutation methods (Anderson 2001). Prior to PERMANOVA and non-metric multidimensional scaling (nMDS) ordination analyses (see below), all variables were normalized without data transformation, and resemble matrices based on the Euclidean distances were calculated (Clarke and Warwick 2001).

Differences between treatments in the organic matter content of the sediment were tested using a one-way PERMANOVA (type-III), with treatment as a fixed factor (five levels: control, rock, closed, live and open). Comparisons between the *C. fluminea* individuals and rock lengths were tested using a one-way PERMANOVA (type-III), with treatment as a fixed factor (four levels: rock, closed, live and open).

The nMDS based on the macrozoobenthic density data followed by the PERMANOVA tests were used to discriminate possible differences between treatments. The ecological indexes of species richness and the Shannon-Wiener diversity index were calculated through the DIVERSE analysis (Clarke and Warwick 2001). Differences in overall macrozoobenthic density, biomass, species richness and the Shannon-Wiener diversity index were tested using a one-way PERMANOVA (type-III), with treatment as a fixed factor (five levels: control, rock, closed, live and open). Comparisons of Annelida, Mollusca and Crustacea density and biomass between treatments were made using a one-way PERMANOVA (type-III), with the same design as described above.

In all PERMANOVA tests, the statistical significance of variance (α= 0.05) was tested using 9999 permutations of residuals within a reduced model. When the number of permutations was lower than 150, the Monte Carlo p-value was considered. One-way PERMANOVA pairwise comparisons were also performed for all PERMANOVA tests.

2.3. Results

2.3.1. Abiotic characterization

The mean [± standard deviation (SD)] values of abiotic factors measured in the water column at high tide during the 2 months experiment were temperature: 20.56 ± 1.44°C; redox potential: 204.31 ± 20.15 mV; salinity: 12.15 ± 3.31 psu; dissolved oxygen: 8.76 ± 0.43 mg l^{-1} and pH: 7.89 ± 0.14. The sediment composition of the study area was very homogeneous, with the mean percentage of each size class frequency as follows: >2 mm, 0.1 ± 0.01%; 1–2 mm, 0.2 ± 0.01%; 0.5–1 mm, 1.0 ± 0.15%; 0.25– 0.5 mm, 5.5 ± 0.65%; 0.125–0.25 mm, 46.5 ± 3.10%; 0.063–0.125 mm, 34.3 ± 2.03%; and <0.063 mm, 12.4 ± 1.08%.

Organic matter content was highest in the live treatment (3.5 ± 0.39%) followed by open (3.2 ± 0.31%), closed (3.2 ± 0.43%), control (3.0 ± 0.33%) and rock (2.9 ± 0.32%) treatments. Significant differences only occurred between the live and rock treatments ($t = 2.54$, $p = 0.04$).

2.3.2. Biotic characterization

The mean (± SD) length of *C. fluminea* individuals was 26.8 ± 4.6 mm in the closed treatment, 26.7 ± 5.8 mm in the live treatment, and 26.8 ± 4.9 mm in the open treatment; the mean length of the rocks in the rock treatment was 26.7 ± 5.0 mm, with no differences between treatments (Pseudo-F = 0.02, $p = 0.99$). There was no *C. fluminea* mortality in the live treatment during the experiment.

A total of 12 macrozoobenthic taxa were recorded in all treatments. The 6 most abundant were *Hediste diversicolor* (Müller, 1776) (36.7%), *Corophium multisetosum* (Stock, 1952) (29.7%), *Cyathura carinata* (Krøyer, 1847) (13.3%), *Spionidae* and *Gammarus* sp. 2 (3.8%) and *Potamopyrgus antipodarum* (Gray, 1843) (3.2%), while the remaining 6 taxa contributed 9.5%. In terms of biomass, the 6 most abundant taxa were *H. diversicolor* (67.5%), *Petromyzon marinus* (Linnaeus, 1758) (6.8%), *C. multisetosum* and *C. carinata* (6.3%), *Nereis cultifera* (Grube, 1840) (5.4%) and *C. fluminea* (3.8%), while the remaining 6 taxa contributed 3.9%.

The nMDS ordination (stress = 0.15; Fig. 2.2) did not reveal any difference in the macrozoobenthic assemblage that colonized each treatment (Pseudo-F = 1.26, $p = 0.10$).

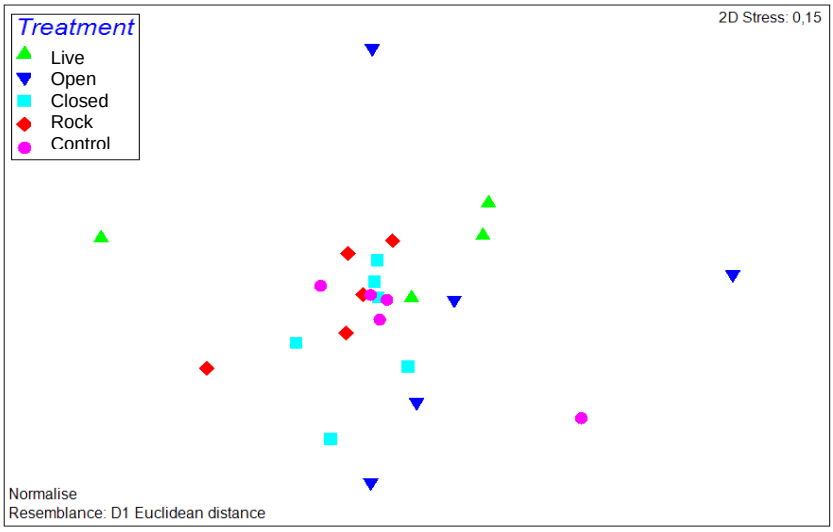

Figure 2.2. Non-metric multidimensional scaling (nMDS) plot of the macrozoobenthos associated with the five experimental treatments (live, open, closed, rock and control).

The mean ± 95% CI macrozoobenthic density (ind. 45 cm^{-2}) was higher in the open (9.2 ± 4.6) and live (8.5 ± 4.6) treatments, followed by closed (4.7 ± 1.4), control (4.5 ± 1.7) and rock (3.8 ± 1.4) (Fig. 2.3A). Significant differences in density between the 5 treatments were found (Pseudo-F = 6.31, $p < 0.01$). Pairwise tests indicated that these differences were in the comparisons of open with closed ($t = 2.80$, $p \leq 0.05$), open with control ($t = 2.82$, $p \leq 0.05$), open with rock ($t = 3.33$, $p \leq 0.01$), live with closed ($t = 2.87$, $p \leq 0.05$), live with control ($t = 2.83$, $p \leq 0.05$) and live with rock ($t = 3.52$, $p \leq 0.01$) treatments.

Similarly, results for biomass (g DW 45 cm^{-2}) showed higher values for the live (0.05 ± 0.056) and open (0.05 ± 0.040) treatments, followed by closed (0.02 ± 0.019), control (0.01 ± 0.007) and rock (0.01 ± 0.010) treatments (Fig. 2.3B). There were significant differences between the 5 treatments (Pseudo-F = 4.19, $p \leq 0.05$). Pairwise tests indicated that these differences were in the comparisons of live with control ($t = 2.74$, $p \leq 0.01$), live with rock ($t = 2.76$, $p \leq 0.05$), open with control ($t = 2.72$, $p \leq 0.01$) and open with rock ($t = 2.76$, $p \leq 0.01$) treatments.

The results for species richness showed higher values for the live (4.5 ± 2.1) and open (4.4 ± 1.9) treatments, followed by control (3.0 ± 0.7) and closed and rock (2.8 ± 0.8) treatments (Fig. 2.3C). There were significant differences between the five treatments (Pseudo-F = 3.69, $p \leq 0.05$). The pairwise tests indicated that significant differences existed between live and closed ($t = 2.61$, $p \leq 0.05$), live and control ($t = 2.48$, $p \leq 0.05$), live and rock ($t = 2.61$, $p \leq 0.05$) and open and rock ($t = 2.24$, p

≤ 0.05) treatments.

No significant differences in Shannon-Wiener diversity index were detected among the five treatments (Pseudo-F = 2.02, p = 0.13) (Fig. 2.3D).

The results for Annelida density (Fig. 2.4A) and biomass (Fig. 2.4B) showed higher values for the live and open treatments. There were significant differences in the density (Pseudo-F = 2.39, p ≤ 0.01), but not in the biomass of Annelida among the five treatments (Pseudo-F = 2.54, p = 0.06). Within the Annelida taxa, the density and biomass of *H. diversicolor* was highest in the live treatment (Table 2.1). Mollusca density (Fig. 2.4C) and biomass (Fig. 2.4D) were also highest in the live treatment. There were significant differences in the biomass (Pseudo-F = 2.09, p ≤ 0.05), but not in the density of Mollusca among the five treatments (Pseudo-F = 2.17, p = 0.07).

Crustacea density (Fig. 2.4E) and biomass (Fig. 2.4F) were highest in the open treatment. There were significant differences in the density (Pseudo-F = 3.88, p ≤ 0.05), but not in the biomass of Crustacea among the five treatments (Pseudo-F = 1.73, p = 0.18). The density of *C. multisetosum* and *Gammarus* sp. 1 were significantly higher in the open treatment (Table 2.1).

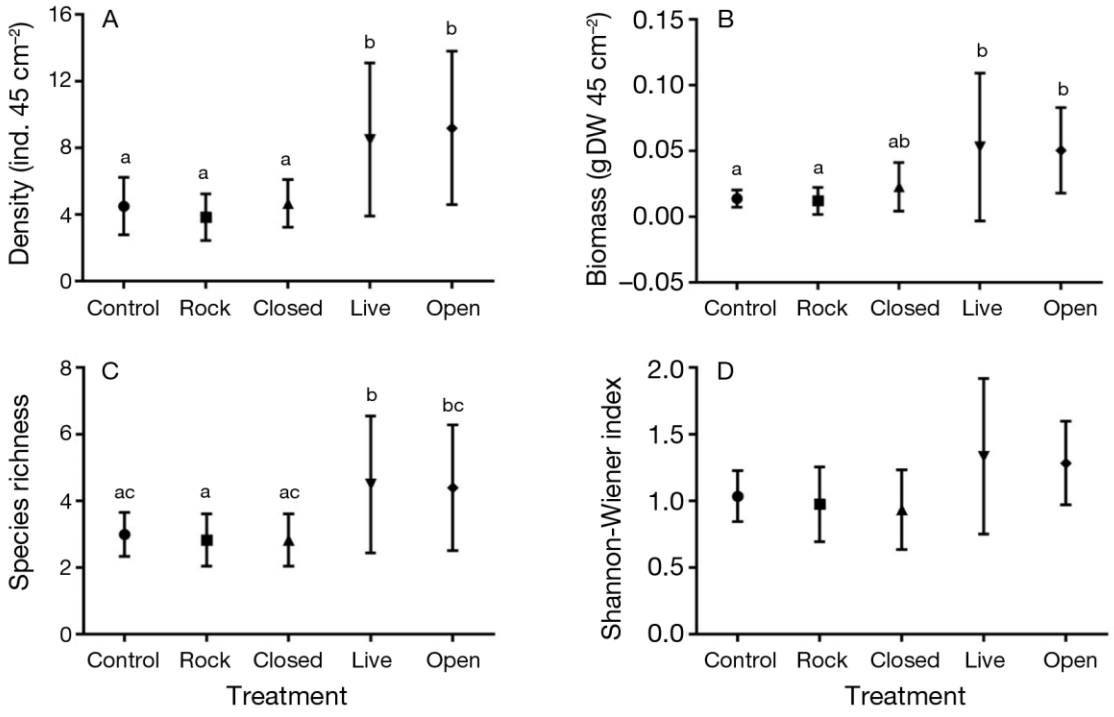

Figure 2.3. Macrozoobenthos mean (±95% CI) (A) density (ind. 45 cm^{-2}), (B) biomass (g DW 45 cm^{-2}), (C) species richness and (D) Shannon-Wiener diversity index for each treatment (control, rock, closed, live and open). Different lowercase letters indicate significant differences among treatments.

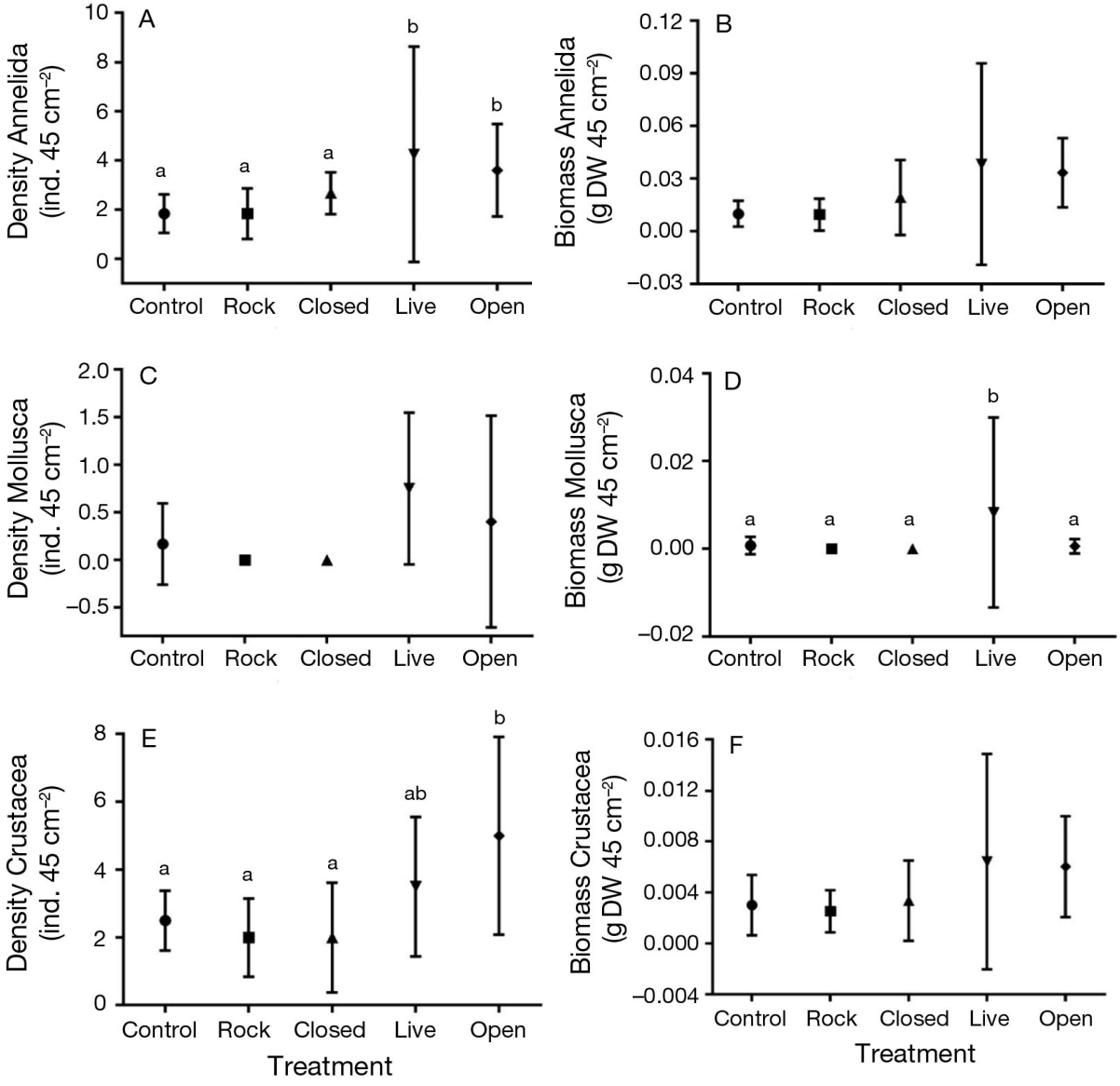

Figure 2.4. Mean (±95% CI) density (ind. 45 cm^{-2}) and biomass (g DW 45 cm^{-2}) per treatment (control, rock, closed, live and open) for (A, B) Annelida, (C, D) Mollusca and (E, F) Crustacea. Different lowercase letters indicate significant differences among treatments.

2.4. Discussion

The studied area is subjected to harsh abiotic conditions during the summer, mainly because salinity can oscillate between 0.05 and 15 psu during low and high tide, respectively. These harsh abiotic conditions do not allow for the establishment of a diverse macrozoobenthic assemblage; only true estuarine organisms with the physiological capacity to tolerate these changing salinity conditions can thrive in this area. However, the number and composition of species recorded in our experiment were similar to that reported in other studies performed in the same area (see Sousa et al. 2008c; Ilarri et al. 2012, 2014). In addition, the nMDS analysis was not able to distinguish any group in the macrozoobenthic assemblage, which was expected, given that it would be unlikely that the macrozoobenthic composition would differ between the five treatments during the 2 months study period.

Despite the similarity in faunal composition among treatments, our experiment clearly showed that treatments with live and open empty shells of *C. fluminea* supported higher macrozoobenthos density, biomass and species richness in comparison to the control, closed and rock treatments. Thus, our results suggest that the presence of *C. fluminea* may actually have positive effects on some macrozoobenthic and epibenthic species in invaded estuarine areas, as recently proposed by Ilarri et al. (2012, 2014). In addition, our experimental approach gave further insights into the identification of possible mechanisms underlying the observed changes in macrozoobenthic colonization in areas with and without *C. fluminea*. Indeed, two main mechanisms seemed to have influenced the results: (1) the production of feces and pseudofeces by *C. fluminea*, which increased organic matter content and food resources for some macrozoobenthic species; and (2) ecosystem engineering activities by *C. fluminea*, which created appropriate conditions for the establishment of other species via shell production and bioturbation of sediments.

In the case of Annelida, the density and biomass of *H. diversicolor* was higher in the presence of live *C. fluminea*. *H. diversicolor* is usually described as a generalist species that consumes the most abundant food resources available at the local scale (Fidalgo e Costa et al. 2006). In fact, this species has different strategies with which to capture its food, but usually behaves as a filter feeder and a deposit feeder, scavenging organic material and detritus on the sediment surface (Fidalgo e Costa et al. 2006; Olivier et al. 1997). According to Batista et al. (2003), *H. diversicolor* is able to increase its

Table 2.1. Mean (±SD) values of density (ind. 45 cm^{-2}) and biomass (g DW 45 cm^{-2}) and one-way PERMANOVA results for the effects of five treatments (control, rock, closed, live and open) on the species collected in the lower Minho estuary, NW Iberian Peninsula. *p < 0.05.

Species	Density							Biomass						
	Control	Rock	Closed	Live	Open	Pseudo-F	p	Control	Rock	Closed	Live	Open	Pseudo-F	p
Hediste diversicolor	1.50 ± 0.55	1.33 ± 0.52	2.17 ± 0.75	3.50 ± 2.38	2.80 ± 0.84	3.44	0.021*	0.0099 ± 0.0070	0.0093 ± 0.0087	0.0136 ± 0.0086	0.0382 ± 0.0360	0.0320 ± 0.0180	3.17	0.023*
Heteromastus filiformis	0.00 ± 0.00	0.33 ± 0.82	0.17 ± 0.41	0.25 ± 0.50	0.00 ± 0.00	0.55	0.807	0.0000 ± 0.0000	0.0001 ± 0.0003	0.0000 ± 0.0000	0.0000 ± 0.0001	0.0000 ± 0.0000	0.74	0.568
Nereis cultifera	0.00 ± 0.00	0.00 ± 0.00	0.17 ± 0.41	0.00 ± 0.00	0.20 ± 0.45	0.74	0.692	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0057 ± 0.0139	0.0000 ± 0.0000	0.0014 ± 0.0031	0.77	0.551
Streblospio benedicti	0.33 ± 0.82	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.20 ± 0.45	0.71	0.693	0.0001 ± 0.0002	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.77	0.548
Spionidae sp.	0.00 ± 0.00	0.17 ± 0.41	0.17 ± 0.41	0.50 ± 0.58	0.40 ± 0.89	0.75	0.554	0.0000 ± 0.0000	0.0001 ± 0.0002	0.0000 ± 0.0000	0.0001 ± 0.0001	0.0001 ± 0.0001	0.53	0.833
Corbicula fluminea	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.25 ± 0.50	0.00 ± 0.00	1.56	0.220	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0072 ± 0.0143	0.0000 ± 0.0000	1.56	0.220
Potamopyrgus antipodarum	0.17 ± 0.41	0.00 ± 0.00	0.00 ± 0.00	0.50 ± 0.58	0.40 ± 0.89	1.14	0.368	0.0008 ± 0.0019	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0012 ± 0.0013	0.0006 ± 0.0013	0.93	0.468
Corophium multisetosum	1.50 ± 1.38	0.83 ± 0.75	1.17 ± 1.33	2.25 ± 0.50	3.40 ± 2.30	2.82	0.049*	0.0012 ± 0.0014	0.0006 ± 0.0006	0.0011 ± 0.0017	0.0031 ± 0.0031	0.0037 ± 0.0033	2.13	0.094
Cyathura carinata	0.67 ± 0.52	1.00 ± 0.89	0.50 ± 0.55	1.00 ± 0.87	0.80 ± 0.84	0.49	0.762	0.0017 ± 0.0019	0.0018 ± 0.0017	0.0013 ± 0.0017	0.0026 ± 0.0022	0.0017 ± 0.0022	0.27	0.892
Gammarus sp. 1	0.00 ± 0.00	0.00 ± 0.00	0.17 ± 0.41	0.00 ± 0.00	0.60 ± 0.55	3.72	0.0211*	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0003 ± 0.0008	0.0000 ± 0.0000	0.0006 ± 0.0006	2.03	0.125
Gammarus sp. 2	0.33 ± 0.52	0.17 ± 0.41	0.17 ± 0.41	0.25 ± 0.50	0.20 ± 0.45	0.14	0.964	0.0002 ± 0.0003	0.0001 ± 0.0003	0.0007 ± 0.0017	0.0008 ± 0.0015	0.0000 ± 0.0001	0.58	0.825
Petromizon marinus	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.20 ± 0.45	1.12	0.371	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0103 ± 0.0230	1.12	0.366

growth and survival rates and to attain higher biomass production in the presence of the clam *Ruditapes decussatus* (Linnaeus, 1758) feces. Therefore, our results suggest that the positive response of *H. diversicolor* to the live treatment may be related to the large amounts of nutrients (in the form of feces and pseudofeces) produced by *C. fluminea* (Vaughn and Hakenkamp 2001). This hypothesis is also supported by the value of organic matter obtained in the live treatment. Although there were no significant differences between treatments (with the exception of live and rocks), the live treatment contained the highest content of organic matter. However, even if changes in organic matter availability serve to increase food resources (which could benefit deposit feeders; Crooks 1998), we cannot discard other explanations for the density increase of *H. diversicolor* in the live treatment. Indeed, bioturbation by *C. fluminea* could also be an important mechanism (Majdi et al. 2014) and it is possible that *H. diversicolor* (being an infaunal organism) responded to the abiotic changes mediated by the sediment reworking of the live clams.

Similar arguments may also apply to organisms belonging to the phylum Mollusca, since the live treatment had the highest values of density and biomass. It is also possible that Molluscan species (of which only two species, *P. antipodarum* and *C. fluminea*, were present) responded to increases in organic matter available in the sediments due to the production of feces and pseudofeces by *C. fluminea*. The density of certain gastropods can increase in response to increases in organic matter content (Osenberg 1989; Brown 1991) and *P. antipodarum* has been described as a consumer of periphyton and fine organic matter (Haynes and Taylor 1984; Broekhuizen et al. 2001; Alonso and Castro-Díez 2012; Krist and Charles 2012). Interestingly, both *C. fluminea* and *P. antipodarum* are non-native in the studied area, and this positive interaction between the two species may be seen as an example of the meltdown hypothesis, in which one introduced species facilitates the presence of another (Simberloff 2006). In the same vein, increases in the organic matter available in the sediments may exert a positive feedback to *C. fluminea* because this species, in addition to being a filter feeder, may also behave as a deposit feeder (see Hakenkamp and Palmer 1999; Vaughn and Hakenkamp 2001). Other mechanisms besides the possible use of organic matter may have influenced our results, namely bioturbation activities and the ability of *C. fluminea* adults to release chemical cues that could influence juvenile settlement in areas where adult clams are present (Sardiña et al. 2009). However, future detailed studies are necessary in order to test these hypotheses.

In the case of Crustacea, particularly *C. multisetosum* and *Gammarus* sp. 1, higher density was detected in the open empty shells treatment. These results indicate that accumulations of empty *C. fluminea* shells may increase structural complexity, with positive consequences for associated amphipods. In fact, the three-dimensional structure provided by open empty shells created new microhabitats which provided substrata for attachment, along with a refuge from water flow, predators, competitors, and physical and/or physiological stress (Gutiérrez et al. 2003; Sousa et al. 2009). Interestingly, higher densities were obtained in open empty shells than closed shells, which may be related to the much higher surface area and habitat heterogeneity provided by open empty shells. Based on these results, it is likely that, even after their death, *C. fluminea* can still affect macrozoobenthic assemblages and it will be important to quantify the decay rates of shells in future studies.

Progress in the study of the impacts of IAS on biodiversity can be facilitated by the implementation of manipulative experiments such as ours, in which an increase in infaunal density, biomass and species

richness was attributed to the presence of *C. fluminea*. Treatments with live clams and open empty shells led to similar increases in density, biomass and species richness; however, the species associated with each treatment differed: polychaetes and molluscs were more important in the live treatment, while crustaceans were more relevant in the open treatment. We are aware that in some faunal groups, this positive effect may be modified in the future if biological and environmental conditions change, and that the current positive effect is not generalized to all species present in the Minho estuary. Indeed, native bivalves have undergone significant declines in density, biomass and spatial distribution following *C. fluminea* introduction (Sousa et al. 2005, 2007a, 2008c,d,e). Interestingly, a recent meta-analysis assessing the impacts of marine invaders on local biodiversity showed that these species typically have negative effects on biodiversity within a trophic level, but positive effects on biodiversity of higher trophic levels (Thomsen et al. 2014). The results of this and earlier studies conducted in the Minho River concerning the impacts of *C. fluminea* on biodiversity seem to follow similar trends to those described by Thomsen et al. (2014).

According to Gutiérrez et al. (2014), assimilation-dissimilation (uptake and release of energy and materials) and ecosystem engineering (physical environmental modifications by organisms) are the two main mechanisms explaining the direct and indirect effects of IAS on ecosystem structure and function. Indeed, assimilation-dissimilation involves the up-take (assimilation) of energy and materials (light, water, nutrients etc.) and their release (dissimilation) in the form of dead tissues and waste products (carbon and nutrients in litter, woody debris, feces, urine, carcasses etc.). In the case of bivalves, given their high filtration rates, these organisms are capable of capturing energy from the water column and releasing feces and pseudofeces rich in organic matter into the sediments. This energy may be consumed by benthic organisms, thereby increasing their density, biomass and species richness, at least for deposit-feeding species (Karatayev et al. 1997; Ward and Ricciardi 2007). On the other hand, ecosystem engineering activities can also promote significant changes in biodiversity (Jones et al. 1994, 1997; Anderson and Rosemond 2007; Altieri and van de Koppel 2014). Concerning IAS, Castilla et al. (2004) found that the introduced ascidian *Pyura praeputialis* (Heller, 1878) (an engineer species) modified the intertidal habitat structure in Antofagasta Bay (northern Chile), by creating broad belts and three-dimensional matrices, resulting in increased species richness at local and seascape scales. This type of facilitation has also been demonstrated with invasive bivalves (Ruesink et al. 2005; Sousa et al. 2009, 2014). For example, non-indigenous zebra *Dreissena*

polymorpha (Pallas, 1771) and golden mussels *Limnoperna fortunei* (Dunker, 1857) were associated with increased benthic macroinvertebrate density, biomass and taxonomic richness due to increases in the provision of refuges and bed substrate complexity (Beekey et al. 2004; Sylvester et al. 2007). However, in contrast to our manipulative experiment, these studies were not able to disentangle the importance of biodeposition of organic matter on sediments or the importance of the ecosystem engineering activities promoted by bivalves.

2.5. Conclusions

Our results clearly indicate that *C. fluminea* can have positive effects on estuarine macrozoobenthos density, biomass and species richness. These effects are most likely due to an enrichment in organic matter via the production of feces and pseudofeces, along with physical ecosystem engineering (e.g. shell production and bioturbation). However, estuaries are highly dynamic systems, often characterized by high environmental disturbance due to natural or human activities (Day et al. 1989; Little 2000); therefore, the results reported in this study may differ from other aquatic ecosystems (including rivers and lakes) that are less disturbed and that support much higher species richness.

Chapter 3

Effects of the invasive clam *Corbicula fluminea* (Müller, 1774) on an estuarine microbial community

Novais A, Souza AT, Ilarri M, Pascoal C, Sousa R (2015) Effects of the invasive clam *Corbicula fluminea* (Müller, 1774) on an estuarine microbial community. Science of the Total Environment 566-567: 1168-1175

Abstract

The Asian clam *Corbicula fluminea* (Müller, 1774) is well recognized for its invasive behaviour and high ecological and economic impacts, being classified as one of the 100 worst IAS in Europe. In this study, we performed a manipulative experiment under natural conditions to assess the effects of *C. fluminea* on sediments biochemistry and on the structure of an estuarine microbial (fungi and bacteria) community. We placed five treatments (control, rock, closed, live and open) for 2 months in the Minho estuary (NW Iberian Peninsula). No differences were detected between treatments regarding the values of carbon (C), nitrite (NO^{-}_{2}), ammonium (NH^{+}_{4}), phosphate (PO^{3-}_{4}) and calcium (Ca) in the sediments; however, potassium (K) had higher values in the open treatment. Furthermore, we found that the presence of live *C. fluminea* stimulated fungal biomass (but not diversity) and bacterial diversity. Bioturbation activities by *C. fluminea* are possibly the main mechanism explaining these results; however, other factors such as the presence of other macroinvertebrate species and/or production of feces and pseudofeces by *C. fluminea* cannot be excluded. To our knowledge, this is the first manipulative experiment under natural conditions that clearly shows the effects of *C. fluminea* on an estuarine microbial community. Given the widespread distribution of this IAS and the paucity of quantitative assessments of invasive bivalves' effects on microbial communities, it will be important that future studies further investigate these processes.

3.1. Introduction

Benthic organisms play a key role in estuarine and marine ecosystems (Snelgrove 1999). For example, numerous benthic vertebrate and invertebrate species modify geochemical gradients, alter hydrodynamics, modify sediment texture, and redistribute food resources, energy, viruses, bacteria, fungi, cysts and eggs due to bioturbation activities (Meysman et al. 2006). Therefore, alterations in benthic biotic composition due to species loss (e.g. due to bottom-trawl fishing, pollution or eutrophication-induced anoxia) and additions (e.g. introduction of IAS) can be responsible for major changes in important ecosystem functions (Levin et al. 2001; Sousa et al. 2011a; Strayer 2012). Indeed, after more than 20 years of research on biodiversity and ecosystem functioning, and into the functional redundancy in particular, it is clear that alterations in species composition modify the efficiency by which ecological communities capture resources, produce biomass, decompose and recycle nutrients (Cardinale et al. 2012). The level of functional redundancy in estuarine and marine benthic ecosystems is yet poorly understood and there is little evidence that high biodiversity is necessary to maintain their ecosystem functions (Snelgrove 1997).

Nowadays, it is very difficult to predict how changes in species composition due to species losses or additions will impact ecosystem functions. This is especially true for changes in biogeochemistry and microbial communities resulting from IAS introductions due to an almost complete lack of quantitative studies. However, and since the structure and dynamics of any community depend on the availability of resources, it is reasonable to think that the introduction of an IAS with great density and biomass may influence microbial communities (Stolp 1988; Gutiérrez and Jones 2006). Indeed, a dominant IAS can add (e.g. litter, exudates, urine, feces, oxygen, C) and/or remove (e.g. C, nutrients, water, oxygen) materials to a particular invaded habitat, also affecting nutrients availability and abiotic conditions (Gutiérrez et al. 2014). In addition, IAS influence on microbial communities might also include physical ecosystem engineering resulting from digging, burrowing and damming (Gutiérrez and Jones 2006).

Bivalves are among the most invasive faunal group in aquatic ecosystems (Sousa et al. 2009, 2014). Through active feeding on particulate organic matter, filter-feeding bivalves can alter water clarity, nutrient cycling, food web structure, and the concentration and composition of suspended particulate matter (Strayer et al. 1999; Phelps 1994; Karatayev et al. 1997; Boltovskoy et al. 2009; Sousa et al.

2014). Furthermore, their capacity to produce a great quantity of feces and pseudofeces can alter biogeochemical cycles and promote sedimentation (Roditi et al. 1997). Several invasive bivalve species can also have high potential for ecosystem engineering since they have key attributes such as production of durable shells, bioturbating and filter-feeding behaviour, relatively large size, high density and widespread distribution (Sousa et al. 2009). Other important ecological impacts mediated by invasive bivalves are related to bioamplification of pollutants along the food web and changes in biotic interactions (e.g. competition, introduction of diseases and parasites) (Sousa et al. 2014; Novais et al. 2016).

One of the most recognized IAS in aquatic ecosystem is the Asian clam *C. fluminea*, which is listed as one of 100 worst invasive species in Europe due to their widespread distribution and high ecological and economic impacts (Sousa et al. 2008a, 2014; Rosa et al. 2011; Crespo et al. 2015). Species belonging to the genus *Corbicula* expanded their distribution in the last decades from Asia to North and South America, Europe and North Africa (reviewed in Ilarri and Sousa 2012 and Crespo et al. 2015). When *C. fluminea* is present in high densities it can cause a wide range of impacts, including modification of biogeochemical cycles and changes to submerged vegetation, phytoplankton, zooplankton and benthic and epibenthic communities (Vaughn and Hakenkamp 2001; Sousa et al. 2008a; Ilarri and Sousa 2012; Ilarri et al. 2012, 2014; Lopes-Lima et al. 2016). This IAS is also capable of bioturbate the sediments by pedal feeding (Sousa et al. 2008b) and excrete large amounts of nutrients in the form of feces and pseudofeces (Strayer et al. 1999). Recent studies have also suggested that periodic massive die-offs of *C. fluminea* can have significant importance on the invaded ecosystem and food web dynamics, by acting as a resource pulse, with effects extending further into the adjacent terrestrial communities (Sousa et al. 2012; Bódis et al. 2014; Ilarri et al. 2015a; Novais et al. 2015a).

Although many studies have addressed the effects of *C. fluminea* in invaded ecosystems, most of them were focused on macrozoobenthic communities (Ilarri et al. 2012, 2015b; Novais et al. 2015b). To date, no data is available on the effect of this IAS on microbial communities in estuarine sediments (but see Zeng et al. 2014 for freshwater sediments). This is also true for many other benthic IAS. Therefore, to better understand the effects of *C. fluminea* on estuarine microbial communities, we carried out a manipulative experiment under natural conditions. It is important to mention that this experiment is part of a larger study that aimed to understand the mechanisms underlying the effects of *C. fluminea* on the structure of estuarine benthic communities. The first part assessed possible effects on macrozoobenthic communities (Novais et al. 2015b). Here we assessed possible effects on sediments biochemistry and

on microbial communities. We hypothesized that both fungal and bacterial communities will positively respond to the presence of live *C. fluminea* as a consequence of the higher excretion (the release of nutrients in the form of feces and pseudofeces) rates and/or as a consequence of possible environmental changes resulting from bioturbation activities mediated by this IAS.

3.2. Materials and Methods

3.2.1. Study area

The experiment was carried out in a sandy intertidal area located 8 km upstream of the mouth of the Minho estuary (41° 54' 37.23'' N, 08° 47' 22.17'' W; NW of Iberian Peninsula). This area was selected because *C. fluminea* presents a very low density (<50 ind. m^{-2}) here (see Sousa et al. 2008c for density comparisons between this and adjacent upstream areas). The Minho estuary has a total length of about 35 km and comprises different habitats with mobile and rocky substrata that favour the occurrence of many species, including some with high conservation and economic importance such as mammals, birds and migratory fish (Sousa et al. 2008c; Costa-Dias et al. 2010; Souza et al. 2013; Mota et al. 2014). Some species were introduced in the last decades, and include mammals, plants, fish, crustaceans, and molluscs such as the Asian clam *C. fluminea* (Sousa et al. 2008e). Currently, the benthic community is dominated by *C. fluminea* that contributes >95% of the total biomass in this estuarine ecosystem (Sousa et al. 2008c,d,e). Since its introduction (at least in 1989; Araujo et al. 1993) in the Minho estuary, the species rapidly spread upstream and nowadays is present in 150 km of the river length (Ferreira-Rodríguez and Pardo 2016). After this IAS introduction, a significant decline in the diversity, abundance and biomass of native bivalves as well some gastropod species, has been observed (Sousa et al. 2008c,d,e).

3.2.2. Experiment setup

A couple of hours before the beginning of the experiment, all adult clams with shell lengths >10 mm were removed from the sandflat. Fifteen 400 cm^2 open-top mesh boxes (mesh size = 10 mm) were

randomly placed in the study area. Five treatments (with three replicates for each) were used: (1) bare sediment (hereafter, control treatment); (2) inanimate substrate consisting of small rocks with a similar oval shape as *C. fluminea* (hereafter, rock treatment); (3) dead *C. fluminea* shells filled with sand and both valves glued together (hereafter, closed treatment); (4) live *C. fluminea* individuals (hereafter, live treatment); and (5) open empty *C. fluminea* shells (hereafter, open treatment). The control treatment was used to recreate a site without *C. fluminea* influence, while the rock treatment was used as a control for the effect of a physically inert substrate similar in size to shells. The closed treatment was used to detect only the physical effect of the shells but not the bioturbation activities, and the live treatment was used to detect the total effect of the presence of live *C. fluminea* (shell as substrate, feces and pseudofeces production and bioturbation activities). Finally, the open treatment was used to detect the effect of open empty shells after the death of individual clams (see Ilarri et al. 2011, 2015b and Sousa et al. 2012 for data on massive mortalities of *C. fluminea* and accumulation of empty shells in the study area). Based on the mean values of *C. fluminea* in the Minho estuary, the treatments had a density of 1200 ind. m^{-2} (Sousa et al. 2008e). All empty *C. fluminea* shells and rocks used were subjected to 60°C during 24 h to avoid possible contamination. Treatments were distributed within a grid of c.a. 1 m interval, chosen to minimize habitat variability and inter-plot interactions. The experiment lasted 2 months (July and August 2013) and a detailed description of the experimental design can be found in Novais et al. (2015b). At the end of the experiment, surface sediment samples (1 cm depth) were collected with a small core (10 cm^2) for nutrient content assessment, fungal biomass quantification and analysis of fungal and bacterial diversity. Sediment samples from each treatment were homogenized (mixed with a spoon and a small sub-sample randomly taken) and deep frozen at -80°C. During the 2 months of the experiment no *C. fluminea* mortality in the live treatment was recorded.

3.2.3. Abiotic characterization

Temperature, redox potential, salinity, dissolved oxygen and pH were measured at two weeks intervals during high tide using a multiparametrical probe YSI EXO 2. In order to characterize the granulometry of the sediment, three samples were collected prior to the beginning of the experiment. In the laboratory, sediment samples were oven-dried for 72 h at 60°C, then sieved with Ro-Tap agitation using a column of sieves with different sizes (for details see Novais et al. 2015b). The concentrations of organic carbon

(C), nitrite (NO^{-}_{2}), ammonium (NH^{+}_{4}), phosphate (PO^{3-}_{4}), calcium (Ca) and potassium (K) in the sediments were determined in Centro de Apoio Científico e Tecnolóxico á Investigación (CACTI), University of Vigo, Vigo, Spain following standard procedures. In detail, the concentration of C was determined by dry combustion using a LECO CN 2000. The concentration of NO^{-}_{2}, NH^{+}_{4} and PO^{3-}_{4}, was quantified by standard colorimetric method using a Bran Lubbe continuous flow analyzer (Brand Luebbe AA3) after an extraction in KCl. Finally, concentrations of Ca and K were quantified by inductively coupled plasma-atomic emission spectrometry (ICP-OES Optima 4300).

3.2.4. Microbial community characterization

Fungal biomass was estimated by ergosterol quantification following Pascoal and Cássio (2004). For this, 1.5 g sediment from each replicate was mixed in 10 mL of 0.8% KOH-methanol for 30 minutes at 80°C. The resulting lipid extract was purified by solid-phase extraction (Sep-Pak cartridges, Waters, Milford, MA, USA) and it was purified and quantified by high-performance liquid chromatography (Beckmann Gold System, Brea, CA, USA), using a LiChrospher RP18 column (250 × 4 mm, Merck). The system was run isocratically with methanol as mobile phase (1.4 mL min^{-1}, 33°C). Ergosterol was detected at 282 nm and quantified using a standard curve of ergosterol (Sigma) in isopropanol.

For microbial diversity a DNA extraction kit (PowerSoil DNA Isolation Kit, MoBio Laboratories, Carlsbad, CA, USA) was used to extract DNA from 200 mg of sediment according to the manufacturer's instructions. Fungal diversity was assessed using the primer pairs ITS3GC/ITS4, which amplify the ITS2 region of fungal rDNA. Bacterial diversity was assessed using the primer pairs 338F_GC/518R, which target the V3 region of bacterial 16S rDNA (Duarte et al. 2010).

For PCR reactions 2 × of Dream GoTaq® Green Master Mix (Promega), 0.4 µM of each primer and 1 µL of DNA were used in a final volume of 25 µL. PCRs were carried out in a MyCycler Thermal Cycler (BioRad Laboratories, Hercules, CA, USA). The amplification program started with a denaturation at 95°C for 2 minutes, 36 cycles of denaturation at 95°C for 30 seconds, primer annealing at 55°C for 30 seconds and extension at 72°C for 1 minute, and final extension at 72°C for 5 minutes (Duarte et al. 2010).

Denaturing gradient gel electrophoresis (DGGE) analysis was performed using a DCodeTM Universal Mutation Detection System (BioRad Laboratories, Hercules, CA, USA). For fungi and bacteria, 700 ng of

the amplified DNA products with 380–400 bp (ITS3GC/ITS4) and 200 bp (338F_GC/518R), respectively, was loaded on 8% (w/v) polyacrylamide gel in 1× Tris-acetate-EDTA (TAE) with a denaturing gradient from 30 to 70% (100% denaturant corresponds to 40% formamide and 7 M urea). All gels were run at 55 V, 56°C for 16 h. Gels were stained with 1× of GelStar (Lonza) for 10 minutes, and gel images were captured under UV light in a ChemiDoc XRS (BioRad).

3.2.5. Data analysis

Differences in fungal biomass and nutrients content between treatments were compared by a one-way PERMANOVA (type-III), with treatment as a fixed factor (five levels: control, rock, closed, live and open). Prior to PERMANOVA, all variables were normalised without data transformation and similarity matrices calculated using Euclidean distances (Clarke and Warwick 2001).

For each group of microbes, DGGE gels were aligned and the relative intensity of each band was analysed with BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium). Each DGGE band was considered one operational taxonomic unit (OTU). The nMDS ordination analysis based on fungal and bacterial communities' data followed by the one-way PERMANOVA (type-III), with treatment as a fixed factor (five levels: control, rock, closed, live and open), were used. All variables were log (X + 1) transformed prior to PERMANOVA and nMDS ordination analysis. Similarity matrices were also calculated using Euclidean distances and Bray Curtis similarity, respectively (Clarke and Warwick 2001). Species richness and the Shannon-Wiener diversity index were calculated using DIVERSE analysis (Clarke and Warwick 2001). Differences in species richness and the Shannon-Wiener diversity index between the five treatments were tested using a one-way PERMANOVA (type-III), following the design described above.

In all PERMANOVA tests, the statistical significance of variance ($\alpha = 0.05$) was tested using 9999 permutations of residuals within a reduced model. When the number of permutations was <150, the Monte Carlo p-value was considered. All analysis that had significant differences were followed by a PERMANOVA a posteriori pairwise comparisons.

PRIMER software (v.6.1.6) with PERMANOVA+ 1.0.1 add-on (Anderson 2001; MJ Anderson et al. 2008) was used for all statistical tests.

3.3. Results

3.3.1. Abiotic characterization

Abiotic factors (mean ± SD) measured in the water column at high tide were, temperature: 20.56 ± 1.44°C; redox potential: 204.31 ± 20.15 mV; salinity: 12.15 ± 3.31; dissolved oxygen: 8.76 ± 0.43 mg $O_2.L^{-1}$ and pH: 7.89 ± 0.14. The sediment composition in the study area was very homogeneous with the two size classes dominating with >80%; >2 mm, 0.1 ± 0.01%; 1–2 mm, 0.2 ± 0.01%; 0.5–1 mm, 1.0 ± 0.15%; 0.25–0.5 mm, 5.5 ± 0.65%; 0.125–0.25 mm, 46.5 ± 3.10%; 0.063–0.125 mm, 34.3 ± 2.03%; and <0.063 mm 12.4 ± 1.08%.

The results for C, NO^{-}_{2}, NH^{+}_{4}, PO^{3-}_{4}, Ca and K in the sediments of each treatment are described in Table 3.1 and Fig. 3.1. Only the concentration of K differed significantly between treatments (Pseudo-F = 4.19, p = 0.04) (Table 3.1 and Fig. 3.1F). Pairwise tests showed significant differences between open and live (t = 3.17, p = 0.03), open and rock (t = 3.01, p = 0.04), and live and closed (t = 3.24, p = 0.03) treatments.

3.3.2. Biotic characterization

3.3.2.1. Fungal biomass

Significant differences in ergosterol values [mean μg g^{-1} sediment ± standard error of the mean (SEM)] were detected (Pseudo-F = 3.87, p = 0.02), with higher values being recorded in the live treatment (0.76 ± 0.13), followed by control (0.69 ± 0.13), open (0.63 ± 0.07), rock (0.35 ± 0.05) and closed (0.34 ± 0.05) treatments (Fig. 3.2). Pairwise tests showed significant differences between live and rock (t = 2.92, p = 0.03), live and closed (t = 3.02, p = 0.02), open and rock (t = 3.22, p = 0.01), and open and closed (t = 3.40, p = 0.01) treatments.

Table 3.1. Mean (±SEM) values of C (%), NO_2^-, NH_4^+, PO_4^{3-}, Ca and K (mg kg^{-1}) in the sediments of each treatment and one-way PERMANOVA results for the effects of the five treatments (control, rock, closed, live and open). * = $p < 0.05$.

Nutrients	Control	Rock	Closed	Live	Open	Pseudo-F	p
C	0.86 ± 0.05	0.79 ± 0.12	0.59 ± 0.08	0.69 ± 0.05	0.86 ± 0.03	2.55	0.12
NO_2^-	1.03 ± 0.37	0.76 ± 0.15	0.78 ± 0.04	1.01 ± 0.03	0.68 ± 0.03	0.75	0.63
NH_4^+	8.91 ± 0.21	6.99 ± 0.28	10.68 ± 1.78	9.85 ± 1.72	17.33 ± 5.59	2.06	0.08
PO_4^{3-}	4.84 ± 0.07	5.33 ± 0.07	5.16 ± 0.14	4.66 ± 0.47	5.44 ± 0.21	1.84	0.16
Ca	22.50 ± 7.13	18.68 ± 4.73	26.94 ± 1.96	27.67 ± 1.94	22.44 ± 3.59	0.73	0.59
K	43.12 ± 2.53	37.20 ± 1.85	42.48 ± 0.92	37.96 ± 1.05	46.59 ± 2.51	4.19	0.04*

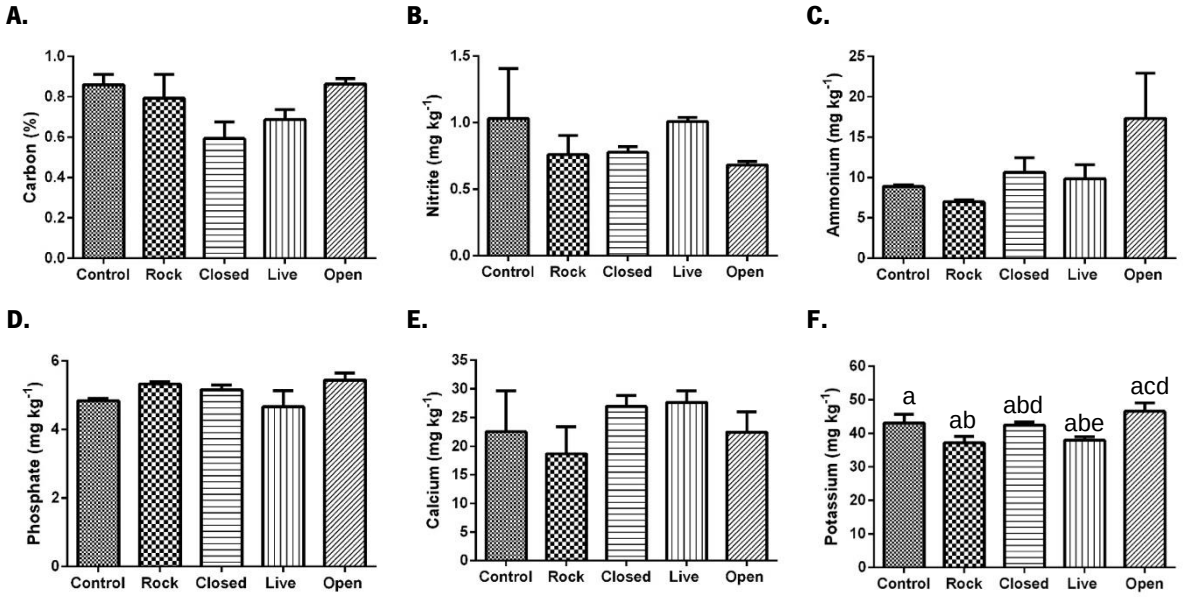

Figure 3.1. Mean (+SEM) nutrients content in the sediments for each treatment (control, rock, closed, live and open). (A) C (%), (B) NO_2^-, (C) NH_4^+, (D) PO_4^{3-}, (E) Ca and (F) K (mg kg^{-1}). Different letters indicate significant differences among treatments ($p < 0.05$).

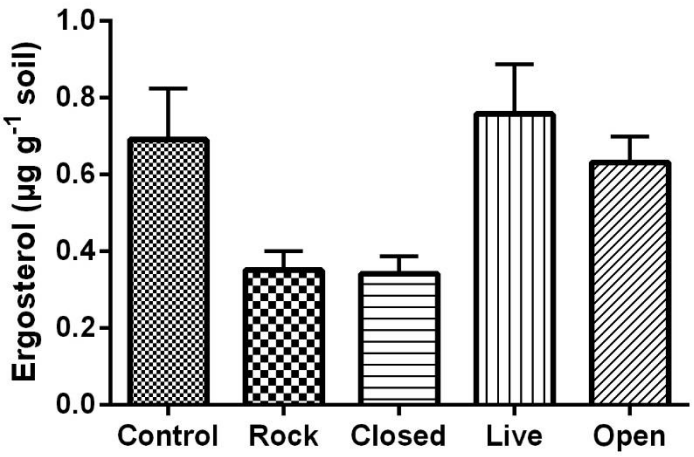

Figure 3.2. Mean (+SEM) ergosterol concentration) (µg g^{-1}sediment) for each treatment (control, rock, closed, live and open). Different letters indicate significant differences among treatments ($p < 0.05$).

3.3.2.2. Microbial diversity

The nMDS ordination based on the fungal community is shown in Fig. 3.3A and significant differences in the structure were detected (Pseudo-F = 1.59, $p \leq 0.01$).

Significant differences in taxon richness values between treatments were detected (Pseudo-F = 8.00, $p \leq 0.01$), with higher values (mean ± SEM) being recorded in control (11.67 ± 1.33) and rock (9.67 ± 0.67), followed by closed (7.00 ± 1.00), live (7.00 ± 0.58) and open (5.67 ± 0.33) treatments (Fig. 3.4A). Pairwise tests showed significant differences between control and live ($t = 3.21$, $p = 0.03$), control and open ($t = 4.37$, $p = 0.01$), rock and live ($t = 3.02$, $p = 0.04$), and rock and open ($t = 5.37$, $p \leq 0.01$) treatments.

Significant differences in the Shannon diversity values between treatments of the fungal community were detected (Pseudo-F = 6.81, $p \leq 0.01$), with the higher values (mean ± SEM) being recorded in control (2.04 ± 0.16), followed by rock (1.70 ± 0.09), closed (1.46 ± 0.16), live (1.18 ± 0.19) and open (1.09 ± 0.12) treatments (Fig. 3.4B). Pairwise tests showed significant differences between control and live ($t = 3.42$, $p = 0.03$), control and open ($t = 4.75$, $p \leq 0.01$), and rock and open ($t = 4.12$, $p = 0.01$) treatments.

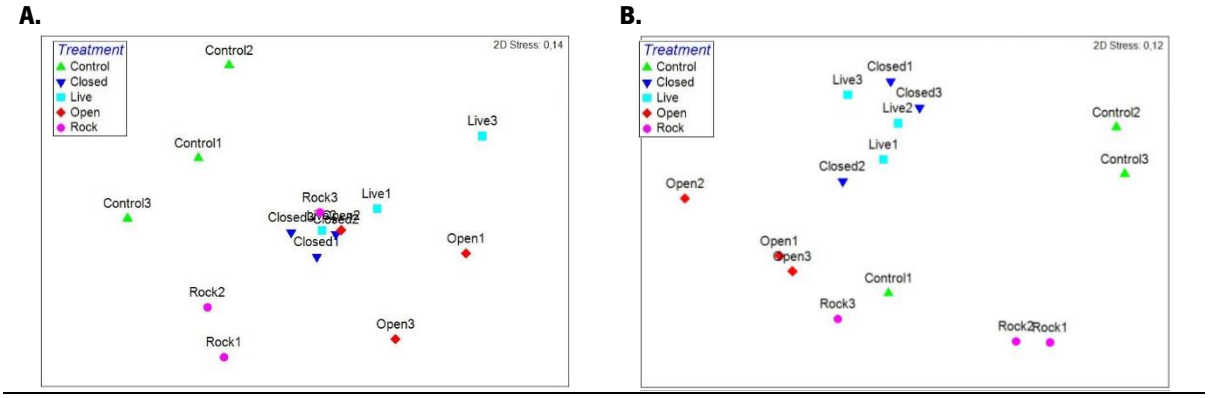

Figure 3.3. Non-metric multidimensional scaling (nMDS) plot of fungal (A) and bacterial (B) communities in the sediment of the five experimental treatments (control, closed, live, open and rock).

The nMDS ordination based on the bacterial community is shown in Fig. 3.3B and significant differences in the structure were detected (Pseudo-F = 3.15, $p \leq 0.01$). Pairwise tests showed significant differences between open and live ($t = 2.02$, $p = 0.03$), rock and closed ($t = 2.16$, $p = 0.04$) and rock and live ($t = 2.14$, $p = 0.04$) treatments.

Significant differences in taxon richness values of the bacterial community between treatments were detected (Pseudo-F = 100.70, $p \leq 0.01$), with higher values (mean ± SEM) being recorded in live (15.33 ± 0.33) and closed (12.67 ± 0.33) treatments, followed by control (9.00 ± 0.58), open (9.00 ± 0.00) and rock (7.00 ± 0.00) treatments (Fig. 3.4C). Pairwise tests showed significant differences between control and closed ($t = 5.50$, $p \leq 0.01$), control and live ($t = 9.50$, $p \leq 0.01$), control and rock ($t = 3.46$, $p = 0.03$), closed and live ($t = 5.66$, $p \leq 0.01$), closed and open ($t = 11.00$, $p \leq 0.01$), closed and rock ($t = 17.00$, $p \leq 0.01$), live and open ($t = 19.00$, $p \leq 0.01$), and live and rock ($t = 25.00$, $p \leq 0.01$) treatments.

Significant differences in the Shannon diversity values of the bacterial community between treatments were detected (Pseudo-F = 5.90, $p = 0.01$), with higher values (mean ± SEM) being recorded in live (2.34 ± 0.04) and closed (2.08 ± 0.12) treatments, followed by control (1.78 ± 0.18), open (1.75 ± 0.13) and rock (1.58 ± 0.11) treatments (Fig. 3.4D). Pairwise tests showed significant differences between control and live ($t = 2.96$, $p = 0.04$), rock and closed ($t = 3.15$, $p = 0.04$), rock and live ($t = 6.69$, $p \leq 0.01$), and open and live ($t = 4.44$, $p = 0.01$) treatments.

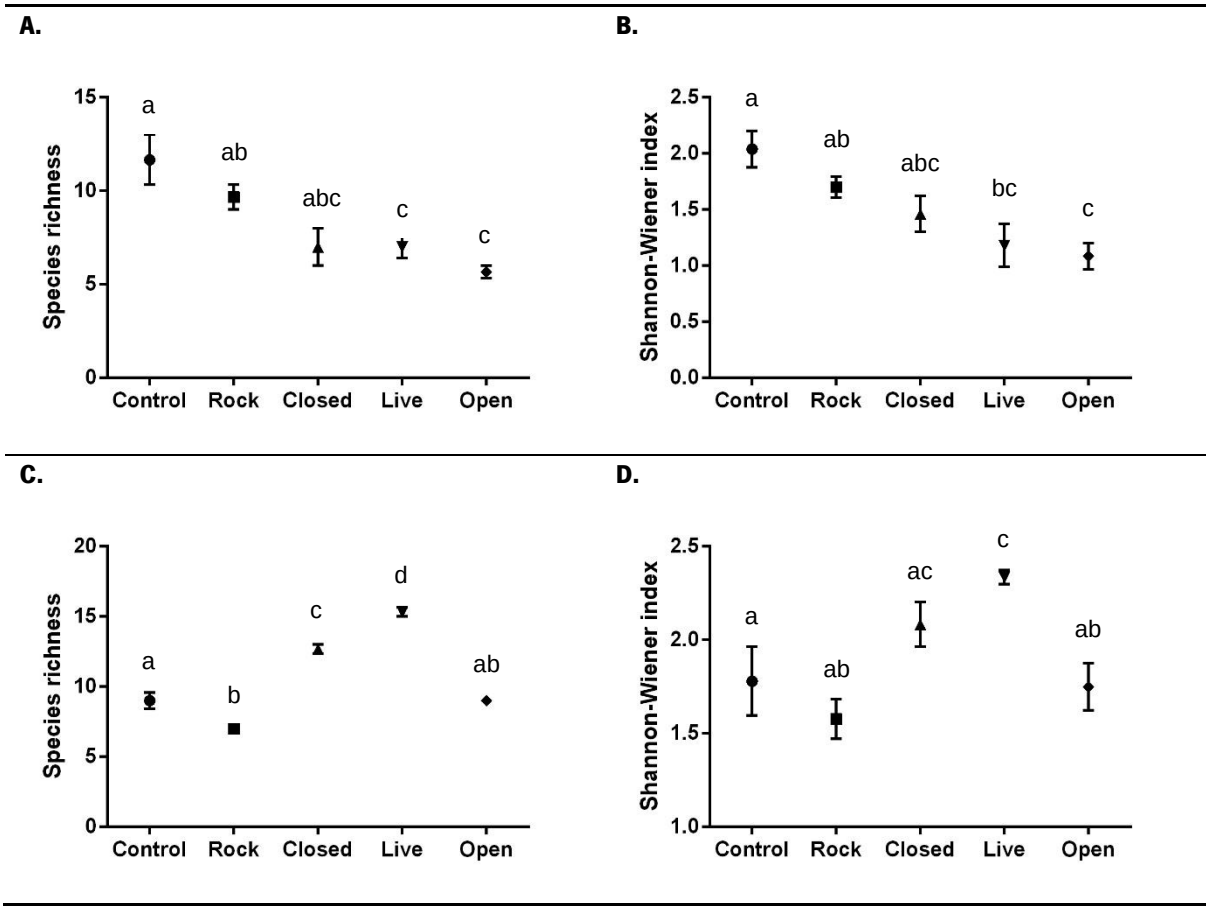

Figure 3.4. Mean (±SEM) of (A) species richness and (B) Shannon-Wiener diversity index of fungal community and (C) species richness and (D) Shannon-Wiener diversity index of bacterial community for each treatment (control, rock, closed, live and open). Different letters indicate significant differences among treatments ($p < 0.05$).

3.4. Discussion

3.4.1. Effects on the sediment biogeochemistry

Among all nutrients analysed only K was significantly affected showing higher values in the open treatment. *Corbicula fluminea* shells are primarily composed of calcium carbonate ($CaCO_3$), usually aragonite (Spann et al. 2010), also incorporating trace amounts of other elements such as Na, Mg, Al, P, S, Cl, and K (Eyster 1986). In an earlier study aiming to assess the decaying rates of empty shells of bivalve species, including *C. fluminea*, Ilarri et al. (2015a) showed a 23.3% mass decay per year for this IAS in the Minho River. Therefore, it is possible that the empty shells used in this experiment were more easily eroded than for example live or closed shells, leading to higher K values in this treatment. Given

this high decay rate we also expected that Ca differed among the five treatments given that this element is the greatest constituent of the shells. However, this prediction was not supported and future detailed studies are needed to clarify these distinct results between K and Ca.

Furthermore, taken into account that live *C. fluminea* are able to excrete large amounts of nutrients in the form of feces and pseudofeces, significant differences in other nutrients were also expected to occur, mainly in C, N and P (Vaughn and Hakenkamp 2001). However, with the exception of K, we were not able to detect any difference in the nutrients' composition between treatments. We expected live treatment to have the highest nutrients content due to the possible effect of feces and pseudofeces accumulation during the 2 months study period. Due to the considerable hydrodynamics in the studied area, often characterized by wide tidal ranges during the summer, it is possible that these nutrients resulting from feces and pseudofeces production were dispersed to adjacent areas and/or the 2 months duration of the experiment was not enough to detect possible differences.

3.4.2. Effects on the fungal community

Our results showed that live *C. fluminea* supported more fungal biomass in comparison to rock and closed treatments. Several environmental factors can affect fungal activity, including dissolved oxygen (Medeiros et al. 2009), nutrients (Gulis and Suberkropp 2003), temperature (Chauvet and Suberkropp 1998), turbulence (Webster 1975) and pH (Dangles et al. 2004). Bioturbation can modify oxygen availability (Majdi et al. 2014), which is a limiting factor to fungal biomass accrual, reproduction and diversity (Medeiros et al. 2009). Through activities such as burrowing, filter, deposit and pedal feeding, bioirrigation, excretion and ventilation (Aller 2001; Vaughn and Hakenkamp 2001; Vanni 2002; Meysman et al. 2006) *C. fluminea* changes the properties of the sediment surface. These changes include modifying the porosity, permeability and spatial heterogeneity enhancing oxygen penetration and increasing sediment water content (Zhang et al. 2011). Since we were not able to detect differences in nutrients between treatments, we suggest that the bioturbation by *C. fluminea* is possibly the most important mechanism explaining the increased fungal biomass in the live treatment. Besides the direct bioturbation activity by *C. fluminea*, other aspects may have also contributed to the increasing of the oxygen content in the live treatment. We have previously found that the presence of live and open empty shells of *C. fluminea* had positive effects on the density, biomass and richness of Annelida, Mollusca and

Crustacea species (Novais et al. 2015b). These species can additionally rework the sediment, constructing and maintaining burrow structures that increase oxygen penetration, as well as the release of nutrients from the sediment (Kristensen and Kostka 2005; Mermillod-Blondin et al. 2005). Polychaetes, bivalves and crustaceans are the three most successful burrowing faunal groups in marine and estuarine sediments (Kristensen and Kostka 2005). Therefore, the presence of other organisms besides *C. fluminea* may also explain the high fungal biomass observed in the open treatment.

Molecular diversity of fungi also varied with treatments. The nMDS plot revealed that control was the most dissimilar group, which can be explained by the higher taxon richness and Shannon diversity values observed for this treatment. On the other hand, the diversity of fungi did not follow the same pattern, with our results showing that treatments without *C. fluminea* were more diverse but had less biomass than treatments with *C. fluminea*. This suggests that the presence of *C. fluminea* led to the exclusion of certain fungal species, but the fewer remaining species were able to produce higher overall biomass. If so, results further support that there is considerable functional redundancy among fungi to maintain their ecological functions (Pascoal et al. 2005).

3.4.3. Effects on the bacterial community

The nMDS ordination of bacterial communities showed differences between treatments being the open treatment most dissimilar compared to the others. Our experiment also showed that treatments with live *C. fluminea* had higher bacterial diversity in comparison to control, rock, closed and open treatments. Similarly to fungal biomass, it appears that bacterial diversity responded to *C. fluminea* bioturbation (Majdi et al. 2014). Species with bioturbation activities increase the sediment-water contact zone improving the supply of oxygen, food particles (e.g. phytoplankton, detritus) and removal of toxic metabolites, which all together may stimulate bacterial activity (Kristensen and Kostka 2005). Biogeochemical evidence and rate measurements supported by viable cell counts and biomass determinations have indicated that microbial activity and abundance can be elevated in or near the burrow zone (Kristensen and Kostka 2005). The high diversity observed in the live treatment may be the result not only of the presence of *C. fluminea* but a combination of this with other organisms, particularly polychaetes, which also have a burrow-dwelling behaviour (Kristensen and Kostka 2005; Novais et al. 2015b). Although we cannot generalize about the direct relationships between burrowing activities and

the community structure or diversity of microorganisms, it is known that the structure of bacterial communities in tubes constructed by polychaetes differed substantially from surface to subsurface sediment (Marinelli et al. 2002; Kristensen and Kostka 2005; Papaspyrou et al. 2006). For example, *H. diversicolor* strongly stimulates bacterial activity in the sediment (Mermillod-Blondin et al. 2005). Some authors also argue that *C. fluminea* can decrease the abundance and biomass of bacteria in the sediments by deposit or pedal feeding (McMahon 1991; Reid et al. 1992; Vaughn and Hakenkamp 2001), whereas others suggest that *C. fluminea* does not consume bacteria (Leff and Leff 2000). Thus, future studies focusing on the origin and resources that are consumed by *C. fluminea* are needed to clarify this aspect.

3.5. Conclusions

Overall, our results showed that the presence of live *C. fluminea* stimulates fungal biomass and bacterial diversity. Surprisingly, and with the exception of K, no effects were detected on the concentrations of C, NO_2^-, NH_4^+, PO_4^{3-} and Ca in the sediments. The effects on fungal biomass and bacterial diversity are most likely due to bioturbation activities directly by *C. fluminea* and/or indirectly by other benthic organisms that attain higher densities in treatments with live or empty shells of this IAS. To our knowledge, this is the first manipulative experiment under natural conditions that clearly shows the effects of the Asian clam *C. fluminea* on the structure of an estuarine microbial community. Given the widespread distribution of this IAS and the paucity of quantitative assessments of invasive bivalves on microbial communities these effects should be further investigated.

Chapter 4

From water to land: How an invasive clam may function as a resource pulse to terrestrial invertebrates

Novais A, Souza AT, Ilarri M, Pascoal C, Sousa R (2015) From water to land: How an invasive clam may function as a resource pulse to terrestrial invertebrates.
Science of the Total Environment 538: 664-671

Abstract

Resource pulses are episodes of low frequency, large magnitude and short duration that result in increased resource availability in space and time, with consequences for food web dynamics. Studies assessing the importance of resource pulses by IAS in the interface between terrestrial and aquatic ecosystems are rare, especially those in the direction from water to land. This study assessed the importance of massive die-offs of the Asian clam *Corbicula fluminea* (Müller, 1774) as a resource pulse to the terrestrial invertebrate community after an extreme climatic event using a manipulative experiment. We used five levels of *C. fluminea* density (0, 100, 500, 1000 and 2000 ind. m^{-2}), with terrestrial invertebrates being censused 7, 30 and 90 days after *C. fluminea* addition. We also assessed the possible effect of plots position, where plots that delimited the experiment were assigned as edge plots and the remaining as core plots. Clear differences were detected in abundance, biomass, richness and diversity of terrestrial invertebrates depending on the *C. fluminea* density, time and position. Interestingly, the highest abundance of adult Diptera was observed 7 days after *C. fluminea* addition, whereas that of the other terrestrial invertebrates was on day 30, both with *C. fluminea* densities higher than 500 ind. m^{-2} located on the edge of the experimental design. This study highlights the importance of major resource pulses after massive die-offs of invasive bivalves, contributing with remarkable amounts of carrion for adjacent terrestrial systems. Part of this carrion can be consumed directly by a great number of invertebrate species while the remainder can enter the detrital food web. Given the high density and biomass attained by several invasive bivalves worldwide and the predicted increase in the number, intensity and magnitude of extreme climatic events, the ecological importance of this phenomenon should be further investigated.

4.1. Introduction

The transport of materials, nutrients and energy across ecosystem boundaries by organisms can have profound consequences for the structure and composition of the recipient community (Polis et al. 1997). These subsidies may arrive regularly or, in certain circumstances, can result from occasional events of ephemeral resource superabundance, termed resource pulses (Yang 2004; WB Anderson et al. 2008). By definition, resource pulses are episodes of low frequency (rarity), large magnitude (intensity) and short duration (brevity) that result in increased resource availability in space and time (Ostfeld and Keesing 2000; Yang et al. 2008). Possible causes of resource pulses include climatic or environmentally driven events (e.g. Jaksic 2001; Letnic et al. 2005), processes of temporal (e.g. Yang 2004) and spatial (e.g. Novak et al. 2003; Kim et al. 2006) resource accumulation and release, outbreak of population dynamics (e.g. Cooper and Smith 1995; Hoi et al. 2004; Hogstad 2005), or a combination of these events (Yang et al. 2008, 2010).

In recent years, ecologists have started to recognize the ecological importance of resource pulses (Yang et al. 2008, 2010). An increasing number of studies have investigated the influence of resource pulses in food web dynamics and suggest that these phenomena can have significant effects at all ecological levels (from individuals to ecosystems). Primarily, resource pulses affect consumer responses at the individual level, as resident consumers (both mobile specialists and opportunistic) take advantage of available resources; mobile specialists can travel long distances to use these resources and opportunistic residents are sufficiently generalists to include them in their diet (Curran and Leighton 2000; Ostfeld and Keesing 2000; Lithner and Jonsson 2002; Meserve et al. 2003). Numerical responses at the population level can also be observed due to behavioural aggregative responses, increases in reproduction, or a combination of both (Ostfeld and Keesing 2000; Yang et al. 2008). Lastly, resource pulses can also be responsible for indirect effects at the community and ecosystem levels, creating a sequence of direct and indirect bottom-up effects that can be followed by strong delayed top-down effects, including changes in biogeochemical cycles (Ostfeld and Keesing 2000; Yang et al. 2008). Although resource pulses are defined as short duration events, sometimes the ecological effects persist over time (Yang et al. 2008).

Both terrestrial and freshwater ecosystems can experience cross-system pulsed subsidies, although aquatic ecosystems receive pulses from terrestrial ecosystems more frequently (Nowlin et al. 2008).

Leaf litter, wood debris, runoff containing nutrients and sediments, deep water entrainment, atmospheric deposition and terrestrial invertebrates that fall into streams are common examples of this type of subsidies (Baxter et al. 2005; Nowlin et al. 2008). However, movements of resources in the opposite direction (water to land) are also possible and recent studies have demonstrated that streams can export matter, nutrients and energy to the surrounding terrestrial landscape via extreme riverine flood pulses (Sousa et al. 2012), migrations of fish (Moore et al. 2007), lateral spread of nutrients by large herbivores (Bump et al. 2009a; Doughty et al. 2013) and aquatic insect emergence (Henschel et al. 2001; Sabo and Power 2002). Interestingly, studies addressing pulsed subsidies from water to land by IAS are rare. Yet, this topic may have significant importance given that aquatic ecosystems are subjected to many IAS introductions, with bivalves being one of the most invasive faunal groups in these ecosystems (Sousa et al. 2009, 2014).

The Asian clam *C. fluminea* is well recognized for its invasive behaviour, as well as for the ecological and economic impacts it causes (Sousa et al. 2008a, 2014; Crespo et al. 2015). Recently, some studies suggested that periodic massive die-offs of *C. fluminea* occur and the available biomass can function as a resource pulse in the invaded ecosystem (Sousa et al. 2012; Bódis et al. 2014; Ilarri et al. 2015a). This extremely high biomass resulting from massive die-offs of *C. fluminea* may have two fundamental outcomes: (1) during great floods, it is relocated from the river bed to the river bank, and this carrion can be viewed as a resource pulse by transferring significant amounts of nutrients and energy to the adjacent terrestrial food web and (2) during intense droughts, part of this biomass stays in the aquatic realm but other part may also subsidize the adjacent terrestrial food web due to low water levels. Yet, nothing is known about how this subsidy influences the structure and dynamics of the recipient food web. Given the limited understanding on how aquatic IAS may subsidize adjacent terrestrial communities, we used a manipulative field experiment simulating a *C. fluminea* mortality event after floods to assess: (1) possible differences in the structure of the terrestrial invertebrate community between distinct *C. fluminea* densities and over time and (2) possible effects of plots position in the experimental design, where plots that delimited the experiment were assigned as edge plots and the remaining as core plots, which may confirm colonization of terrestrial invertebrates from adjacent areas.

4.2. Material and methods

4.2.1. Study area

The experiment was carried out in the Minho River (NW of Iberian Peninsula), 40 km upstream the mouth of the estuary (42° 04' 28.12'' N, 08° 31' 29.14'' W; Fig. 4.1). *Corbicula fluminea* was first reported in the Minho River in 1989 and now represents more than 95% of the total benthic faunal biomass in the international section of the river and has been responsible for several ecological and economic impacts (Sousa et al. 2008c,d). During major floods (e.g. winters of 2000/2001 and 2009/2010) a significant amount of bivalves was transported from the river bed to the river banks, where the empty shells still remain (for details see Sousa et al. 2012; Ilarri et al. 2015a). When the river flow returned to normal by the end of spring, freshwater bivalve species (*A. anatina*, *P. littoralis*, *U. delphinus* and *C. fluminea*) got stranded on the banks and died, reaching up to 2280 ind. m^{-2} and 10 225 g wet weight. m^{-2} (Sousa et al. 2012), where *C. fluminea* represented more than 99% of the total bivalve biomass. The studied area was therefore selected due to its reliability in reproducing the magnitude of these extreme climatic events in order to understand the importance of massive die-offs of *C. fluminea* as a resource pulse to the terrestrial invertebrate community.

4.2.2. Experimental design

Corbicula fluminea individuals were collected in the Minho River and used later (as dead clams) in a manipulative field experiment. The experiment was conducted approximately 250 m inland of the river bank in a randomized complete block design with eight blocks. Each block contained five plots (1 m^2; delimited with four stakes at the corners) corresponding to five levels of manipulated *C. fluminea* density: 0, 100, 500, 1000 and 2000 ind. m^{-2}, totalizing 40 plots. Of these, 22 plots that delimited the experimental design were assigned as edge plots and the remaining 18 as core plots, and they were considered an additional treatment (Fig. 4.1). Plots were distributed within a grid of ca. 1 m intervals, chosen to minimize habitat variability and inter-plot interactions. The density levels were chosen to reflect a well-documented range of naturally occurring *C. fluminea* densities after die-offs resulting from major floods (Sousa et al. 2012; Ilarri et al. 2015a). A standard biomass (mean ± SD) was used for

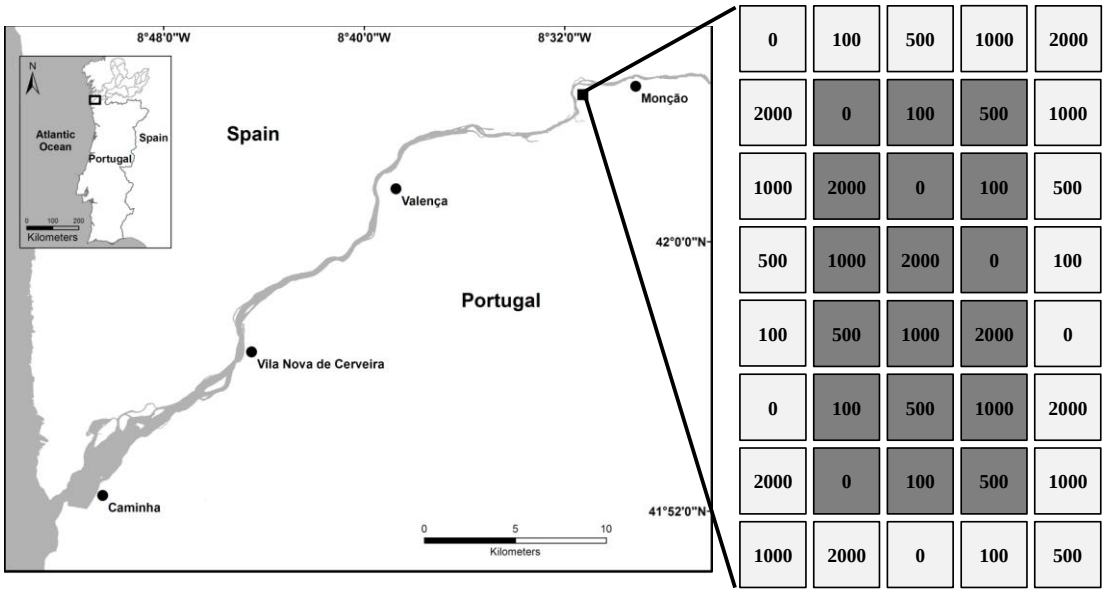

Figure 4.1. Study area showing the selected sampling site in the river bank of the Minho River (NW Iberian Peninsula) and the scheme of the manipulative field experiment. The 0, 100, 500, 1000 and 2000 ind. m^{-2} values are the levels of *C. fluminea* density; light gray corresponds to the edge plots and dark gray to the core plots.

each of the five density levels, accomplished through similar shell lengths (24.45 ± 7.63 mm): 0 ind. m^{-2}, 0 ± 0 g; 100 ind. m^{-2}, 870.7 ± 62.9 g; 500 ind. m^{-2}, 4020.5 ± 189.9 g; 1000 ind. m^{-2}, 7850.3 ± 258.8 g and 2000 ind. m^{-2}, 16,481.5 ± 328.5 g. The experiment lasted 3 months (June to September) and samples were collected 7, 30 and 90 days after *C. fluminea* addition.

Adult Diptera individuals were visually counted in each experimental plot during 1 minute. Other terrestrial invertebrates were censused using two non-baited circular pitfall traps (7 cm diameter) placed at two opposite sides of each experimental plot during 24 h (following a similar methodology as described in Angulo et al. 2011). These pitfall traps provided an index of the relative abundance of invertebrates and have been extensively used in the sampling of terrestrial invertebrates (e.g. Hocking et al. 2009; Angulo et al. 2011). Samples with biological material were sieved through a 500-µm mesh and were preserved in 70% ethanol. The organisms were counted, identified to the species level, whenever possible, and assigned to the following functional groups: carnivores/scavengers, omnivores, herbivores and detritivores (Zahradník and Severa 1981; Chinery 1984; Jones 1985; Barrientos 1987; Tilling 1987; Zahradník 1990; Ilharco 1992; Mingo 1994). To quantify biomass, organisms were oven-dried for 72 h at 60°C and weighed on a precision scale.

4.2.3. Data analysis

All statistical tests were conducted using the PRIMER analytical software (v.6.1.6, PRIMER-E) with PERMANOVA + 1.0.1 add-on (MJ Anderson et al. 2008). PERMANOVA tests the simultaneous response of one or more variables to one or more factors in an ANOVA experimental design on the basis of any distance measure, using permutation methods (Anderson 2001). Prior to PERMANOVA analyses, all variables were normalized without data transformation, and resemble matrices based on the Euclidean distances were calculated (Clarke and Warwick 2001).

Three-way PERMANOVAs (type-III) were used in a three-way crossed design to test for fixed effects of *C. fluminea* densities (five levels: 0, 100, 500, 1000 and 2000 ind. m^{-2}), time (three levels: 7, 30 and 90 days) and position (two levels: core and edge) used as fixed factors on adult Diptera abundance, other terrestrial invertebrates (excluding adult Diptera) abundance, biomass, species richness and Shannon-Wiener diversity index, and on functional groups (carnivores/ scavengers, omnivores, herbivores and detritivores) abundance and biomass. Adult Diptera were analysed separately from the other terrestrial invertebrates given the different sampling approach used (described above).

Species richness and the Shannon-Wiener diversity index were calculated through the DIVERSE analysis (Clarke and Warwick 2001).

In all PERMANOVA tests, the statistical significance of variance (α= 0.05) was tested using 9999 permutations of residuals within a reduced model. When the number of permutations was lower than 150, the Monte Carlo p-value was considered. Three-way PERMANOVA pairwise comparisons were also performed for all PERMANOVA tests.

4.3. Results

4.3.1. Adult Diptera

The relative abundance (mean ± SD) of adult Diptera was highest at day 7 for *C. fluminea* density of 2000 (core: 9.00 ± 2.65, edge: 14.60 ± 5.41) and 1000 (core: 4.67 ± 1.53, edge: 8.40 ± 2.58), followed by 500 (core: 2.75 ± 1.71, edge: 3.00 ± 0.82), 100 (core: 1.00 ± 0.82, edge: 1.50 ± 0.58)

and 0 ind. m^{-2} (core and edge: 0.00 ± 0.00) (Fig. 4.2). Adult Diptera abundance differed significantly between *C. fluminea* density (Pseudo-F = 30.14, $p < 0.001$), time (Pseudo-F = 105.83, $p < 0.001$), and position (Pseudo-F = 5.98, $p \leq 0.01$) (Table S1). Significant interactions between *C. fluminea* density and time (Pseudo-F = 23.12, $p < 0.001$) and between time and position (Pseudo-F = 5.61, $p \leq 0.01$) were also found (Table S1). Pairwise tests indicated that these differences were related to the high abundance observed at day 7, at higher *C. fluminea* densities (1000 and 2000 ind. m^{-2}) and at the plots located on the edge of the experimental design.

4.3.2. Terrestrial invertebrates

A total of 573 individuals belonging to 75 invertebrate taxa were recorded. The most abundant taxa were Formicidae (18.0%), Phalangiidae sp. 1 (10.3%), Sciaridae (8.6%), Microcoryphia (7.7%), Gryllidae (7.2%), Carabidae sp. 1 (5.6%), Staphylinidae (3.1%), Anthomyiidae (3.0%), while the remaining taxa represented 36.5% of the total individuals (Table 1). In terms of biomass, Carabidae sp. 1 (39.3%), Julida (10.7%), Carabidae sp. 5 (8.3%), Gryllidae (7.6%), Carabidae sp. 4 (6.6%), Lycosidae (6.1%), Lucanidae (3.7%) and Araneae sp. 2 (3.0%) were the dominant taxa, while the remaining taxa had a total biomass of 14.7% (Table 4.1).

The relative abundance (mean ± SD) of invertebrates was highest at day 30, at *C. fluminea* density of 2000 ind. m^{-2} and at the core (18.33 ± 9.07), and lowest at day 7, at *C. fluminea* density of 0 ind. m^{-2} and at the edge (0.50 ± 1.00) (Fig. 4.3A). Invertebrate abundance differed significantly between *C. fluminea* density, time and position (Pseudo-F = 2.70, $p \leq 0.01$) (Table S2A). Pairwise tests revealed that these differences were related to the abundance values observed at day 30, at higher *C. fluminea* densities (500, 1000 and 2000 ind. m^{-2}) and at the plots located on the edge of the experimental design.

The highest biomass (mg DW) was obtained at day 30, at *C. fluminea* density of ind. m^{-2} and at the edge (121.29 ± 100.89), and lowest at day 90, at a density level of 0 ind. m^{-2} and at the core (1.58 ± 1.60) (Fig. 4.3B). Significant differences in biomass between *C. fluminea* density (Pseudo-F = 4.29, $p \leq 0.01$) and position (Pseudo-F = 9.93, $p \leq 0.01$) were found (Table S2B). Pairwise tests revealed that these differences were associated with the high biomass observed at higher *C. fluminea* densities (1000 and 2000 ind. m^{-2}) and at the plots located on the edge of the experimental design.

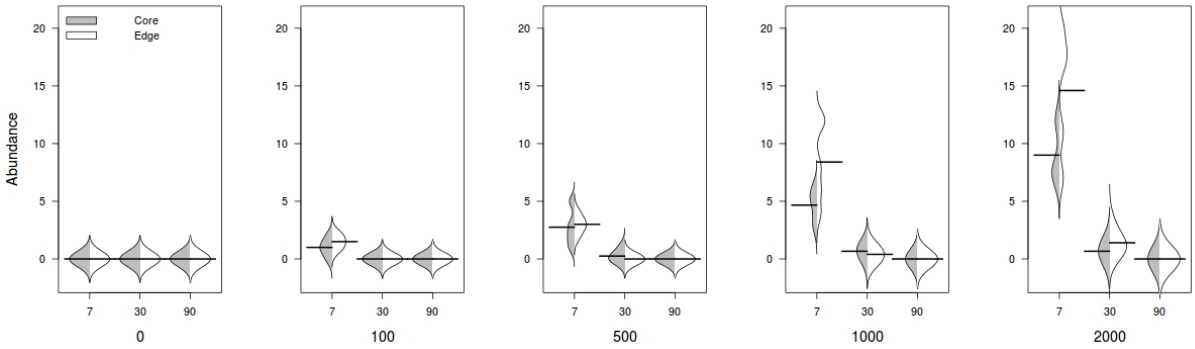

Figure 4.2. The relative abundance (mean ± SD) of adult Diptera in treatments with increasing *C. fluminea* density levels (0, 100, 500, 1000 and 2000 ind. m^{-2}), at three sampling time periods (7, 30 and 90 days) and at different positions (core and edge) of the selected sampling site in the river bank of the Minho River (NW Iberian Peninsula).

The highest species richness was obtained at day 30, at *C. fluminea* density of 500 ind. m^{-2} and at the edge (8.25 ± 1.50), and the lowest richness was found at day 7, at *C. fluminea* density of 0 ind. m^{-2} and at the edge (0.50 ± 1.00) (Fig. 4.3C). Species richness differed significantly between *C. fluminea* density (Pseudo-F = 19.67, $p < 0.001$), time (Pseudo-F = 79.54, $p < 0.001$) and position (Pseudo-F = 6.88, $p \leq 0.01$) (Table S3A). Significant interactions between *C. fluminea* density and time (Pseudo-F = 2.51, $p < 0.05$) and between *C. fluminea* density and position (Pseudo-F = 2.51, $p < 0.05$) were also found (Table S3A). Pairwise tests revealed that higher species richness was observed at day 30, at higher *C. fluminea* densities (500, 1000 and 2000 ind. m^{-2}) and at the plots located on the edge of the experimental design.

The highest values for Shannon-Wiener diversity index were obtained at day 30, at *C. fluminea* density of 500 ind. m^{-2} and at the edge (1.96 ± 0.18), and the lowest values were found at day 7, at *C. fluminea* density of 0 ind. m^{-2} and at the core (0.00 ± 0.00) (Fig. 4.3D). Shannon-Wiener diversity index differed significantly between *C. fluminea* density (Pseudo-F = 16.82, $p < 0.001$), time (Pseudo-F = 51.98, $p < 0.001$) and position (Pseudo-F = 7.94, $p \leq 0.01$), and significant interactions between *C. fluminea* density and position (Pseudo-F = 2.59, $p < 0.05$) were found (Table S3B). Pairwise tests revealed that these differences were associated with the high diversity observed at day 30, at higher *C. fluminea* densities (500, 1000 and 2000 ind. m^{-2}) and at the plots located on the edge of the experimental design.

Table 4.1. Abundance and biomass of the most abundant terrestrial invertebrates of each functional group (carnivores/scavengers, omnivores, herbivores and detritivores).

		Abundance (%)		Biomass (%)
Carnivores/Scavengers				
	Microcoryphia	7.7	Carabidae sp. 1	39.3
	Carabidae sp. 1	5.6	Carabidae sp. 5	8.3
	Staphylinidae	3.1	Carabidae sp. 4	6.6
	Lycosidae	2.4	Lycosidae	6.1
	Trombidiidae	2.3	Araneae sp. 2	3.0
	Araneae sp. 2	2.1	Araneae sp. 1	1.6
	Araneae sp. 1	1.6		
Omnivores				
	Formicidae	18.0	Julida	10.7
	Phalangiidae sp. 1	10.3		
	Julida	2.8		
	Isotomidae	1.4		
Herbivores				
	Gryllidae	7.2	Gryllidae	7.6
	Cicadellidae	2.1	Lucanidae	3.7
	Psyllidae	1.6		
Detritivores				
	Sciaridae	8.6	Isopoda	1.6
	Anthomyiidae	3.0	Calliphoridae	1.3
	Agromyzidae	1.9		
	Chironomidae	1.0		
	Phoridae	1.0		

Analysis of invertebrate functional groups showed that relative abundance of carnivores/ scavengers (represented mainly by the Microcoryphia and Carabidae sp. 1) differed significantly between *C. fluminea* density (Pseudo-F = 8.44, $p < 0.001$), time (Pseudo-F = 28.81, $p < 0.001$) and position (Pseudo-F = 10.10, $p \leq 0.01$), with significant interactions between *C. fluminea* density and time (Pseudo-F = 2.49, $p < 0.05$) (Fig. S1A and Table S4A). Biomass of carnivores/scavengers differed significantly between *C. fluminea* density (Pseudo-F = 3.11, $p < 0.05$), time (Pseudo-F = 4.10, $p < 0.05$) and position (Pseudo-F = 7.31, $p \leq 0.01$) (Fig. S1B and Table S4B). Pairwise tests, for both abundance and biomass, suggested that these differences were associated with the high values observed at day 30 and at higher *C. fluminea* densities (1000 and 2000 ind. m^{-2}). In most cases, the highest values were observed at the plots located on the edge of the experimental design.

The relative abundance of omnivores (represented mainly by the Formicidae and Phalangiidae sp. 1) differed significantly between *C. fluminea* density, time and position (Pseudo-F = 3.93, $p < 0.001$) (Fig.

S1C and Table S5A). Pairwise tests suggested that these differences were mainly related to the high abundance observed at day 30, at higher *C. fluminea* densities (500, 1000 and 2000 ind. m^{-2}) and at the plots located on the core of the experimental design. No significant differences in biomass were detected among *C. fluminea* density (Pseudo-F = 0.72, p = 0.61), time (Pseudo-F = 1.83, p = 0.17) and position (Pseudo-F = 1.87, p = 0.20) (Fig. S1D and Table S5B).

The relative abundance of herbivores (represented mainly by the Gryllidae and Cicadellidae) differed significantly between *C. fluminea* density (Pseudo-F = 3.49, $p \leq 0.01$) and time (Pseudo-F = 19.78, $p < 0.001$) (Fig. S1E and Table S6A). Pairwise tests suggested that these differences were associated with the high abundance observed at day 30 and at higher *C. fluminea* densities (1000 and 2000 ind. m^{-2}). The biomass of herbivores differed significantly between time (Pseudo-F = 3.50, $p < 0.05$) (Fig. S1F and Table S6B). The pairwise tests suggested that these differences were associated with the high biomass observed at day 90.

The relative abundance of detritivores (represented mainly by the Sciaridae and Anthomyiidae) differed significantly between *C. fluminea* density (Pseudo-F = 3.49, $p \leq 0.01$) and time (Pseudo-F = 10.29, $p < 0.001$) (Fig. S1G and Table S7A). Pairwise tests suggested that these differences were related to the high abundance observed at day 30 and at higher *C. fluminea* densities (500, 1000 and 2000 ind. m^{-2}). No significant differences in detritivores biomass were detected among *C. fluminea* density (Pseudo-F = 2.00, p = 0.09), time (Pseudo-F = 2.00, p = 0.14) and position (Pseudo-F = 0.98, p = 0.33) (Fig. S1H and Table S7B).

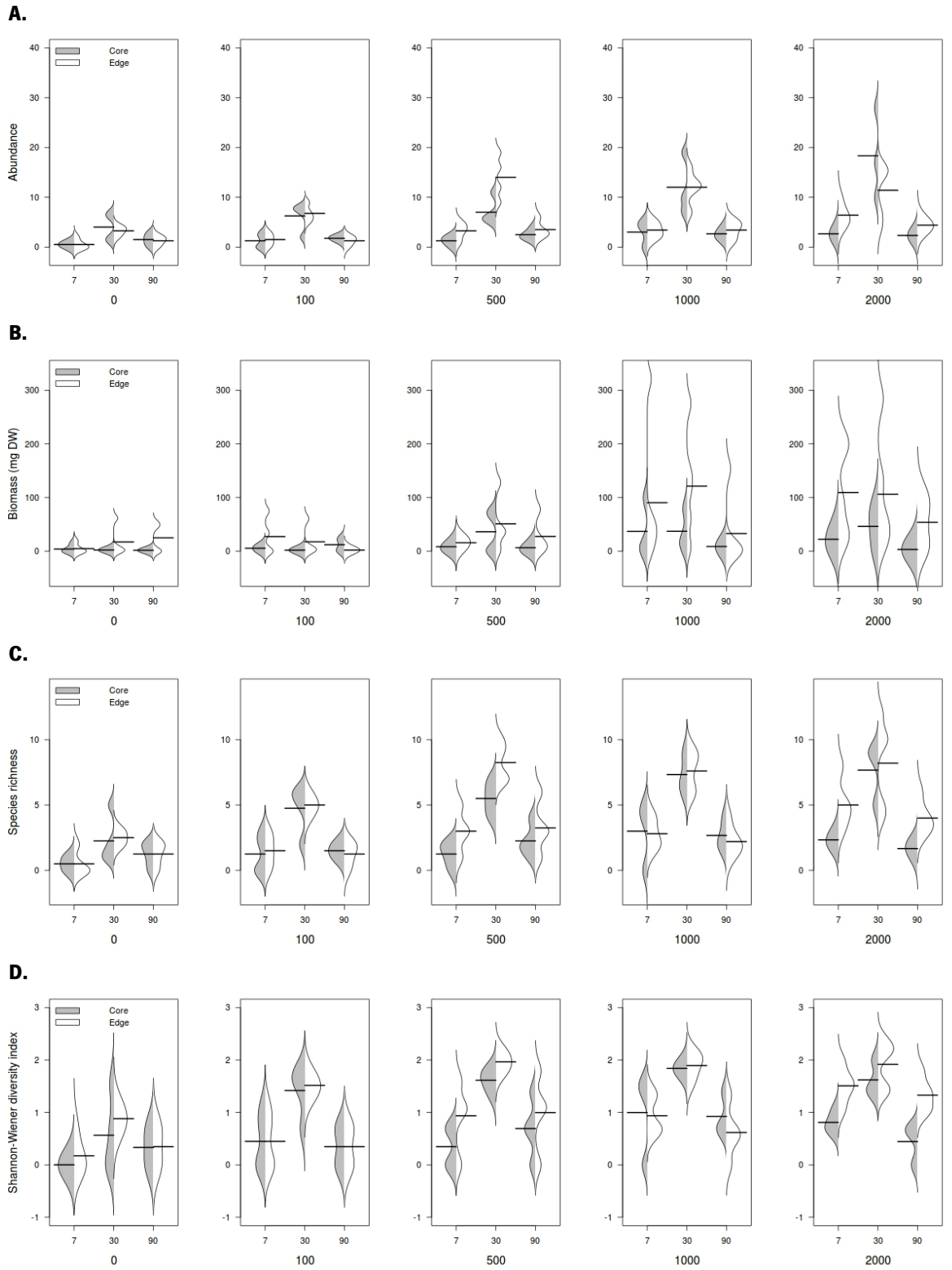

Figure 4.3. Terrestrial invertebrates' (mean ± SD) (A) relative abundance, (B) biomass (mg DW), (C) species richness and (D) Shannon-Wiener diversity index in treatments with increasing *C. fluminea* density levels (0, 100, 500, 1000 and 2000 ind. m^{-2}), at three sampling time periods (7, 30 and 90 days) and at different positions (core and edge) of the selected sampling site in the river bank of the Minho River (NW Iberian Peninsula).

4.4. Discussion

4.4.1. Corbicula fluminea as a resource pulse to terrestrial invertebrates

Earlier studies have shown that *C. fluminea* may be subjected to massive mortalities during extreme climatic events (floods and droughts) and the resulting biomass can be seen as a rare, high magnitude event, which may function as a resource pulse to consumers (Mouthon and Daufresne 2006; Werner and Rothhaupt 2008; Ilarri et al. 2011; Sousa et al. 2012; Bódis et al. 2014). However, those studies were merely descriptive, just assessed the *C. fluminea* mortality and speculated about the possible effects on the recipient community. By contrast, our manipulative experiment was the first to assess the effects of massive *C. fluminea* mortality after floods on the terrestrial invertebrate community. Our results revealed that the abundance, biomass, richness and diversity of terrestrial invertebrates responded to the *C. fluminea* addition and effects depended on density levels, time and position of the plots in the experimental design. Unfortunately, there are very few studies to which our results can be compared, and we are not aware of a similar study assessing the effects of a massive mortality event of an aquatic IAS on the structure of a terrestrial invertebrate community. We were only able to partly compare our results with a study by Hocking et al. (2009) addressing the colonization of terrestrial invertebrates on salmon carcasses. Even considering that they were dealing with a native fish and not an invasive bivalve and that habitat characteristics were fairly different, some similarities in the colonization process did existed. Indeed, in that study carried out in Clatse and Neekas rivers (British Columbia, Canada), authors were able to identify 60 species of terrestrial invertebrates out of 36 families on salmon carcasses (Hocking et al. 2009). The community was dominated by Coleoptera (21 spp.), Diptera (10 spp.) and Hymenoptera (6 spp.), and consisted mainly of saprophagous, predators and detritivores. In our 3-months long study, we were able to identify 75 taxa, 16 belonging to Diptera, 12 to Coleoptera and 5 to Hymenoptera, which consisted mainly of carnivores/scavengers, detritivores and omnivores. In addition to these, taxa belonging to Aranea (9 spp.) and Hemiptera (6 spp.) were also present.

In our study, the abundance of adult Diptera progressively increased with higher *C. fluminea* density; so a positive relationship between *C. fluminea* density and adult Diptera abundance was established. Strong variations were also detected over time as the highest abundance was observed at day 7,

declined by day 30 and by day 90 no measurable abundance could be accounted for. It is known that necrophagous flies are the first animals to arrive during a decomposition process consuming the carrion at the initial stages of decomposition and playing a fundamental role in the breakdown of dead organic matter and in nutrient cycling (Prado e Castro et al. 2012). Diptera easily move from one site to another usually without any particular pattern; yet, our results showed that Diptera responded to position as evidenced by the higher values in plots located on the edge of the experimental design. The higher abundance obtained on the edge may be related to the colonization process from adjacent areas and in this way flies were more abundant in plots near the edge than in the core. However, and given the great mobility of these organisms, this situation may be related to other factors such as temperature. In fact, the plots positioned at the edge had more shadow than the plots in the core and so temperature could be slightly different contributing to a possible difference in the colonization process of certain organisms, including Diptera. Unfortunately, we did not monitor temperature throughout the experiment or even between core and edge plots, and so future investigations should be conducted to clarify this aspect.

Our experiment also showed that abundance, biomass, richness and diversity of terrestrial invertebrates significantly responded to the *C. fluminea* addition as the highest values were observed in *C. fluminea* densities higher than 500 ind. m^{-2}. The abundance and biomass of the functional group carnivores/scavengers were higher when *C. fluminea* densities were higher than 500 ind. m^{-2}, but for omnivores, herbivores and detritivores only the abundance was higher and no effect was detected for biomass. Although the decomposition process is not the same for all species, usually it follows a sequential stage (Payne 1965; Braig and Perotti 2009). Following necrophagous flies and others initial decomposers, predators/scavengers can also respond rapidly to the increase in an unusual food resource. Decomposers and detritivores complete this process by consuming the remains left by scavengers (Prado e Castro 2011). Given their opportunistic behaviour, omnivores can also participate in this process. Although it is uncommon to detect herbivores during decomposition (Payne 1965), our study showed that some species might be present. However, their occurrence may be accidental given that herbivores are anatomically and physiologically not adapted to consume carrion. So, it is important to note that the presence of a particular organism or group cannot be synonymous to that the resource has been consumed and/or assimilated. On the other hand, it may be that some of these herbivores used the accumulation of carrion and shells to deposit their eggs. Overall, the response to higher

density of *C. fluminea* may be explained by the species-energy theory (Wright 1983), where higher resource availability increases the abundance and richness of local communities. Indeed, the recipient terrestrial community is characterized by limited resources and so the availability of this massive carrion may provide an important resource to consumers, allowing an increase in abundance, biomass, richness and diversity.

Our experiment showed that abundance, richness and diversity of terrestrial invertebrates significantly responded to time, with higher values obtained 30 days after the addition of dead clams. In the case of carnivores/scavengers and herbivores, both abundance and biomass were higher at day 30. For omnivores and detritivores only the abundance was higher at day 30. As mentioned above, the decomposition is a continuous process but it can be divided into different stages. In a decaying corpse, many species colonize it only during a limited period of time, arriving in a more or less predictable sequence, and representing an ecological succession (Payne 1965; Rodriguez and Bass 1983; Smith 1986). It is known that colonization by terrestrial invertebrates is not immediate and occurs in the later stages (Prado e Castro 2011). For example, many carrion beetles occurred on carcasses at a later, drier stage of decomposition (Smith 1986). Also Prado e Castro et al. (2013) demonstrated that the highest abundance and richness of Coleoptera was found between 7 and 20 days (advanced decay stage) in spring and summer conditions.

Our experiment also showed that terrestrial invertebrate biomass, richness and diversity varied according to the position, with highest values observed in the plots located on the edge of the experimental design. This may suggest that most organisms migrate from adjacent areas to consume the available resources. In this way, and considering that most of the captured organisms (excluding adult Diptera) have low mobility it is plausible that plots located on the edge of the experimental design have higher values than plots located on the core. This situation was more pronounced on carnivores/scavengers, as the abundance and biomass of this functional group was higher in the plots on the edge. As explained above, temperature may also have had some influence on these results since it is possible that plots on the edge had lower temperature due to higher shadow.

4.4.2. Ecological significance

Several studies have demonstrated that freshwater and terrestrial ecosystems are not independent and they are open to the movement of materials, nutrients and energy (Polis et al. 1997). Examples of cross-habitat flows between freshwater and terrestrial ecosystems have often described the movement of materials, nutrients and energy from land to water (but see Ben-David et al. 1998; Bastow et al. 2002; Bartz and Naiman 2005; Baxter et al. 2005). Much less attention has been devoted to rare phenomena such as resource pulses resulting from massive die-offs of invasive aquatic species to the terrestrial community. Indeed, the few studies that have addressed the influence of IAS on trophic subsidies assessed how these species decrease the emergence of insects to riparian consumers and how this translates in a reduction of consumers such as amphibians, birds, mammals and spiders (Finlay and Vredenburg 2007; Epanchin et al. 2010; Benjamin et al. 2011) or how invasive predators disrupt nutrient subsidies vectored by seabirds from sea to land (Croll et al. 2005; Maron et al. 2006). Although some studies assessed how an IAS reduces the magnitude of a possible subsidy, we are not aware of a study similar to ours aimed at assessing how an invasive clam (or other invasive aquatic organism) could function as an important resource pulse to the adjacent terrestrial community.

Since global warming is expected to increase the occurrence of extreme climatic events, in particular an intensification of the global water cycle with a consequent increase in flood risk (Milly et al. 2002), including in the North of Portugal (Santos et al. 2015), it is important to assess how these rare extreme climatic events and IAS could interact and lead to resource pulses (Yang et al. 2008; Diez et al. 2012). Recently, Fey et al. (2015) showed that the magnitude of massive mortality events has been intensifying for some groups of animals, with extreme examples comprising the removal of more than 90% of the population producing millions of tons of dead biomass in a single event. These massive die-offs have also been reported in invasive bivalves with values comprising dozens of kilograms per m^2 (e.g. Sousa et al. 2012; Bódis et al. 2014), but no data were available about possible changes in community structure and ecosystem functioning. Our study provides the first empirical evidence on how massive die-offs of *C. fluminea*, as a consequence of extreme floods, can cause substantial pulsed enrichment of nutrients and energy to the adjacent terrestrial ecosystems, with clear effects on the invertebrate community. As shown in this study, the availability of bivalves' carrion can trigger effects across multiple trophic levels, as the increase in resources increase the abundance, biomass and

species richness of consumers, particularly of adult Diptera and carnivores/scavengers. Although not quantified in this study, it is possible that birds, mammals and other organisms may also respond to the exogenous input of nutrients and energy. In the same way, and although a great part of the carrion was consumed aboveground, it is possible that significant levels of biomass resulting from these massive die-offs enter the detrital food web driving changes in microbial biomass and nutrient cycles (Yang 2004). Therefore, future studies should also quantify the effects of massive die-offs on the structure of microbial communities.

4.5. Conclusions

This study provides the first demonstration of an aquatic-to-terrestrial connection resulting from a resource pulse to terrestrial invertebrates generated by a freshwater invasive clam. Contrary to the usual low quality subsidies (e.g. leaf litter and wood debris) moved from terrestrial to aquatic ecosystems, our example comprises a high quality subsidy moving in the water to land direction after an extreme climatic event. Given the high density and biomass attained by several aquatic invasive bivalves worldwide (e.g. *C. fluminea*, *D. polymorpha*, *L. fortunei* and *S. woodiana*) and the predicted increase in the number and intensity of extreme climatic events (floods and droughts) the ecological importance of these massive die-offs, including other invasive faunal groups such as fish, and the subsequent response of organisms other than invertebrates should be further investigated.

Chapter 5

Effects of invasive aquatic carrion on soil chemistry and terrestrial microbial communities

Novais A, Pascoal C, Sousa R (accepted) Effects of invasive aquatic carrion on soil chemistry and terrestrial microbial communities.
Biological Invasions

Abstract

Carrion plays a crucial role in the recycling of nutrients and organic matter in ecosystems. Yet, despite their ecological importance, studies addressing the relevance of carrion originated from invasive alien species (IAS) in the interface between aquatic and terrestrial ecosystems are uncommon, especially those assessing belowground effects. In this study, we carried out a manipulative experiment to assess the impact of massive mortalities of the Asian clam *Corbicula fluminea* (Müller, 1774) as a carrion subsidy evaluating possible effects on the terrestrial soil chemistry and the structure of a microbial (bacteria and fungi) community. We placed five levels of *C. fluminea* density (0, 100, 500, 1000 and 2000 ind. m^{-2}) and samples were collected 7, 30 and 90 days after clams' addition. The results revealed that *C. fluminea* carrion have a significant effect belowground, specifically on nutrients content (mainly NH_4^+, NO_2^-, NO_3^- and PO_4^{3-}), fungal biomass and fungal and bacterial diversity. Given the predicted increase and intensification of extreme climatic events and the widespread distribution of several aquatic IAS (including bivalve species such as *C. fluminea*) the ecological importance of these massive mortalities (and resulting carrion) cannot be ignored because they may affect microbial communities with significant impacts on nutrient cycling, even in adjacent terrestrial habitats.

5.1. Introduction

Decomposition of detritus plays a crucial role in the recycling of nutrients and organic matter (Swift et al. 1979; Moore et al. 2004). Defined as any resource of non-living organic matter, detritus is considered the basal trophic level of many terrestrial and aquatic food webs (Swift et al. 1979; Moore et al. 2004; Benbow et al. 2015). In terrestrial ecosystems, detritus may consist of plant-derived matter (e.g. leaf litter, dead wood, root exudates), dead microbes, faecal matter and animal tissue (carrion) (Swift et al. 1979). Plant-derived detritus comprises the majority of the resources that undergo decomposition in terrestrial ecosystems but they are nutrient poor and very recalcitrant (Swift et al. 1979; Carter et al. 2007). On the other hand, carrion is much more nutrient-rich and decomposes at much faster rates than plant detritus, and, as such, its role in nutrient cycling may be very relevant (Swift et al. 1979; Barton et al. 2013). Indeed, carrion decomposition is usually associated with the activity of microbes, invertebrate and vertebrate detritivores and scavengers (Carter et al. 2007; Barton et al. 2013). In one hand, the combined activity of microbes and invertebrates increases the nutrients release from the carrion into the soil; on the other hand, vertebrates may reduce this contribution to the soil by direct consumption or dispersion (Carter et al. 2007; Barton et al. 2013). In terrestrial ecosystems where carrion releases nutrients into the soil, plants may ultimately mobilize these nutrients entering the belowground detrital pathway (Moore et al. 2004; Carter et al. 2007). Despite its potential importance, only very recently scientists have started to acknowledge the role of carrion in the overall decomposition process (DeVault et al. 2003; Carter et al. 2007; Wilson and Wolkovich 2011).

Detrital inputs enter any ecosystem via allochthonous or autochthonous sources (Moore et al. 2004). Allochthonous inputs are resources that originate in one habitat but move into another, while autochthonous inputs originate and are consumed in the same habitat (Polis et al. 1997). Many ecosystems experience spatial subsidies as regular seasonal events, but subsidies can also result from sporadic episodes of resource superabundance, named resource pulses (Ostfeld and Keesing 2000; WB Anderson et al. 2008; Yang et al. 2008). Examples of resource pulses include periodical cicadas' emergence in North American forests (e.g. Yang 2004, 2008), El Niño rainfalls in arid ecosystems (e.g. Polis et al. 1997; Meserve et al. 2003; Letnic et al. 2005), seed or fruit mast events (e.g. Wolff 1996; Curran and Leighton 2000), and massive spawning events by migratory fish (e.g. Wold and

Hershey 1999; Yanai and Kochi 2005). Despite their ecological importance, these studies focused mostly on aboveground processes and just a few assessed belowground effects (Yang 2004; Yang et al. 2010). For example, Yang (2004) investigated the belowground effects of cicada massive mortalities and reported increases on nitrogen availability and microbial biomass in forest soils and on plants growth and reproduction.

Terrestrial and freshwater ecosystems can receive both autochthonous and allochthonous subsidies, although autochthonous inputs are more common in terrestrial ecosystems and allochthonous inputs in freshwater ecosystems (Nowlin et al. 2008). Due to the geographic position of freshwater ecosystems in the landscape, usually the allochthonous inputs are in the land-water direction (Shurin el at. 2006). However, some studies have verified that freshwater ecosystems can also transfer resources to the adjacent terrestrial ecosystems. Aquatic insect emergence (Henschel et al. 2001; Sabo and Power 2002), lateral spread of nutrients by large herbivores (Bump et al. 2009a; Doughty et al. 2013), migrations of fish (Moore et al. 2007) and extreme riverine flood pulses (Junk et al. 1989; Sousa et al. 2012) are some examples. In addition, and since freshwater ecosystems are subject to numerous introductions of IAS, allochthonous inputs in the water-land direction mediated by IAS may also occur (Bódis et al. 2014). Nevertheless, very few studies report these phenomena and even less assess their possible ecological impacts (Sousa et al. 2014). Recently, Novais et al. (2015a) found that the biomass resulting from massive mortalities of the Asian clam *C. fluminea* functions as a resource pulse to aboveground consumers, namely terrestrial invertebrates. Interestingly, abundance, biomass, richness and diversity of terrestrial invertebrates responded positively to *C. fluminea* carrion addition and clear temporal differences were also detected (Novais et al. 2015a). Similarly to the aboveground effects, *C. fluminea* carrion may also result in significant belowground effects with possible changes on soil chemistry and terrestrial microbial communities.

Given the limited understanding of carrion in belowground processes, mainly carrion derived from IAS, we carried out a manipulative experiment under natural conditions simulating a *C. fluminea* mortality event. It is important to mention that this experiment is part of a larger study that aimed at understanding the impact of massive mortalities of *C. fluminea* as a resource pulse to terrestrial communities. The first part of the study assessed possible effects on a terrestrial invertebrate community (Novais et al. 2015a). Here we assessed possible effects on soil chemistry and on the structure of a terrestrial microbial community. We hypothesized that *C. fluminea* carrion would increase

nutrients content and the biomass and diversity of a microbial (fungi and bacteria) community; however, this increase would be time dependent.

5.2. Material and methods

5.2.1. Study area and experimental setup

The Minho River (NW of Iberian Peninsula) was selected to carry out this experiment since in recent years massive mortalities of *C. fluminea* after extreme climatic events have been reported (see Ilarri et al. 2011; Sousa et al. 2012). The selected area is 40 km upstream the river mouth and approximately 250 m inland in the left river bank (42° 04' 28.12'' N, 08° 31' 29.14'' W). Although relatively further inland, earlier data (Ilarri et al. 2015a) confirm the reliability of this site in reproducing the magnitude of massive mortalities of *C. fluminea* after a great flood. Indeed, during the 2001 flood a great accumulation of dead *C. fluminea* [average (± SD) density values of 2367.5 ± 1023.90 ind. m^{-2}] was reported in the studied area (Ilarri et al. 2015a).

In the last decades, several IAS were introduced in the downstream area of the Minho River with *C. fluminea* being especially problematic (for details see Sousa et al. 2005, 2007a, 2008c,e; Costa-Dias et al. 2010; Mota et al. 2014). Currently, the presence of *C. fluminea* dominates the benthic community, contributing with more than 95% to the total benthic biomass in the Minho River international section (Sousa et al. 2008c,d). During the winters of 2000/2001 and 2009/2010 major floods occurred in the Minho River and a substantial quantity of bivalves (*C. fluminea* and other native species such as *P. littoralis*, *U. delphinus* and *A. anatina*; for details see Sousa et al. 2012; Ilarri et al. 2015a) was moved to the adjacent river banks. These bivalves suffer massive mortalities when water levels return to normal at the end of spring/beginning of summer. For example during the major floods of 2009/2010, Sousa et al. (2012) reported mean density and biomass values of dead bivalves of 1043 ind. m^{-2} and 5726 g wet weight. m^{-2}, respectively, along five sites on the left bank of the Minho River, where *C. fluminea* represented approximately 99% of the total biomass found.

In order to assess the possible effects of the massive mortalities of *C. fluminea* on soil chemistry and on the terrestrial microbial community a manipulative field experiment was conducted. *C. fluminea*

individuals were collected 48 h before the experiment, frozen and posteriorly used in the experiment using a randomized complete block design with three blocks. Each block contained five 1 m^2 plots corresponding to five levels of manipulated *C. fluminea* density: 0, 100, 500, 1000 and 2000 ind. m^{-2}. These levels were selected to mimic a range of *C. fluminea* densities observed when massive mortalities occur resulting from major floods (Sousa et al. 2012; Ilarri et al. 2015a). In order to minimize inter-plot interactions and habitat variability, plots were distributed within a grid of c.a. 1 m interval. The experiment lasted 3 months (June to September 2013) and samples were collected 7, 30 and 90 days after *C. fluminea* addition. A more detailed description of the experimental design can be found in Novais et al. (2015a).

Surface soil samples (1 cm depth) were collected with a small core (3-5 cm^2) for nutrient assessment, fungal biomass quantification and analysis of microbial (fungal and bacterial) diversity. Multiple surface soil samples in each plot were randomly collected, homogenized (mixed and a small sub-sample randomly taken) and deep frozen at -80°C.

5.2.2. Soil chemistry characterization

Concentrations of organic C, total nitrogen (N), NH_4^+, NO_2^-, nitrate (NO_3^-), PO_4^{3-}, Ca and K were measured in Centro de Apoio Científico e Tecnolóxico á Investigación (CACTI), University of Vigo, Vigo (Spain) following standard procedures. Concentrations of organic C and total N were quantified by dry combustion using a LECO CN 2000. Concentrations of NH_4^+, NO_2^-, NO_3^- and PO_4^{3-} were quantified by standard colorimetric methods using a Bran Lubbe continuous flow auto analyzer (Brand Luebbe AA3) after an extraction in KCl. Finally, concentrations of Ca and K were quantified by inductively coupled plasma-atomic emission spectrometry (ICP-OES Optima 4300).

5.2.3. Terrestrial microbial community characterization

Fungal biomass was estimated from 1.5 g of soil from each replicate by ergosterol quantification, following Pascoal and Cássio (2004). Lipids extraction was performed by heating (30 min at 80°C) the sample in 10 mL of 0.8% KOH-methanol and the resulting extract was partially purified by solid-phase extraction (Sep-Pak cartridges, Waters, Milford, MA, USA). Ergosterol was quantified by high-

performance liquid chromatography (Beckmann Gold System, Brea, CA, USA) using a LiChrospher RP18 column (250×4 mm, Merck), where the system ran isocratically with methanol as mobile phase (1.4 mL min^{-1}, 33°C). Ergosterol was detected at 282 nm and its concentration was estimated based on a standard curve of ergosterol (Sigma) in isopropanol.

For microbial diversity assessment, DNA was extracted from 200 mg of soil using a DNA extraction kit (PowerSoil DNA Isolation Kit, MoBio Laboratories, Carlsbad, CA, USA), following the manufacturer's instructions. The ITS2 region of fungal rDNA and the V3 region of bacterial 16S rDNA were amplified with the primer pairs ITS3GC/ITS4 and 338F_GC/518R, respectively (following Duarte et al. 2010).

For polymerase chain reaction (PCR) 2x of Dream GoTaq® Green Master Mix (Promega), 0.4 μM of each primer and 1 μL of DNA were used in a final volume of 25 μL. A MyCycler Thermal Cycler (BioRad Laboratories, Hercules, CA, USA) was initially used for amplification with a denaturation for 2 minutes at 95°C, 36 cycles of denaturation for 30 seconds at 95°C, primer annealing for 30 seconds at 55°C and extension for 1 minute at 72°C, and a final extension for 5 minutes at 72°C (following Duarte et al. 2010).

The DGGE analysis was performed using a DCodeTM Universal Mutation Detection System (BioRad Laboratories, Hercules, CA, USA). For fungi and bacteria, samples of 700 ng from the amplified DNA products with 380-400 bp (ITS3GC/ITS4) and 200 bp (338F_GC/518R), respectively, was loaded on 8% (w/v) polyacrylamide gel in 1x Tris-acetate-EDTA (TAE) with a denaturing gradient from 30 to 70% (100% denaturant corresponds to 40% formamide and 7 M urea). All gels were run at 55 V, 56°C for 16 h and then were stained with 1× of GelStar (Lonza) for 10 minutes. Gel images were captured under UV light using a ChemiDoc XRS (BioRad).

5.2.4. Data analysis

Two-way PERMANOVA (type-III) were used in a two-way crossed designed to test for fixed effects of *C. fluminea* density (five levels: 0, 100, 500, 1000 and 2000 ind. m^{-2}) and time (three levels: 7, 30 and 90 days) on fungal biomass and nutrients content. Variables were standardized without transformation prior to PERMANOVA analyses, with the exception of NO_3^- and PO_4^{3-} that were log (X+1) transformed. Similarity matrices were also calculated using Euclidean distances (Clarke and Warwick 2001).

For each group of microbes, DGGE gels were aligned and the relative intensity of the band was analyzed with BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium). Each DGGE band was considered one OTU. Differences in the structure of microbial (fungal and bacterial) community were tested by nMDS ordination analysis followed by the two-way PERMANOVAs (type-III), with the same design as described above. Variables were standardized without transformation prior to nMDS ordination analyses and similarity matrices were calculated using the Bray Curtis similarity index (Clarke and Warwick 2001).

In PERMANOVA tests, the statistical significance of variance (α = 0.05) was tested using 9999 permutations of residuals within a reduced model. When the number of permutations was <150, the Monte Carlo p-value was considered. PERMDISP was used in all data to test the homogeneity of multivariate dispersions.

PRIMER analytical software (v.6.1.6, PRIMER-E) with the PERMANOVA + 1.0.1 add-on (Anderson 2001; MJ Anderson et al. 2008) was used for all statistical tests described above.

5.3. Results

5.3.1. Soil chemistry characterization

The results for organic C and total N (%), NH_4^+, NO_2^-, NO_3^-, PO_4^{3-}, Ca and K (mg kg^{-1}) in the soil are shown in Fig. 5.1 and Table S8. Concentrations of NH_4^+ and NO_3^- differed significantly between *C. fluminea* density (Pseudo-F = 15.51, p = 0.01 and Pseudo-F = 4.99, p = 0.01, respectively) and time (Pseudo-F = 6.31, p = 0.01 and Pseudo-F = 42.87, p = 0.01, respectively) (Fig. 5.1 C, E and Table S8). Carbon and NO_2^- differed significantly only between time (Pseudo-F = 3.95, p = 0.03 and Pseudo-F = 4.74, p = 0.01, respectively), and PO_4^{3-} only between *C. fluminea* density (Pseudo-F = 5.66, p = 0.01) (Fig. 5.1 A, D, F and Table S8). No significant differences in N, Ca and K were detected among the *C. fluminea* density and time (Fig. 5.1 B, G, H and Table S8).

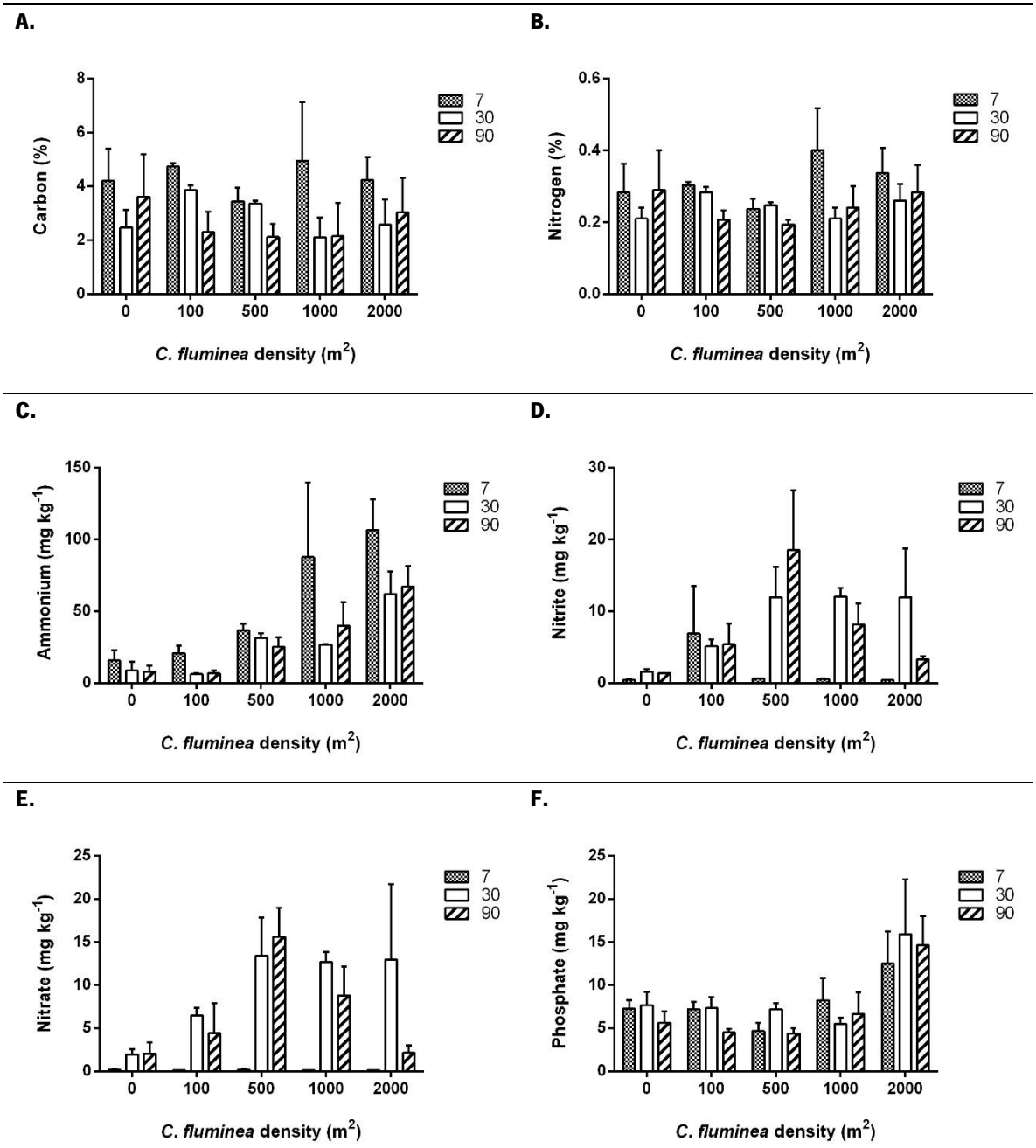

Figure 5.1. Mean (± SEM) values of organic C (A), total N (B) (%), NH_4^+ (C), NO_2^- (D), NO_3^- (E), PO_4^{3-} (F), Ca (G) and K (H) (mg kg^{-1}) at different densities of *C. fluminea* (0, 100, 500, 1000 and 2000 ind. m^{-2}) and sampling times (7, 30 and 90 days).

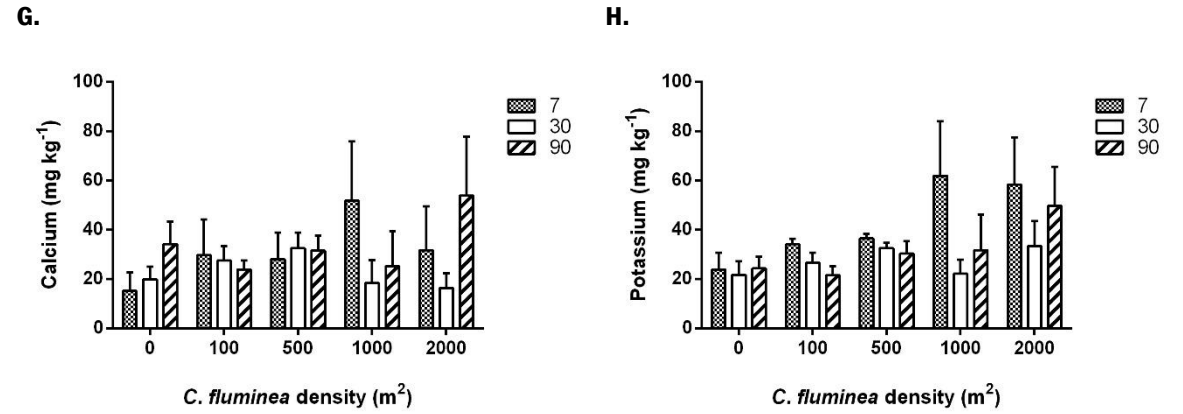

Figure 5.1. Continued.

5.3.2. Terrestrial microbial community

5.3.2.1. Fungal biomass

The mean (± SEM) ergosterol concentration was highest at *C. fluminea* density of 2000 ind. m^{-2} (14.78 ± 4.65 µg g^{-1} soil) at day 7, and lowest at *C. fluminea* density of 1000 ind. m^{-2} at day 90 (2.08 ± 0.91 µg g^{-1} soil) (Fig. 5.2). Ergosterol concentration differed significantly only between *C. fluminea* density (Pseudo-F = 3.10, p = 0.02).

5.3.2.2. Microbial diversity

Fungal taxon richness based on DGGE OTUs varied from 13 to 23 OTUs and showed a tendency to increase with *C. fluminea* densities and time (not shown). The nMDS ordination based on the fungal community is shown in Fig. 5.3A and significant differences in *C. fluminea* density (Pseudo-F = 1.92, p = 0.01) and time (Pseudo-F = 2.92, p = 0.01) were detected.

Bacterial taxon richness based on DGGE OTUs varied from 7 to 15 OTUs and had a tendency to increase with *C. fluminea* densities and time (not shown). The nMDS ordination based on the bacterial community is shown in Fig. 5.3B and significant differences in *C. fluminea* density (Pseudo-F = 3.08, p = 0.01) and time (Pseudo-F = 3.37, p = 0.01) were detected.

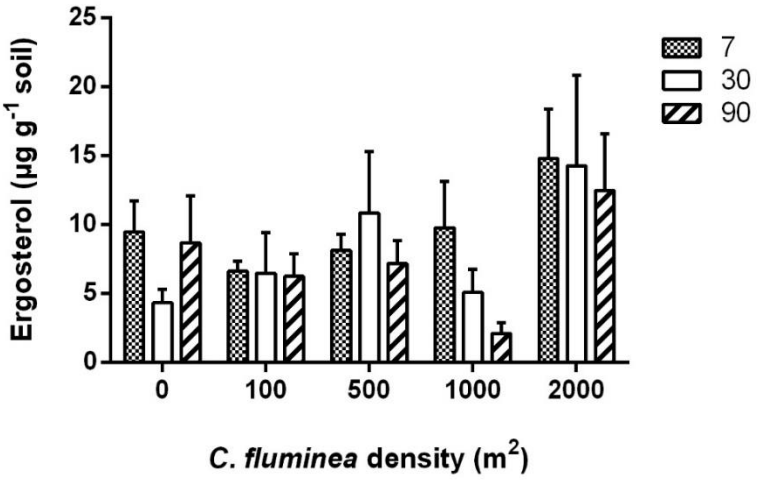

Figure 5.2. Mean (± SEM) values of ergosterol concentration (µg g^{-1} soil) at different densities of C. fluminea (0, 100, 500, 1000 and 2000 ind. m^{-2}) and sampling times (7, 30 and 90 days).

5.4. Discussion

Carrion is a higher quality resource that can have significant effects on soil properties (e.g. nutrients content) and biological communities (Carter et al. 2007; Barton et al. 2013). Nutrients entering the soil through the releases of fluids and transfer of carrion tissues are posteriorly recycled by belowground microbial decomposers (Barton et al. 2013). In the particular case of *C. fluminea*, the carrion resulting from massive mortalities can release some nutrients into the soil via leaching of the shells and decomposition of the soft tissues, also having a significant effect on fungal biomass and diversity of microbial communities.

5.4.1. Effects on soil chemistry

Corbicula fluminea shells are predominantly made of calcium carbonate ($CaCO_3$) in the crystal form of aragonite (Spann et al. 2010) but also contain trace amounts of many other chemical elements such as Na, Mg, Al, P, S, Cl, and K (Eyster 1986). Although we expected that Ca and K would differ significantly between *C. fluminea* density and time, no differences were detected (Fig. 5.1 G and H). Considering that the manipulative experiment took place during the summer, which is often

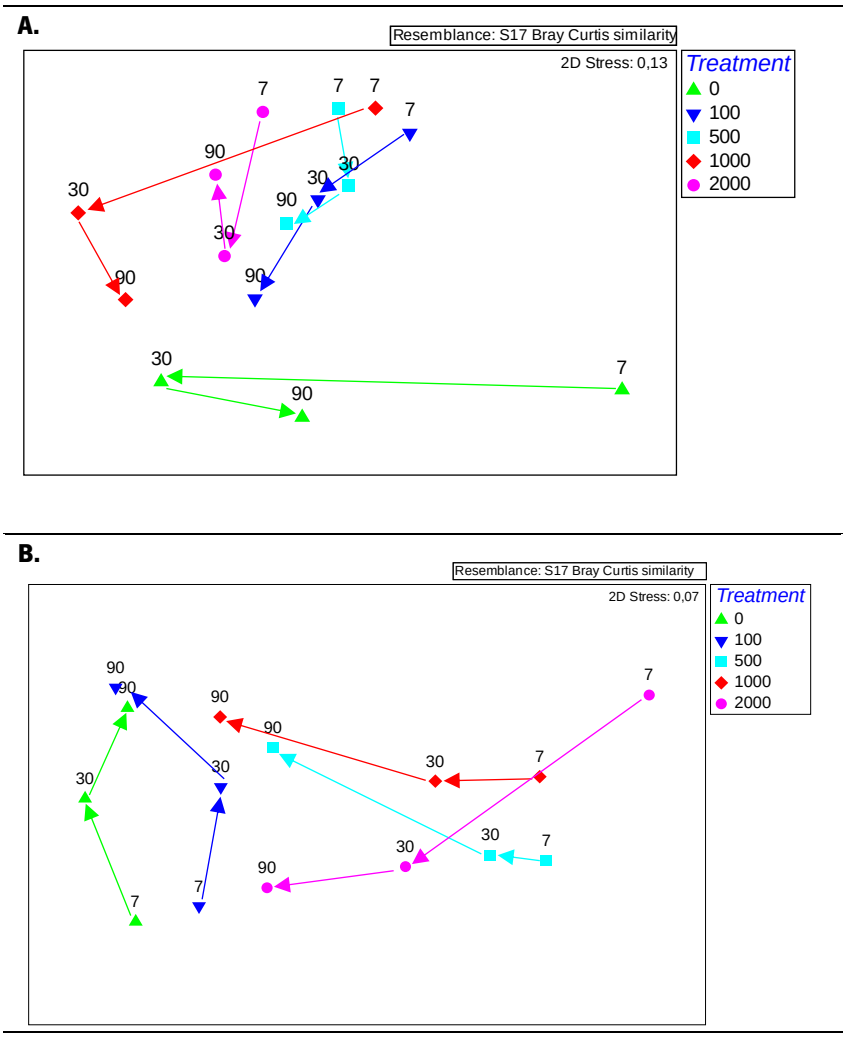

Figure 5.3. Non-metric multidimensional scaling (nMDS) plot of fungal (A) and bacterial (B) community at different densities of *C. fluminea* (0, 100, 500, 1000 and 2000 ind. m^{-2}) and sampling times (7, 30 and 90 days).

characterized by high temperatures and low precipitation in the study area, it is possible that the shells demineralization/leaching process was not enough for these nutrients to accumulate in the soil. In addition, the 3 months' duration of the experiment was probably insufficient to detect these differences. Nitrogen is present in the environment in a wide variety of chemical forms and it is one of the main constituents of many biopolymers, such as amino and nucleic acids of living organisms (Cammack et al. 2015). Thus, during the decomposition of *C. fluminea* carrion, we expected the release of inorganic N into the soil. Although the percentage of total N did not differ between *C. fluminea* density and time, significant differences in the N chemical forms, such as NH_4^+, NO_2^- and NO_3^- were detected (Fig. 5.1 B, C, D and E).

The NH_4^+ content significantly responded to *C. fluminea* addition as the highest values were observed in densities higher than 500 ind. m^{-2} and variations over time were also detected as the highest value was obtained at day 7 and declined at days 30 and 90 (Fig. 5.1 C). In contrast, the values of NO_2^- and NO_3^- were low at day 7 independently of *C. fluminea* density, with the exception of 100 ind. m^{-2} in NO_2^-. At day 30, both NO_2^- and NO_3^- values progressively increased mainly for the treatments containing densities of *C. fluminea* higher than 100 ind. m^{-2}. Lastly, at day 90, the values of NO_2^- and NO_3^- remained high for the density of 500 ind. m^{-2}, but progressively decreased in the next two higher *C. fluminea* densities (Fig. 5.1 D and E). These temporal differences in the concentrations of NH_4^+, NO_2^- and NO_3^- seems to be related to the natural process of N cycling, which transforms N from one form to another. When an animal dies, the organic N available in the soil is converted into NH_4^+ by fungi and both aerobic and anaerobic bacteria (Bothe et al. 2007). Thereafter, the nitrification occurs through nitrifying bacteria in two stages: first, the NH_4^+ is converted into NO_2^- and second, NO_2^- is oxidized into NO_3^-. Indeed, pulses of nitrogen-rich detritus result in a momentary acceleration of nitrogen mineralization (Wardle 2002). Similarly to our manipulative experiment, Yang (2004) observed temporal variations in NH_4^+ content. In that study, and using carcasses of cicadas, soil NH_4^+ significantly increased in treatment plots with 240 cicadas m^{-2} when compared to control in the first 30 days of the experiment, while in the subsequent 70 days no effects were detected. In the case of NO_3^-, the effects were more persistent over time, the NO_3^- availability significantly increased relatively to control during the first 30 days and this pattern was prolonged for the 70 subsequent days (Yang 2004).

Other examples in literature also showed increased content of NH_4^+ and NO_3^- in the soil around animal carcasses (see for example Hopkins et al. 2000; Towne 2000; Bump et al. 2009b; Parmenter and MacMahon 2009). However, in most studies, measurements were performed one or more years after carcasses addition, making the comparison with our results challenging (Hopkins et al. 2000; Towne 2000; Parmenter and MacMahon 2009). For example, Bump et al. (2009b) placed several ungulate carcasses in a North American hardwood forest and found that the content of $NH4^+$ and NO_3^- (but not of P, K, Mg and Ca) in soil significantly increased after 3 months. Interestingly, the $NH4^+$ values observed in our results for *C. fluminea* density of 2000 ind. m^{-2} at day 90 were significantly higher than the control and were similar to those obtained by Bump et al. (2009b) (67.27 ± 14.14 and ≈ 46 mg kg^{-1}, respectively). Hence, our experiment suggests that the decomposition of *C. fluminea* carrion

releases nitrogenous compounds into the soil, which had an effect on N cycle and consequently on $NH4^{+}$, NO_2^{-} and NO_3^{-} availability.

Our manipulative experiment showed that significant differences in C were only detected over time with values at day 7 higher than those observed on days 30 and 90 (Fig. 5.1A). Carcasses may release a significant pulse of C into to the soil during decomposition, but this pulse results in a localized microbial activity, which in turn rapidly mineralized organic C into CO_2 (Carter et al. 2007). Indeed, soils with animal carcasses contain more C compared to control but also had higher CO_2 (Hopkins et al. 2000; Carter and Tibbett 2006). According to Carter and Tibbett (2006), a soil incubated at 12 and 22°C caused an immediate release of CO_2 that peaked on day 2. Unfortunately, we did not measure CO_2 during our experiment and despite the absence of significant differences our results seem to follow the sequence of events described above: first, as a consequence of the decomposition process, C content increased in the soil, and, subsequently, organic C was possibly mineralized and released into the atmosphere contributing to decreased C content in the soil over time.

Our manipulative experiment also showed that PO_4^{3-} content responded to the *C. fluminea* addition as the highest values were observed in *C. fluminea* density of 2000 ind. m^{-2} at day 90 (Fig. 5.1F). Our results showed that *C. fluminea* carrion might have an effect on PO_4^{3-} by increasing their availability in the soil when present in high densities and similarly to that described for mammalian carrion (Bump et al. 2009b; Parmenter and MacMahon 2009). According to Parmenter and MacMahon (2009), who measured nutrient cycling and decomposition rates in a semiarid shrub-steppe ecosystem, phosphorous (P) increased in the soil after 15 months of carrion addition, representing up to 18.3% of the total P available in the carcasses. Also, Melis et al. (2007) reported that content of PO_4^{3-} was higher in soils with bison carrion than control soils.

In general, our results were clear enough to recognize that the decomposition of *C. fluminea* carrion can have a significant effect on several soil nutrients increasing their availability and this effect was more effective in some nutrients (e.g. NH_4^{+}, NO_2^{-}, NO_3^{-} and PO_4^{3-}) than others (e.g. Ca, K and N). However, our results should be interpreted with caution since several factors may affect carrion decomposition rates and may turn the comparisons with other studies challenging. First, the carrion origin and nature: our target species is an invertebrate aquatic species, so it is nutritionally different from most of the studied species available in the literature that usually comprise terrestrial mammals (e.g. pig, sheep, dog, bison, human) (Hopkins et al. 2000; Carter and Tibbett 2006; Carter et al. 2007;

Melis et al. 2007; Parmenter and MacMahon 2009). Second, carcass size, which is important in the amount of nutrients able to be transferred into the soil: our study used many small specimens, as opposed to the majority of studies that usually use a unique large specimen (Barton et al. 2013). Third, environmental variables such as moisture, temperature and soil type have important effects on decomposition rates (Forbes et al. 2005; Carter et al. 2007): our manipulative experiment was conducted during summer conditions (high temperature and low precipitation) and in sandy soils. Fourth, belowground activity by vertebrate and invertebrate species: sites may be colonized by different organisms that could exert different controls in the amount of carrion that enters into the soil (Putman 1983; Carter et al. 2007). Besides nutrients, oxygen availability is a major factor controlling microbial decomposition (Medeiros et al. 2009). In our study, we did not measure dissolved oxygen due to technical difficulties in monitoring this abiotic factor in soil samples; however, future studies should take dissolved oxygen into account when investigating the drivers of microbial decomposition in soils.

5.4.2. Effects on terrestrial microbial communities

Our study detected differences in fungal biomass only between *C. fluminea* density, with higher values always observed in treatments with a *C. fluminea* density of 2000 ind. m^{-2} (Fig. 5.2). These results were similar to those obtained by Yang (2004) that showed that the abundance of fungal phospholipid fatty acids (PLFAs) in treatment plots increased 28% after 28 days compared with control plots. Also Bump et al. (2009c) showed that fungal PLFAs were 81% more abundant in the presence of moose carcasses after 40 months compared to control sites. In addition, other studies also showed increased soil fungal biomass in the presence of carrion (see for example Parkinson et al. 2009).

Furthermore, the soil fungal community showed some succession in response to the decomposition process (Carter and Tibbett 2003; Parkinson et al. 2009). Interestingly, our results of molecular diversity of fungi showed variations in *C. fluminea* density along time mainly noted between day 7 and 90 (Fig. 5.3A). Although the DGGE technique did not allow us to identify the species involved in the process, the initial decomposition stages usually comprise zygomycetes, deuteromycetes, saprotrophic basidiomycetes and ascomycetes, while ectomycorrhizal basidiomycetes are often present in later stages (Sagara 1992; Yamanaka 1995a,b; Tibbett and Carter 2003). However, this succession was described for time intervals much higher than those used in our experiment, from 1-10 months to 1-4

years (Sagara 1992; Yamanaka 1995a,b). Despite advances made to understand the succession patterns of fungal communities during decomposition (Duarte et al. 2010), the effects of carrion are still largely unknown (Stokes et al. 2009). In an attempt to close this gap, our results demonstrated significant effects on fungal biomass and diversity supporting the idea that the presence of the Asian clam carrion has a significant effect on soil fungal community.

On the other hand, our experiment showed that molecular diversity of bacteria varied according to the *C. fluminea* density and over time. Differences in community composition were pronounced for *C. fluminea* densities ≥ 500 ind. m^{-2} at day 7 (Fig. 5.3B). This may be partially explained by the high turnover rates of bacteria, which responded rapidly to nutrient addition. Moreover, bacterial composition in plots with *C. fluminea* addition tended to become similar to the control plots at day 90 (Fig. 5.3B), suggesting that bacterial community recovered over time, after the declining effect of *C. fluminea* carrion. Interestingly, bacterial composition became more similar with control along time for plots with less carrion addition. This suggests that the higher the density of *C. fluminea* carrion the higher the impact on bacterial community, which would probably take longer to recover. Ecological succession of bacteria during the decomposition of organic matter depends on nutrient availability and undergoes functional and structural changes throughout the decomposition process until complete mineralization (Parkinson et al. 2009; Crippen et al. 2015). However, very few studies have addressed the importance of carrion decomposition on soil microbial communities, mainly on bacteria, and so, additional work is fundamental to better understand the responses of soil bacteria to carrion inputs.

5.5. Conclusion

Overall, our results revealed that the decomposition of *C. fluminea* carrion has significant effects belowground, including on nutrients content, fungal biomass and fungal and bacterial diversity. These results are particularly important when viewed across entire landscapes. Indeed, in highly invaded aquatic ecosystems, massive mortalities of *C. fluminea* may change soil chemistry, nutrient cycling and microbial communities even in adjacent terrestrial areas. Although our approach tried to mimic an extreme climatic event resulting from a flood, recent studies showed that massive mortalities of *C. fluminea* also occurred during drought events (Bódis et al. 20014; McDowell et al. 2017) and this

situation can also significantly affect aquatic ecosystem functioning. Given the predicted increase and intensification of extreme climatic events (e.g. heatwaves, floods and droughts) and the widespread distribution of several aquatic IAS in the future, the ecological importance of these massive mortalities (and resulting carrion) cannot be ignored and should be investigated in more detail.

Chapter 6

Effects of *Corbicula fluminea* die-offs on the structure and functioning of freshwater ecosystems

Novais A, Batista D, Cássio F, Pascoal C, Sousa R Effects of *Corbicula fluminea* die-offs on the structure and functioning of freshwater ecosystems.
Submitted

Abstract

1. Freshwater ecosystems are susceptible to the impacts of global climate change including extreme events such as floods and droughts. These impacts may be influenced by the presence of widespread invasive alien species, such as the Asian clam *Corbicula fluminea* (Müller, 1774). The high density and biomass attained by this species in the Minho River (NW Iberian Peninsula) is responsible for several ecological impacts.

2. By using a manipulative experiment under natural conditions, we assessed the effects of *C. fluminea* die-offs on the structure of microbial (fungi and bacteria) and invertebrate communities and leaf litter decomposition.

3. Results suggested that *C. fluminea* die-offs did not affect the structure of microbial and invertebrate communities neither leaf decomposition rate. However, differences in temporal dynamics were detected and followed an ecological succession during the course of the experiment.

4. Our study revealed that the presence of live *C. fluminea* stimulated fungal biomass and leaf mass loss, probably due to an increased availability of nutrients via production of feces and pseudofeces.

6.1. Introduction

Global climate change has impacts on biodiversity with possible consequences for ecosystem functioning (Bellard et al. 2012). In response to climate change, several species have changed their distribution (i.e. latitudinal and/or altitudinal range shifts), phenology (i.e. the time at a determined lifecycle event occurs), and physiology (Parmesan 2006; Bellard et al. 2012). These responses are common to both terrestrial (Parmesan and Yohe 2003) and aquatic (Hoegh-Guldberg and Bruno 2010; Brown et al. 2016) ecosystems. Some well-known examples are the disruption in plant-pollinator (Parmesan 2006) and predator-prey interactions (Barbraud and Weimerskirch 2006), the increase in coral bleaching and mortality (Hoegh-Guldberg et al. 2007), and the decline of kelp forests cover (Ling 2008).

Global climate change also impacted freshwater biodiversity (Kundzewicz et al. 2008) at all levels of biological organization. At the individual level, physiological and behavioural responses can occur and include changes on insect emergence (Jonsson et al. 2015), invertebrate shredders feeding behaviour and body elemental composition (Ferreira et al. 2010; Fernandes et al. 2015). Consequently, individuals' responses can lead to several responses at the population level; for example, increased temperature can stimulate the growth and reproduction of some aquatic hyphomycete species with impact to overall community performance (Rajashekar and Kaveriappa 2000; Dang et al. 2009; Batista et al. 2012). Additionally, climate change can be responsible for indirect effects at the community level; for example, warming significantly reduced the number of emergent Chironomidae resulting in a shift in the community structure (Jonsson et al. 2015). Finally, ecosystem functioning can also be affected, such as the acceleration in litter decomposition as a response to higher water temperature (Fernandes et al. 2009; Batista et al. 2012).

Alterations in freshwater ecosystems are determined by the patterns of changes in temperature, evaporation and precipitation (Kien and Verdon-Kidd 2010) and their interactions with local biophysical and anthropogenic factors (McAlpine et al. 2009). In addition to gradual shifts in mean climate conditions, future scenarios also predict a change in the hydrological cycle, including the increase of extreme climatic events such as floods and droughts (Huntington 2006; Kundzewicz et al. 2008).

Species introductions are numerous in freshwater ecosystems and they are a major threat to biodiversity (Strayer 2010; Simberloff et al. 2013). Climate change, including extreme climatic events,

can modify the impacts of IAS on freshwater ecosystems (Diez et al. 2012). However, studies addressing this subject are rare. Extreme climatic events may put IAS at a competitive advantage but the opposite has also been observed (Diez et al. 2012). Additionally, extreme climatic events can cause mass mortality events on IAS (Sousa et al. 2008c; Ilarri et al. 2011; Bódis et al. 2014). In the Hungarian section of the Danube River, massive mortalities of two invasive bivalves, *S. woodiana* and *C. fluminea*, were observed during the autumn of 2011 (Bódis et al. 2014). In the Iberian Peninsula, die-off events of both native and invasive species have also been observed in freshwater ecosystems due to strong heatwaves (Sousa et al. 2008c; Ilarri et al. 2011). For example, extreme abiotic changes, such as low river flow, high temperature, low dissolved oxygen and lower redox potential, occurred in the Minho River (NW of Iberian Peninsula) during the summers of 2005 and 2009, and high mortalities in several macrozoobenthic estuarine species, including *C. fluminea*, were reported (Sousa et al. 2008c; Ilarri et al. 2011).

Given the widespread distribution and the high density and biomass attained by *C. fluminea* in the Minho River, and the predictable increase in the number and intensity of extreme climatic events in the Iberian Peninsula (and elsewhere), it is crucial to investigate the impacts of *C. fluminea* die-offs on key freshwater processes, such as leaf litter decomposition as well as on the organisms that drive this process. Thus, we carried out a manipulative experiment under natural conditions simulating a *C. fluminea* mortality event to assess possible effects on (1) the structure of microbial communities (both fungi and bacteria), (2) the structure of an invertebrate community, and (3) leaf litter decomposition. In addition, possible temporal differences were also assessed. We hypothesized that an increase in *C. fluminea* biomass resulting from massive mortalities will provide an additional resource that will enhance the abundance, biomass and diversity of microbial and invertebrate communities and may accelerate plant litter decomposition.

6.2. Material and methods

6.2.1. Study area

The experiment was carried out in a lateral arm of the Minho River, with 1 m depth and 20 m width, located approximately 38 km upstream of the mouth of the estuary (42° 03' 04.40'' N, 08° 33' 39.62'' W; NW of Iberian Peninsula). The Minho River originates in Serra da Meira (Spain) and drains a hydrological basin of approximately 17 000 km^2, 95% of which being located in Spain and 5% in Portugal (Sousa et al. 2005, 2007a). This river has 300 km and its mesotidal estuary extends approximately 40 km upstream (Sousa et al. 2005, 2007a). The international section of the Minho River (last 70 km, including the estuary) has important habitats favouring the maintenance of a high biodiversity (for a review see Sousa et al. 2008e; Costa-Dias et al. 2010; Mota et al. 2014). In the last years, several IAS were introduced in this estuary, where *C. fluminea* has a prominent role (Sousa et al. 2008c,e; Souza et al. 2013). Nowadays, *C. fluminea* is present in 150 km of the river length (Ferreira-Rodríguez and Pardo 2016), contributing with approximately 95% for the total benthic biomass in downstream areas (Sousa et al. 2008c,d).

Extreme climatic events seem to have an important role triggering the massive mortalities of *C. fluminea* in the Minho estuary (Ilarri et al. 2011). According to Ilarri et al. (2011), that measured the annual density of *C. fluminea* in this river from 2004 to 2009, the lowest mean densities were recorded in 2005 (956 ind. m^{-2}) and 2009 (777 ind. m^{-2}), years where heatwaves occurred. This situation (i.e. the high biomass and consequent decomposition resulting from the *C. fluminea* massive mortalities) was possibly responsible for significant changes in the structure of the molluscan assemblages (for a review see Sousa et al. 2008e; Ilarri et al. 2011).

6.2.2. Experimental design

The experiment was conducted in a complete randomized block design (eight blocks). Each block contained five baskets (38 × 29 × 21.5 cm) corresponding to five treatments: (1) bare sediment (control treatment); (2) live *C. fluminea* individuals (live treatment); (3) open empty *C. fluminea* shells (open treatment); (4) dead *C. fluminea* soft parts (soft treatment); and (5) dead *C. fluminea* individuals

including shells and soft parts (total treatment). Five baskets from each block were covered with a coarse mesh net (10 mm) to allow colonization by microbes and invertebrates, and other five baskets with the same five treatments were covered with a fine mesh net (0.5 mm) to allow microbial colonization, excluding the presence of invertebrates. All treatments included 5 g of alder leaves. The control treatment was used to recreate a site without *C. fluminea* influence and the live treatment was used to recreate a site with no *C. fluminea* mortality. The open treatment was used to detect the effect of the empty shells as a substrate, while the soft treatment was used to detect the effect of the *C. fluminea* dead tissues as a resource. Finally, the total treatment was used to detect the total effect of dead *C. fluminea* (shells as a substrate and dead tissues as a resource). All treatments, except the control (no clams), had a density of 1000 ind. m^{-2} with a similar size structure, reflecting a well-documented range of naturally occurring *C. fluminea* mortalities resulting from drought events (Sousa et al. 2008c; Ilarri et al. 2011). Baskets were distributed within a grid of ca. 1 m intervals, chosen to minimize habitat variability and inter-plot interactions. The experiment took place at the end of the summer in 2015 (September 18) and samples were collected 11 and 33 days of immersion after the beginning of the experiment. Abiotic factors were measured at the beginning and end of the experiment. Temperature, conductivity, total dissolved solids, redox potential, salinity, dissolved oxygen and pH were measured in situ with a multiparametrical probe YSI EXO 2.

In the laboratory, leaves and invertebrates were rinsed with tap water to remove sediment and processed. Leaf disks were cut with a cork borer (12 mm diameter); one set of three leaf disks was lyophilized for DNA extraction, and one set of six leaf disks was used for fungal biomass quantification.

6.2.3. Microbial community characterization

6.2.3.1. Fungal biomass

Six leaf disks from each replicate were used for quantification of ergosterol concentration as a surrogate for fungal biomass (Gessner 2005). Lipids were extracted from leaf disks by heating (80°C, 30 min) in 0.8% of KOH/methanol, and the extract was partially purified by solid-phase extraction. Ergosterol was quantified by high-performance liquid chromatography (HPLC) using a LiChrospher RP18 column (250 mm x 4 mm, Merck), connected to a liquid chromatographic system (UltiMate 3000 LC Systems, Thermo Scientific, CA, USA). The system was run isocratically with HPLCgrade methanol at 1.4 mL

min^{-1} and 33°C. Ergosterol was detected at λ=282 nm and its concentration was estimated using standard series of ergosterol (Fluka) in isopropanol.

6.2.3.2. Microbial diversity from DNA fingerprints

DNA was extracted from three leaf disks from each replicate using an Ultra Clean Soil DNA isolation kit (MoBio Laboratories, Solana Beach, CA), according to the manufacturer instructions. The ITS2 region of fungal rDNA was amplified with the primer pairs ITS3GC and ITS4 and the V3 region of bacterial 16SrDNA was amplified with the primer pairs 518R and 338F_GC as described by Duarte et al. (2008). For polymerase chain reaction (PCR) of fungal and bacterial DNA, 0.3 µL GoTaq G2 Flexi DNA Polymerase, 1 µL of each primer, 2 µL of DNA and 28.7 µL of ultra-pure water were used in a final volume of 50 µL. PCR reagents were purchased from Promega except primers that were from Stabvida. DNA amplification was carried out in a MyCycler Thermal Cycler (BioRad Laboratories, Hercules, CA, USA) using the following program: initial denaturation at 95°C for 2 minutes; followed by 36 cycles of denaturation at 95°C for 30 seconds, primer annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute. Final extension was at 72°C for 5 minutes (Duarte et al. 2008).

The DGGE analysis was performed using a DCodeTM Universal Mutation Detection System (BioRad Laboratories). For fungi and bacteria, samples of 20 µL from the amplification products of 380-400 bp (ITS3GC/ITS4) and 200 bp (338F_GC/518R), respectively, were loaded on 8% (w/v) polyacrylamide gel in 1x Tris-acetate-EDTA (TAE) with a denaturing gradient from 30 to 70% for fungal DNA and 40 to 70% for bacteria DNA. Gels were run at 55 V, 56°C for 16 h and stained with Midori Green (Grisp) for 10 min in a shaker at 40 rpm. Gel images were captured under UV light in a ChemiDoc XRS (BioRad).

6.2.4. Invertebrate community characterization

Invertebrates from each replicate were sieved through a 500-µm mesh and were preserved in 70% ethanol. The organisms were counted and identified to the lowest possible taxonomic level (following Serra et al. 2009 and Tachet et al. 2010). For biomass quantification, organisms were over-dried for 72 h at 60°C and weighed on a precision scale.

6.2.5. Leaf mass loss

To determine leaf mass loss, the remaining leaves from each treatment were washed and oven-dried for 72 h at 60°C and subsequently weighed on a precision scale. Leaf mass loss was determined as the difference in weight between initial and final measurements.

6.2.6. Data analysis

Three-way PERMANOVA (type-III) were used in a three-way crossed design to test for fixed effects of treatments (five levels: control, live, open, soft and total), time (two levels: 11 and 33 days) and presence of invertebrates (two levels: with and without) used as fixed factors on fungal biomass and leaf mass loss. Prior to PERMANOVA analyses, similarity matrices were calculated using Euclidean distances (Clarke and Warwick 2001).

For each group of microbes, DGGE gels were aligned and the relative intensity of the band was analyzed with BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium). Each DGGE band was considered one OTU. The nMDS ordination analysis based on fungal and bacterial communities' data followed by a three-way PERMANOVA (type-III) were used to discriminate possible differences between treatments, time and presence of invertebrates, with the same design described above. Prior to nMDS ordination and PERMANOVA, similarity matrices were calculated using Bray Curtis similarity and Euclidean distances, respectively (Clarke and Warwick 2001).

The nMDS ordination analysis based on the invertebrate abundance data followed by a two-way PERMANOVA (type-III) were used in a two-way crossed designed to test for fixed effects of treatments (five levels: control, live, open, soft and total) and time (two levels: 11 and 33 days). Prior to nMDS ordination and PERMANOVA analysis, similarity matrices were calculated using Bray Curtis similarity and Euclidean distances, respectively (Clarke and Warwick 2001).

Species richness and the Shannon-Wiener diversity index were calculated through the DIVERSE analysis (Clarke and Warwick 2001). Comparisons of invertebrate abundance, biomass, species richness and Shannon-Wiener diversity index between treatments and time were done using a two-way PERMANOVA (type-III), with the design described above. Prior to PERMANOVA analyses, similarity matrices were calculated using Euclidean distances (Clarke and Warwick 2001).

In all PERMANOVA tests, the statistical significance of variance (α = 0.05) was tested using 9999 permutations of residuals within a reduced model. When the number of permutations was lower than 150, the Monte Carlo p-value was considered. All analysis that had significant differences were followed by a PERMANOVA a posteriori pairwise comparisons.

PRIMER analytical software (v.6.1.6, PRIMER-E) with the PERMANOVA + 1.0.1 add-on (Anderson 2001; MJ Anderson et al. 2008) was used for all statistical tests described above.

6.3. Results

6.3.1. Abiotic characterization

Abiotic factors (mean ± SD) values measured in the water column during the 33 days of the experiment were: temperature: 17.94 ± 1.97°C; conductivity: 72.00 ± 7.07 μScm^{-1}; total dissolved solids: 0.06 ± 0.01 mg l^{-1}; redox potential: 65.70 ± 7.50 mV; salinity: 0.04 ± 0.00; dissolved oxygen: 9.44 ± 0.15 mg l^{-1} and pH 7.75 ± 0.11.

6.3.2. Microbial community characterization

In the treatments with invertebrates, fungal biomass (mean µg ergosterol g^{-1} leaf dry mass ± SD) was highest at live treatment at day 33 (419.61 ± 112.76), and lowest at control treatment at day 11 (119.79 ± 51.45) (Fig. 6.1; Table S9). In the treatments without invertebrates, the highest fungal biomass was obtained at live treatment at day 33 (435.80 ± 103.66), and the lowest at soft treatment at day 11 (20.22 ± 8.16) (Fig. 6.1; Table S9). Fungal biomass differed significantly between treatments (Pseudo-F = 12.39, $p \leq 0.001$), time (Pseudo-F = 64.51, $p \leq 0.001$) and the presence of invertebrates (Pseudo-F = 114.70, $p \leq 0.001$) (Table S9). Significant interactions between treatments and time (Pseudo-F = 10.82, $p \leq 0.001$) and between time and the presence of invertebrates (Pseudo-F = 14.49, $p \leq 0.001$) were also found (Table S9). Pairwise tests indicated that these differences were associated

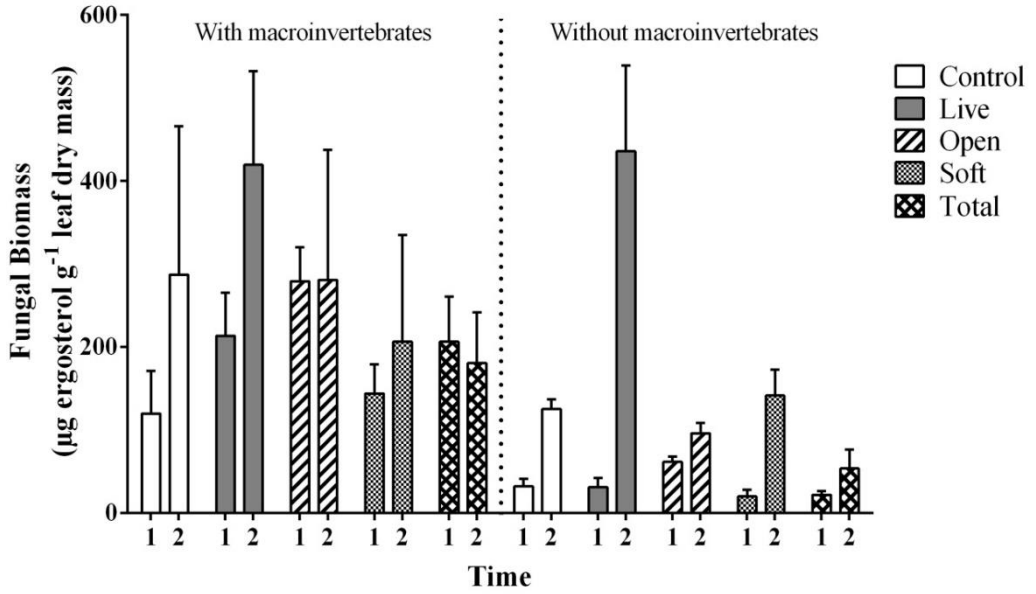

Figure 6.1. Fungal biomass (mean µg ergosterol g^{-1} leaf dry mass ± SD) in the five treatments (control, live, open, soft and total), two sampling times (11 and 33 days) in the presence or absence of invertebrates (with and without).

with the high biomass observed at day 33, particularly at live treatments, with or without invertebrates (Table S9).

The nMDS ordination based on the fungal data (Fig. 6.2) showed that the structure of the community differed only between time (Pseudo-F = 8.67, p = 0.006), with a clear discrimination between two groups: treatments with 11 days and treatments with 33 days.

The nMDS ordination based on the bacterial data (Fig. 6.2) showed that the structure of the community was only significantly affected by the interaction between treatments and the presence of invertebrates (Pseudo-F = 2.72, p = 0.04).

6.3.3. Invertebrate community characterization

A total of 7550 individuals belonging to 69 macrozoobenthic taxa were recorded in all treatments. The most abundant taxa were *Dugesia* sp. (14.43%), *C. fluminea* (11.31%), Chironomidae sp. 1 (9.85%), Planorbidae sp. (7.92%), *Ferrissia clessiniana* (Jickeli, 1882) (5.92%), *Prostoma* sp. (5.50%), *Daphnia*

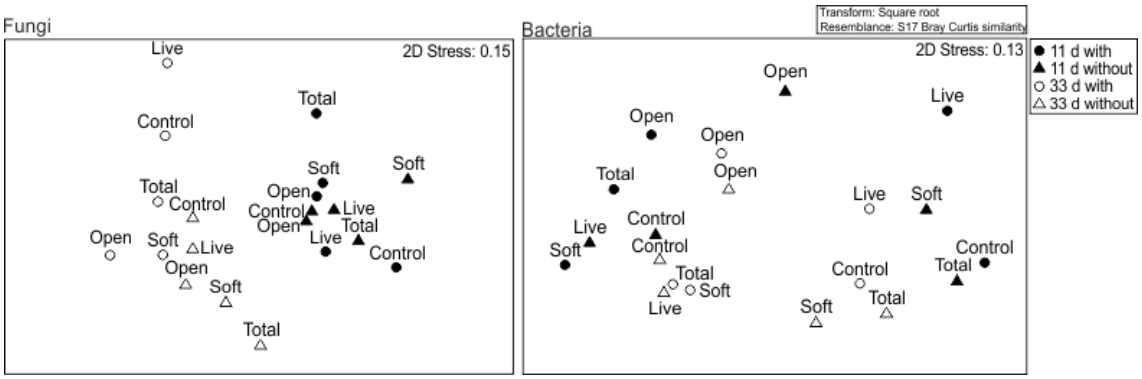

Figure 6.2. Non-metric multidimensional scaling (nMDS) plot of fungal and bacterial communities in the leaves in the five treatments (control, live, open, soft and total), two sampling times (11 and 33 days) in the presence or absence of invertebrates (with and without).

sp. (4.82%), *Physella acuta* (Draparnaud, 1805) (4.38%), *P. antipodarum* (4.03%), while the remaining taxa represented 31.84% of the total macrozoobenthic individuals. In terms of biomass, *C. fluminea* (53.67%), *Bithynia tentaculata* (Linnaeus, 1758) (15.57%), *P. clarkii* (8.57%), *P. antipodarum* (7.97%), *P. acuta* (5.13%), *Radix* sp. (2.14%), Planorbidae sp. (1.25%) and *Dugesia* sp. (1.00%) were the dominant taxa, while the remaining taxa had a total biomass of 4.7%.

The nMDS ordination based on the relative abundance of invertebrates showed significant differences between time (Pseudo-F = 4.10, p = 0.003), with a clear discrimination between treatments with 11 days and 33 days (Fig. 6.3).

The relative abundance (mean ± SD) of invertebrates was highest at control treatment at day 33 (77.12 ± 16.80 individuals), and lowest at live treatment at day 11 (43.38 ± 15.73 individuals) (Fig. 6.4A). Invertebrate abundance was only significantly affected by time (Pseudo-F = 11.67, p = 0.001). Pairwise tests indicated that these differences were between day 11 and 33 at control (t = 3.33, p = 0.02) and soft (t = 3.02, p = 0.03) treatments.

Invertebrate biomass (mg DW) was highest at soft treatment at day 33 (473.30 ± 367.94), and lowest at total treatment at day 11 (96.23 ± 86.28) (Fig. 6.4B). Invertebrate biomass differed significantly only between time (Pseudo-F = 16.57, p ≤ 0.001). Pairwise tests indicated that these differences were between day 11 and 33 at total (t = 2.89, p = 0.03) treatment.

Species richness was highest at control treatment at day 33 (29.75 ± 2.99), and lowest at live treatment at day 11 (20.75 ± 4.79) (Fig. 6.4C). Species richness was only significantly affected by

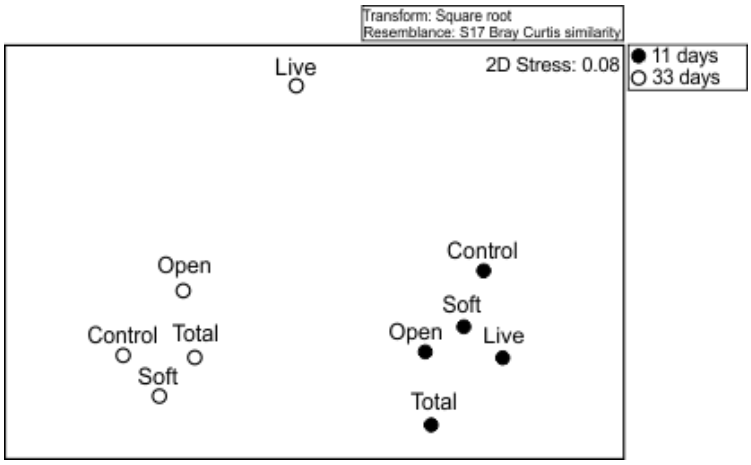

Figure 6.3. Non-metric multidimensional scaling (nMDS) plot of the invertebrate community associated with the five treatments (control, live, open, soft and total) and two sampling times (11 and 33 days).

time (Pseudo-F = 11.09, p = 0.002). Pairwise tests indicated that these differences were between day 11 and 33 at control (t = 3.49, p = 0.01) treatment.

Shannon-Wiener diversity index was highest at control treatment at day 33 (3.19 ± 0.08), and lowest at live treatment at day 11 (2.85 ± 0.21) (Fig. 6.4D). Shannon-Wiener diversity index differed significantly only with time (Pseudo-F = 7.75, p = 0.007). Pairwise tests indicated that these differences were between day 11 and 33 at control (t = 3.27, p = 0.02) treatment.

6.3.4. Leaf mass loss

In the treatments with invertebrates, the percentage of leaf mass loss (mean ± SD) was highest at live treatment at day 33 (62.68 ± 2.81), and lowest at the total treatment at day 11 (38.47 ± 1.98) (Fig. 6.5; Table S10). In the treatments without invertebrates, the highest leaf mass loss was obtained at live treatment at day 33 (54.32 ± 5.22), and the lowest at soft treatment at day 11 (33.40 ± 0.38) (Fig. 6.5; Table S10). Leaf mass loss differed significantly between treatments (Pseudo-F = 4.80, $p \leq 0.01$), time (Pseudo-F = 460.48, $p \leq 0.001$) and the presence of invertebrates (Pseudo-F = 54.89, $p \leq 0.001$) (Table S10). Pairwise tests indicated that these differences were associated with the high leaf mass loss observed at day 33, particularly at live treatments, with or without invertebrates (Table S10).

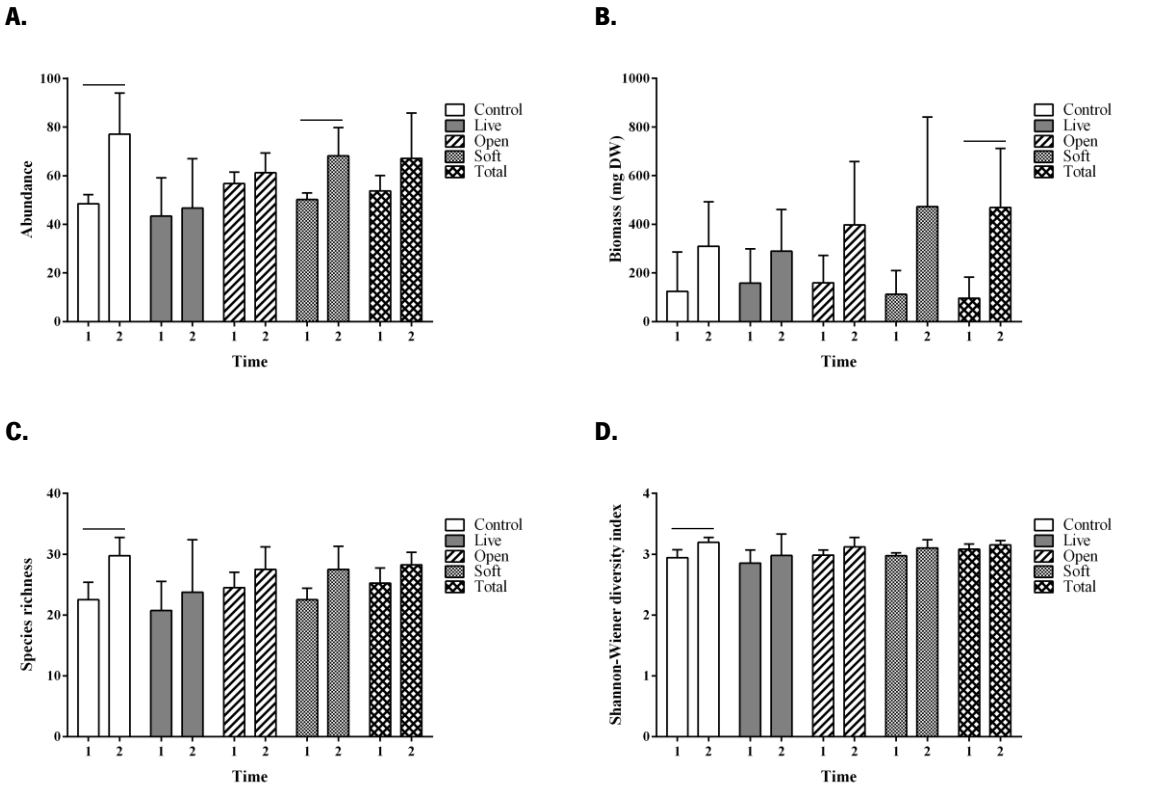

Figure 6.4. Invertebrate (mean ± SD) (A) relative abundance, (B) biomass (mg DW), (C) species richness and (D) Shannon-Wiener diversity index in the five treatments (control, live, open, soft and total) and two sampling times (11 and 33 days).

6.4. Discussion

Clear temporal differences were detected following a classical ecological succession. Typically, microbial biomass is low at early stages of leaf litter decomposition and increase at middle decomposition stages with fungal community contributing with more than 95% to the total microbial biomass (Mora-Gomez et al. 2016; Pascoal and Cássio 2004). Furthermore, invertebrates appear to demonstrate preference for leaves colonized by a well-developed microbial community (Graça 2001). In our experiment, fungal biomass significantly increased over time, with higher values observed 33 days after the beginning of the experiment. Invertebrates followed the same trend with abundance, biomass, richness and diversity significantly increasing over time, with higher values observed at day 33. Consistently, the nMDS plots of fungal and invertebrate communities were also able to distinguish

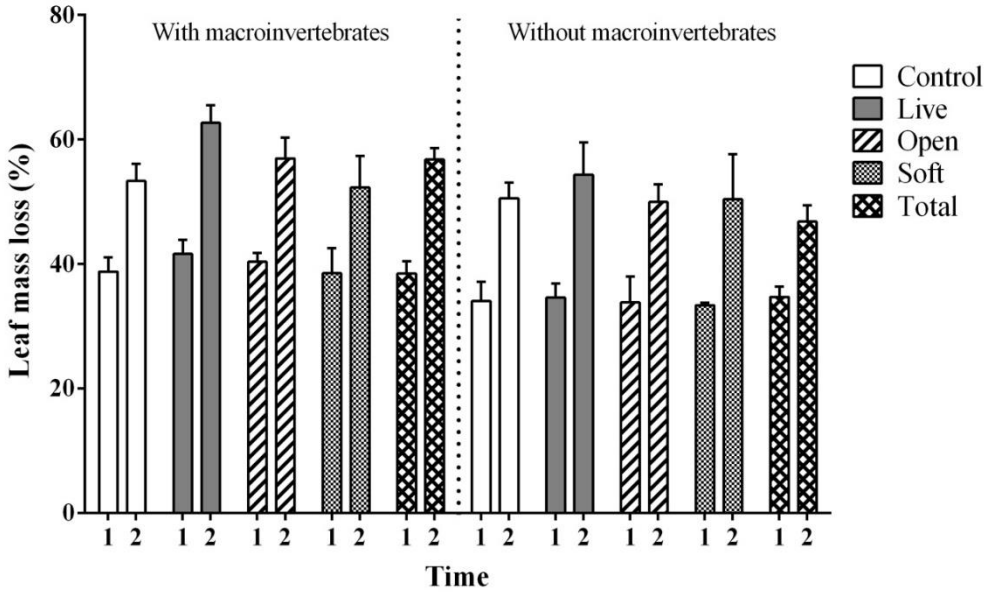

Figure 6.5. Percentage of leaf mass loss (mean ± SD) in the five treatments (control, live, open, soft and total), two sampling times (11 and 33 days) in the presence or absence of invertebrates (with and without).

two groups based on time. Finally, as a result of fungi and invertebrate activities, leaf mass loss was significantly higher at day 33. These results suggest that up to day 11 the leaves suffered leaching, physical abrasion and began to be colonized, while at day 33 microbial colonization was well-established, therefore the higher values for fungal biomass.

In our study, fungal biomass was higher in the treatments where *C. fluminea* were live, which may be related to the feces and pseudofeces produced by this IAS (Vaughn and Hakenkamp 2001). Earlier studies have demonstrated that nutrient enrichment stimulates fungal biomass (Pascoal and Cássio 2004). On the other hand, the response of fungal biomass in soft and total treatments were lower than expected, which may be explained by the presence of high ammonia levels and decreased dissolved oxygen that is frequently associated with tissue decay during *C. fluminea* mortalities (Cherry et al. 2005; Cooper et al. 2005). According to Cooper et al. (2005) dead clams' density up to 1000 ind.m^{-2} results in ammonia production and dissolved oxygen reductions that can exceed toxic levels for some species. Furthermore, it is known that reductions in dissolved oxygen in streams negatively affect fungal biomass (Pascoal and Cássio, 2004; Medeiros et al. 2009).

We expected that the presence of *C. fluminea* would affect the abundance and diversity of invertebrates. In a previous study, Ilarri et al. (2012) found that the presence of *C. fluminea* has a positive influence

on the density, biomass and diversity of some faunal groups such as Gastropoda, Crustacea and Insecta, both in brackish and freshwater conditions. Recently, Novais et al. (2015b) also reported increases in density, biomass and species richness of estuarine macrozoobenthos such as Annelida, Mollusca and Crustacea in the presence of live specimens and open empty shells of *C. fluminea*. However, in the present study, we found no significant differences in abundance, biomass, species richness and Shannon-Wiener diversity index of invertebrates between treatments. These differences between past and present results may be related to variations in local abiotic factors included those derived from the discrepancy in the period of the year in which the experiments were performed and/or to the lower period of time available for invertebrate colonization in the present study.

Leaf mass loss was higher in the treatment where *C. fluminea* was live in comparison with other treatments. Although phytoplankton is considered the main food of freshwater bivalves, some species may also depend on other resource types, such as zooplankton, algae, bacteria, detritus and particulate organic matter to meet their nutritional needs (Vaughn et al. 2008). For instance, Kasai and Nakata (2005) found that a species belonging to the genus *Corbicula* could assimilate terrestrial particulate organic matter. This may be especially true for oligotrophic ecosystems, such as Minho River, where phytoplankton growth is limited due to low concentration of nutrients (Dias et al. 2014). Recently, Dias et al. (2014) found that specimens of *C. fluminea* in Minho River, in addition to phytoplankton, consumed other sources of organic matter, such as, terrestrial organic matter, sediment organic matter and microphytobenthos to support its high abundance in this ecosystem. Thus, live specimens of *C. fluminea* may have contributed to increased leaf mass loss in the live treatment. Besides, at nutrient-enriched sites high leaf decomposition rates were associated with maximum fungal biomass (Pascoal and Cássio 2004). These results suggest that the presence of live *C. fluminea* stimulated fungal biomass resulting in an increased leaf mass loss. Although there were significant differences in leaf mass loss between treatments, differences between live versus soft and total treatments should be more pronounced taking into account fungal biomass results. This suggests that other organisms such as bacteria and invertebrates may have contributed to the increased leaf mass loss in soft and total treatments. Although the abundance and biomass of invertebrates did not differ significantly between treatments, the values observed in the soft and total treatments were slightly higher than those obtained for live treatment. Unfortunately, we did not measure bacterial decomposition activity on leaves, but this effect cannot be ignored and should be studied in the future.

The presence of invertebrates enhanced the response of fungal biomass and also leaf mass loss. Invertebrate excretion can stimulate fungal biomass by increasing ammonia concentration in leaf substrata (Villanueva et al. 2012). These results are consistent with those obtained in the treatment where *C. fluminea* was live. Indeed, excretion of *C. fluminea* and other invertebrates appears to stimulated fungal biomass.

In our study, we expected a higher leaf mass loss in treatments with invertebrates mainly for two reasons: (1) many invertebrates are key players in leaf litter decomposition contributing to accelerate leaf fragmentation and consequently the decomposition process (Graça 2001), and (2) fungal biomass was highest in treatments with invertebrates. In this case, we need to take into account that bacteria may have played an important role contributing to increase leaf mass loss in treatments without invertebrates. The lowest values observed without invertebrates can be explained taking into account that bacteria can have a negative effect on fungi causing suppression of their growth and biomass (Romaní et al. 2006).

6.5. Conclusion

Overall, *C. fluminea* die-offs did not affect aquatic microbial and invertebrate communities and leaf litter decomposition. In addition, our study reinforces the hypothesis that the presence of live *C. fluminea* could affect native communities. In particular, fungal community is benefited from the presence of live *C. fluminea* and also from the presence of other invertebrates. These effects were most likely due to an increased availability of nutrients via production of feces and pseudofeces by *C. fluminea* and other invertebrates.

Since *C. fluminea* is a non-native species with a well-known invasive behaviour, we expected that its massive mortalities would be crucial for important processes such as leaf litter decomposition, but it was not the case. Yet, with this study we conclude that leaf litter decomposition is a complex process that depends on many other factors.

Chapter 7

Conclusion and future directions

It is well-know that *C. fluminea* can cause a wide range of negative impacts on the invaded ecosystems and numerous studies have addressed these effects. In contrast, the studies presented in this thesis showed that *C. fluminea* could also interact positively with some native species, even in highly invaded ecosystems. Results revealed that the presence of live and open empty shells of *C. fluminea* increased the density, biomass and species richness of estuarine macrozoobenthos. Although the presence of live and open shells of *C. fluminea* led to similar results, the species associated with each treatment differed: polychaetes and molluscs significantly responded to the presence of live *C. fluminea*, while crustaceans significantly responded to the presence of open empty shells of *C. fluminea*. This study gave further insights into the identification of the two possible mechanisms underlying the observed results: (1) the production of feces and pseudofeces by *C. fluminea*, which increases organic matter content and food resources for some deposit feeding macrozoobenthic species, such as some polychaetes and molluscs; and (2) ecosystem engineering activities by *C. fluminea*, which can create conditions for the establishment of other species (mainly amphipods) via shell production and bioturbation in the sediments (Chapter 2). Furthermore, belowground assessments showed that the presence of live *C. fluminea* stimulated fungal biomass (but not diversity) and bacterial diversity. Since no differences were detected in sediment nutrients, bioturbation activities by *C. fluminea* are possibly the main mechanism explaining these results. Despite the direct bioturbation activity by *C. fluminea* influencing fungal and bacteria communities, other factors such as the presence of other macroinvertebrate species such as polychaetes and molluscs and/or production of feces and pseudofeces by *C. fluminea* cannot be excluded (Chapter 3). Overall, the results of Chapters 2 and 3 suggest that *C. fluminea* have positive effects on some species belonging to Annelida, Mollusca and Crustacea, also influencing microbial communities in invaded estuarine ecosystems. In addition, these results support earlier theoretical hypotheses advanced by Gutiérrez et al. (2014) suggesting that assimilatory-dissimilatory and physical ecosystem engineering are two mechanisms by which IAS can affect ecosystems structure and functioning. It is important to note that for some faunal groups these positive effects may be different in the future if biological and environmental conditions change, and that current positive effects are not generalized to all species present in the Minho River estuary. Indeed, estuaries have high environmental disturbance due to its natural or human activities (Day et al. 1989; Little 2000), and therefore the results reported here may differ from those in other aquatic ecosystems, such as rivers and lakes that are less disturbed and that support much higher species

richness.

Studies carried out in this thesis also revealed that *C. fluminea* may function as a resource pulse to adjacent terrestrial ecosystems after massive die-offs resulting from extreme floods. This availability of bivalves' carrion in terrestrial ecosystems can trigger effects across multiple trophic levels, as the increase in resource availability led to an increase in the abundance, biomass and species richness of consumers. Adult Diptera were the first organisms to significantly respond to *C. fluminea* addition, and a positive relationship between *C. fluminea* density and adult Diptera abundance was established. Then, other terrestrial invertebrates responded to *C. fluminea* addition, mainly the functional group of carnivores/scavengers that attained higher values at *C. fluminea* densities higher than 500 ind. m^{-2} located on the edge of the experimental area (Chapter 4). *Corbicula fluminea* carrion addition had also significant belowground effects, specifically on nutrients content (mainly NH_4^+, NO_2^-, NO_3^- and PO_4^{3-}), fungal biomass and fungal and bacterial diversity (Chapter 5). Overall, the results of Chapters 4 and 5 provided the first empirical evidence on how massive die-offs of *C. fluminea* can cause a substantial pulse of energy and nutrients to the adjacent terrestrial ecosystems, with clear effects on the above and belowground communities. These results are especially important and novel because earlier examples of cross-habitat flows between freshwater and terrestrial ecosystems have often described the movement of materials, nutrients and energy from land to water direction, and much less attention has been devoted to rare phenomena such as resource pulses resulting from massive die-offs of IAS in the opposite direction (i.e. from water to the land and affecting the terrestrial community). Moreover, since global warming is expected to increase the occurrence and magnitude of extreme climatic events, in particular an intensification of the global water cycle with a consequent increase in flood and drought risk (Huntington, 2006; Kundzewicz et al. 2008), it is important to assess how these rare extreme climatic events and IAS could interact and lead to resource pulses in the future (Yang et al. 2008; Diez et al. 2012).

A final manipulative experiment was also conducted to assess the possible effect of *C. fluminea* die-offs on the structure of microbial and invertebrate communities and on leaf decomposition after droughts in the aquatic realm. Although results have suggested that *C. fluminea* die-offs did not affect the structure of microbial and invertebrate communities neither leaf decomposition, temporal differences were detected and followed an ecological succession (Chapter 6). In addition, Chapter 6 reinforces the hypothesis that the presence of live *C. fluminea* could have strong effects on native

communities, given that fungal biomass and leaf mass loss were benefited by the presence of live *C. fluminea*, probably due to an increased availability of nutrients via production of feces and pseudofeces. Overall, the studies presented in this thesis confirmed that *C. fluminea* could affect native communities and revealed that massive die-offs of *C. fluminea*, contributing with remarkable amounts of carrion for adjacent terrestrial ecosystems, may function as a resource pulse. Despite the Asian clam *C. fluminea* may benefit certain organisms, it is necessary to be aware that this IAS can cause serious negative impacts on invaded ecosystems. Indeed, since the introduction of *C. fluminea* in the Minho River estuary, native bivalves have undergone significant declines in density, biomass and spatial distribution (Sousa et al. 2005, 2007a, 2008c,d,e). Given the high density and widespread distribution of *C. fluminea*, and the predicted increase and intensification of extreme climatic events, the ecological importance of these phenomena should be addressed in future studies.

Despite many studies in the last years addressed several aspects about the genetics, physiology and ecology of this species in the invaded range (including studies in this thesis) many issues still to be addressed. Because the manipulative study in the Minho River estuary only lasted 2 months (Chapters 2 and 3), further research should include a broader time scale, with the purpose of comprising other seasons of the year, in order to clarify if the positive effects of *C. fluminea* on estuarine macrozoobenthic and microbial communities persist over time. Moreover, similar studies should be done in other aquatic ecosystems equally invaded by *C. fluminea* to determine if results follow the patterns found in this thesis. Possibly ecosystem engineering activities and the production of feces and pseudofeces by *C. fluminea* are not the only ecological mechanisms through which the *C. fluminea* can change structure and functioning of the invaded ecosystems, so further research should include other important ecological processes such as competition, filtration and shells durability. General models should also be developed including all these ecological processes as well as the main taxonomic groups affected, including possible models comprising trophic interactions and food-webs. Furthermore, since aquatic ecosystems are susceptible to the impacts of global climate change, manipulative studies under both baseline and extreme conditions are necessary to understand the ecological role of *C. fluminea* in future climatic scenarios.

Studies of this thesis provide the first demonstration that an invasive bivalve species can act as a pulsed trophic subsidy moved from aquatic to terrestrial ecosystems. So, it opened several hypotheses that should be addressed in future studies. Because our manipulative studies only focused on

invertebrate and microbial communities, further investigation should take account if *C. fluminea* carrion can also subsidize other consumers such as mammals, amphibians and birds as well as plant species. Manipulative studies using a broader temporal scale are also needed to resolve several uncertainties about the time required for total decomposition of *C. fluminea* carrion and shells, and about the time that *C. fluminea* carrion and shells stop to have an effect on terrestrial communities. In addition, manipulative studies in other terrestrial ecosystems with different abiotic conditions (e.g. moisture, temperature, light/shadow and soil type) should be done aiming to understand how the decomposition of *C. fluminea* carrion and shells varies and if *C. fluminea* carrion addition has the same effects as described in Chapters 4 and 5. More interesting would be the modeling of these effects at different ecological levels (from individuals to ecosystems) to get a better knowledge of the impacts of die-offs of *C. fluminea* at the landscape level. Although the studies present in this thesis showed that several species responded to *C. fluminea* carrion addition, they do not guarantee that these species have consumed the available carrion. Thus, future studies should be done encompassing new methodologies (i.e. stable isotope and/or fatty acid analyses) to clarify this issue. It would also be interesting to extend the knowledge about the effects of *C. fluminea* carrion addition on microbial communities, as well as to investigate the possibility of competitive interactions between fungal and bacterial communities or between these and the macroinvertebrate communities. Given that massive die-offs of *C. fluminea* resulting from extreme droughts did not affect the structure of aquatic microbial and invertebrate communities neither leaf litter decomposition, further studies in other aquatic ecosystems with different abiotic conditions should be done to investigate if the results are similar. Despite many studies with *C. fluminea* have to be done to understand these and other aspects, similar studies with other IAS should also be performed using for example zebra (*D. polymorpha*) or golden (*L. fortunei*) mussels. Although there are not many studies addressing IAS as a pulsed subsidy mainly in the interface between aquatic and terrestrial ecosystems, it is clear that the *C. fluminea* is not the only IAS acting as a resource pulse. For example, carcasses of invasive fish species (e.g. Pacific salmonids) resulting from mortalities in the course of reproductive events in many rivers and lakes can be transported to adjacent terrestrial ecosystems resulting in an important subsidy for terrestrial communities.

As mentioned above, more research is still to be done to better understand and integrate all the effects mediated by *C. fluminea* on the structure and functioning of the invaded ecosystems. However, the

results reported here contributed to increase the knowledge about the main ecological processes and mechanisms through which the *C. fluminea* can change the abiotic properties and associated biota. The use of a multidisciplinary integrated approach combining ecology, microbiology and sediment chemistry contributed to increase the understanding of the *C. fluminea* impacts at the ecosystem level, which is particularly useful for the management and mitigation of the *C. fluminea* effects on both aquatic and adjacent terrestrial ecosystems.

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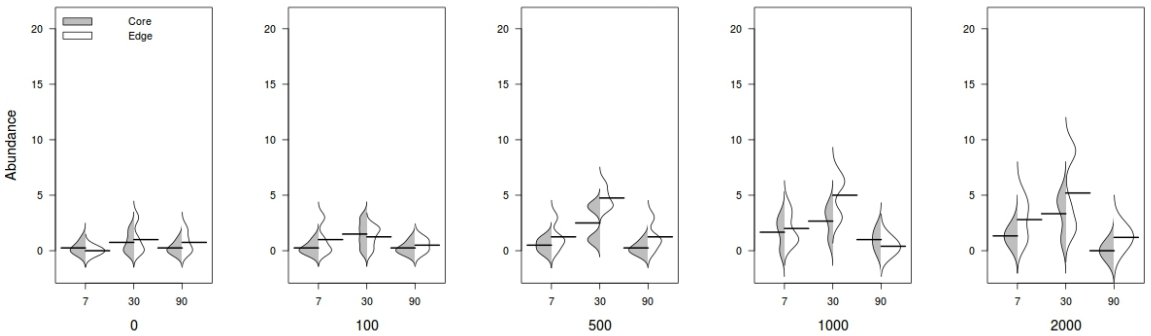
Supplementary material

A.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Abundance
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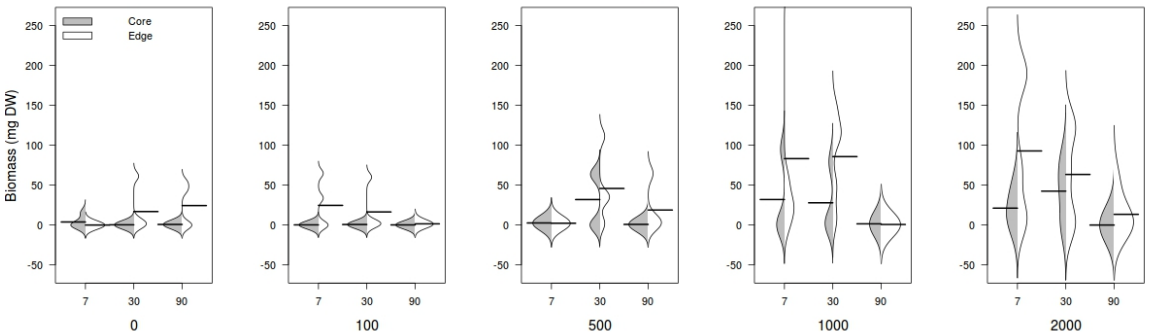
B.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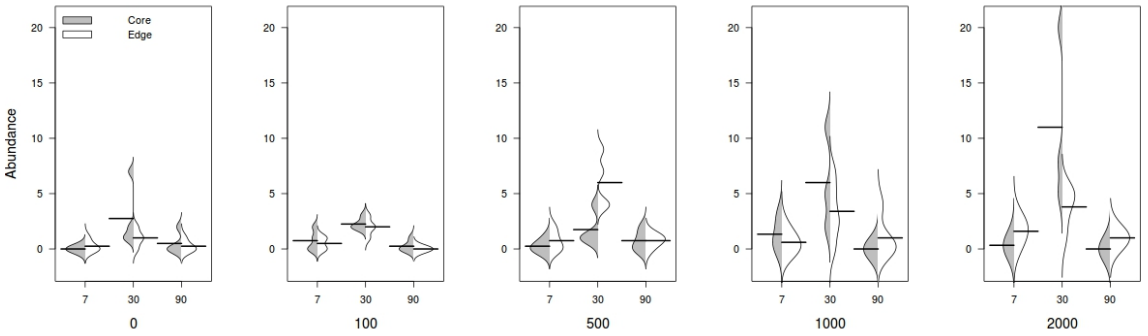
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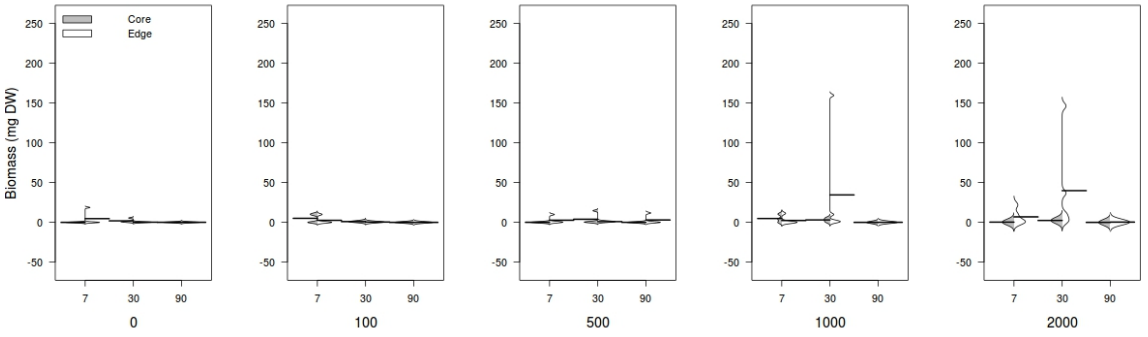
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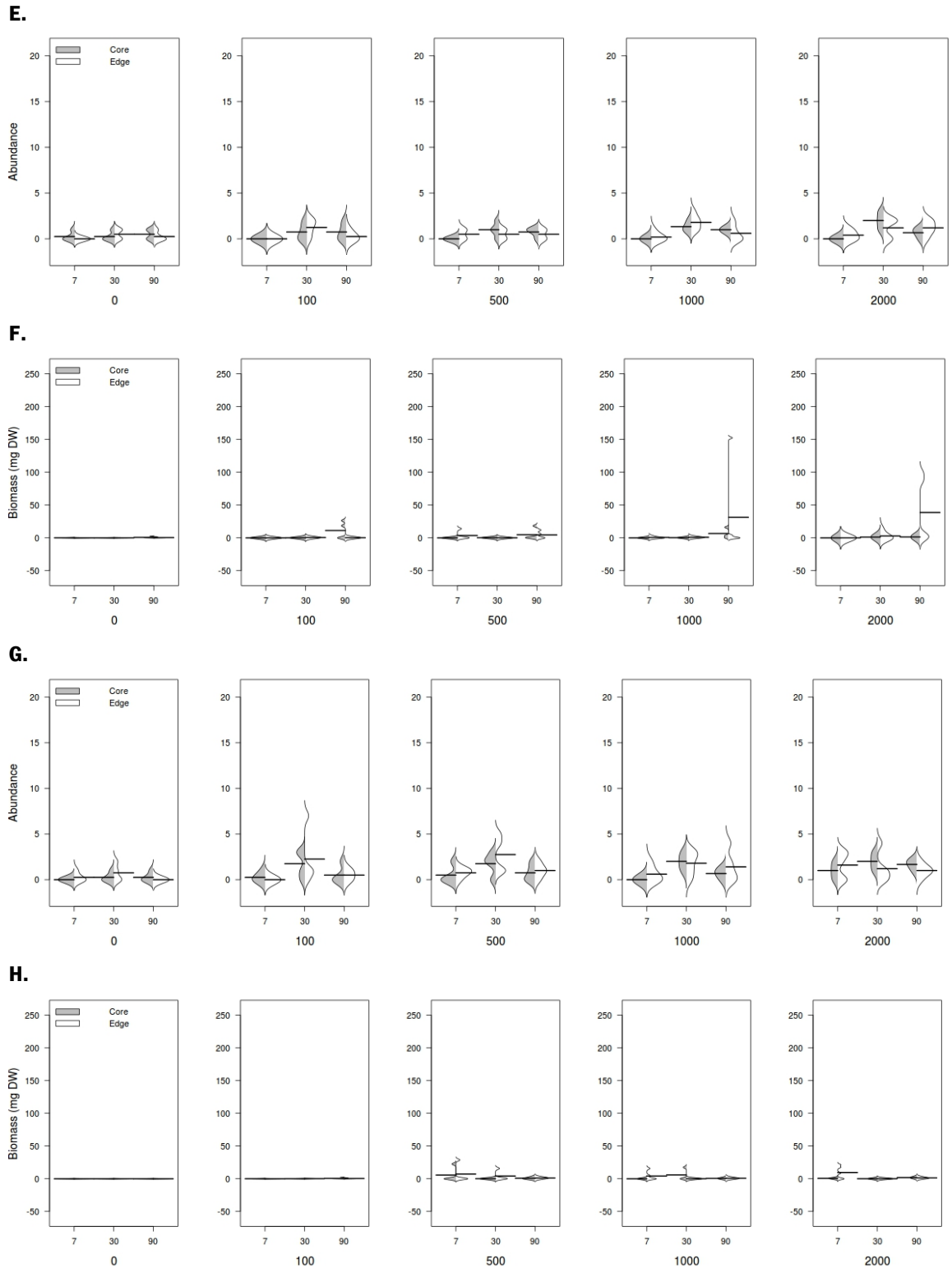

Figure S1. Relative abundance and biomass (mean ± SD) of each functional group (A) carnivores/scavengers' relative abundance, (B) carnivores/scavengers' biomass (mg DW), (C) omnivores' relative abundance, (D) omnivores' biomass, (E) herbivores' relative abundance, (F) herbivores' biomass, (G) detritivores' relative abundance and (H) detritivores' biomass in treatments with increasing *C. fluminea* density levels (0, 100, 500, 1000 and 2000 ind.m^{-2}), at different positions (core and edge) of the selected sampling site in the river bank of the Minho River (NW Iberian Peninsula) at 3 sampling time (7, 30 and 90 days).

Table S1. Three-way PERMANOVA results comparing the adult Diptera relative abundance among treatments with increasing *C. fluminea* density levels (0, 100, 500, 1000 and 2000 ind.m^{-2}) in the river bank of the Minho River (NW Iberian Peninsula) at different positions (core and edge) throughout sampling time (7, 30 and 90 days). ns = non-significant p-value. Different letters indicate significant differences among treatments.

Source	df	SS	MS	Pseudo-F	p-value
Density	4	19.63	4.91	30.14	< 0.001
Time	2	34.46	17.23	105.83	< 0.001
Position	1	0.97	0.97	5.98	≤ 0.01
Density x Time	8	30.11	3.76	23.12	< 0.001
Density x Position	4	1.41	0.35	2.16	ns
Time x Position	2	1.83	0.91	5.61	≤ 0.01
Density x Time x Position	8	2.11	0.26	1.62	ns
Residual	90	14.65	0.16		
Total	119	119			

Pairwise comparison

Time	Position	Density				
		0	100	500	1000	2000
7	core	0.00 ± 0.00^{a}	1.00 ± 0.82^{b}	2.75 ± 1.71^{bc}	4.67 ± 1.53^{cd}	9.00 ± 2.65^{de}
	edge	0.00 ± 0.00^{a}	1.50 ± 0.58^{b}	3.00 ± 0.82^{c}	8.40 ± 2.58^{d}	14.60 ± 5.41^{de}
30	core	0.00 ± 0.00	0.00 ± 0.00	0.25 ± 0.50	0.67 ± 0.58	0.67 ± 0.58
	edge	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.40 ± 0.55^{ab}	1.40 ± 0.89^{b}
90	core	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	edge	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Table S2. Three-way PERMANOVA results comparing the terrestrial invertebrate (A) relative abundance and (B) biomass among treatments with increasing *C. fluminea* density levels (0, 100, 500, 1000 and 2000 ind.m^{-2}) in the river bank of the Minho River (NW Iberian Peninsula) at different positions (core and edge) throughout sampling time (7, 30 and 90 days). ns = non-significant p-value. Different letters indicate significant differences among treatments.

A.

Source	df	SS	MS	Pseudo-F	p-value
Density	4	494.45	123.61	16.72	< 0.001
Time	2	1304.00	651.98	88.18	< 0.001
Position	1	11.11	11.11	1.50	ns
Density x Time	8	228.10	28.51	3.86	< 0.001
Density x Position	4	57.28	14.32	1.94	ns
Time x Position	2	8.40	4.20	0.57	ns
Density x Time x Position	8	159.87	19.98	2.70	≤ 0.01
Residual	90	665.42	7.39		
Total	119	2840.90			

Pairwise comparison

Time	Position	Density 0	100	500	1000	2000
7	core	0.50 ± 0.58^{a}	1.25 ± 1.50^{ab}	1.25 ± 0.96^{ab}	3.00 ± 2.65^{ab}	2.67 ± 1.15^{b}
	edge	0.50 ± 1.00^{a}	1.50 ± 1.29^{ab}	3.25 ± 1.70^{bc}	3.40 ± 1.52^{bcd}	6.40 ± 2.30^{ce}
30	core	4.00 ± 2.94^{a}	6.25 ± 2.87^{a}	7.00 ± 2.71^{ab}	12.00 ± 6.24^{ab}	18.33 ± 9.07^{b}
	edge	3.25 ± 0.96^{a}	6.75 ± 1.71^{b}	14.00 ± 4.40^{c}	12.00 ± 3.24^{cd}	11.40 ± 5.50^{bcde}
90	core	1.50 ± 1.29	1.75 ± 0.50	2.50 ± 1.00	2.67 ± 1.15	2.33 ± 0.58
	edge	1.25 ± 0.96^{a}	1.25 ± 0.96^{a}	3.50 ± 1.73^{ab}	3.40 ± 1.82^{ab}	4.40 ± 0.89^{b}

B.

Source	df	SS	MS	Pseudo-F	p-value
Density	4	49566.00	12392.00	4.29	≤ 0.01
Time	2	13675.00	6837.60	3.37	ns
Position	1	28661.00	28661.00	9.93	≤ 0.01
Density x Time	8	15548.00	1943.50	0.67	ns
Density x Position	4	16292.00	4073.00	1.41	ns
Time x Position	2	1377.10	688.56	0.24	ns
Density x Time x Position	8	5287.40	660.93	0.23	ns
Residual	90	2.5989E5	2887.70		
Total	119	4.1581E5			

Pairwise comparison

Time	Position	Density 0	100	500	1000	2000
7	core	3.93 ± 7.59	5.38 ± 6.21	8.30 ± 9.88	36.83 ± 54.14	22.03 ± 22.81
	edge	4.68 ± 9.36	27.20 ± 35.49	15.75 ± 12.10	90.34 ± 131.26	109.32 ± 87.22

Table S2. Continued.

30	core	2.20 ± 2.42	1.85 ± 1.41	36.21 ± 40.83	37.04 ± 37.95	46.14 ± 42.96
	edge	17.18 ± 29.75	17.46 ± 28.77	50.99 ± 53.84	121.29 ± 100.89	106.20 ± 109.63
90	core	1.58 ± 1.60	11.93 ± 12.86	6.43 ± 10.13	8.60 ± 8.72	3.10 ± 1.55
	edge	24.88 ± 28.68	2.05 ± 2.63	27.50 ± 34.73	32.80 ± 67.64	53.69 ± 47.39

Table S3. Three-way PERMANOVA results comparing the terrestrial invertebrate (A) species richness and (B) Shannon-Wiener index among treatments with increasing *C. fluminea* density levels (0, 100, 500, 1000 and 2000 ind.m^{-2}) in the river bank of the Minho River (NW Iberian Peninsula) at different positions (core and edge) throughout sampling time (7, 30 and 90 days). ns = non-significant p-value. Different letters indicate significant differences among treatments.

A.

Source	df	SS	MS	Pseudo-F	p-value
Density	4	183.94	45.99	19.67	< 0.001
Time	2	371.95	185.98	79.54	< 0.001
Position	1	16.10	16.10	6.88	≤ 0.01
Density x Time	8	46.93	5.87	2.51	< 0.05
Density x Position	4	23.50	5.87	2.51	< 0.05
Time x Position	2	0.73	0.37	0.16	ns
Density x Time x Position	8	8.19	1.02	0.44	ns
Residual	90	210.43	2.34		
Total	119	885.33			

Pairwise comparison

Time	Position	Density 0	100	500	1000	2000
7	core	0.50 ± 0.58^{a}	1.25 ± 1.50^{ab}	1.25 ± 0.96^{ab}	3.00 ± 2.65^{ab}	2.33 ± 0.58^{b}
	edge	0.50 ± 1.00^{a}	1.50 ± 1.29^{ab}	3.00 ± 1.63^{bc}	2.80 ± 1.10^{bcd}	5.00 ± 1.87^{ce}
30	core	2.25 ± 1.89^{a}	4.75 ± 1.89^{ab}	5.50 ± 1.29^{b}	7.33 ± 1.53^{b}	7.67 ± 2.31^{b}
	edge	2.50 ± 0.58^{a}	5.00 ± 0.82^{b}	8.25 ± 1.50^{c}	7.60 ± 1.52^{cd}	8.20 ± 3.49^{bcde}
90	core	1.25 ± 0.96	1.50 ± 0.58	2.25 ± 1.59	2.67 ± 1.15	1.67 ± 0.58
	edge	1.25 ± 0.96^{a}	1.25 ± 0.96^{a}	3.25 ± 2.06^{ab}	2.20 ± 1.10^{ab}	4.00 ± 1.22^{b}

B.

Source	df	SS	MS	Pseudo-F	p-value
Density	4	12.84	3.21	16.82	< 0.001
Time	2	19.84	9.92	51.98	< 0.001
Position	1	1.52	1.52	7.94	≤ 0.01
Density x Time	8	2.11	0.26	1.38	ns
Density x Position	4	1.98	0.49	2.59	< 0.05
Time x Position	2	4.965E-2	2.4896E-2	0.13	ns
Density x Time x Position	8	0.62	7.6896E-2	0.40	ns
Residual	90	17.18	0.19		
Total	119	58.01			

Pairwise comparison

Time	Position	Density 0	100	500	1000	2000
7	core	0.00 ± 0.00^{a}	0.45 ± 0.54^{ab}	0.35 ± 0.40^{ab}	1.00 ± 0.87^{ab}	0.81 ± 0.20^{b}
	edge	0.17 ± 0.35^{a}	0.45 ± 0.54^{ab}	0.94 ± 0.67^{abc}	0.94 ± 0.36^{b}	1.51 ± 0.34^{c}

Table S3. Continued.

30	core	0.56 ± 0.74^{a}	1.42 ± 0.50^{ab}	1.61 ± 0.21^{b}	1.84 ± 0.10^{b}	1.62 ± 0.34^{ab}
	edge	0.88 ± 0.22^{a}	1.52 ± 0.18^{b}	1.96 ± 0.18^{c}	1.89 ± 0.25^{cd}	1.92 ± 0.45^{bcde}
90	core	0.33 ± 0.38	0.35 ± 0.40	0.69 ± 0.57	0.92 ± 0.40	0.44 ± 0.38
	edge	0.35 ± 0.40^{a}	0.35 ± 0.40^{a}	1.00 ± 0.74^{ab}	0.62 ± 0.48^{a}	1.33 ± 0.31^{b}

Table S4. Three-way PERMANOVA results comparing the carnivores/scavengers' (A) relative abundance and (B) biomass among treatments with increasing *C. fluminea* density levels (0, 100, 500, 1000 and 2000 ind.m^{-2}) in the river bank of the Minho River (NW Iberian Peninsula) at different positions (core and edge) throughout sampling time (7, 30 and 90 days). ns = non-significant p-value. Different letters indicate significant differences among treatments.

A.

Source	df	SS	MS	Pseudo-F	p-value
Density	4	16.81	4.20	8.44	< 0.001
Time	2	28.68	14.34	28.81	< 0.001
Position	1	5.03	5.03	10.10	≤ 0.01
Density x Time	8	9.93	1.24	2.49	< 0.05
Density x Position	4	2.43	0.61	1.22	ns
Time x Position	2	1.03	0.52	1.04	ns
Density x Time x Position	8	2.61	0.33	0.65	ns
Residual	90	44.80	0.45		
Total	119	119			

Pairwise comparison

Time	Position	Density 0	100	500	1000	2000
7	core	0.25 ± 0.50^{a}	0.25 ± 0.50^{a}	0.50 ± 0.58^{ab}	1.67 ± 1.53^{ab}	1.33 ± 0.58^{b}
	edge	0.00 ± 0.00^{a}	1.00 ± 1.41^{ab}	1.25 ± 1.26^{ab}	2.00 ± 1.41^{b}	2.80 ± 1.79^{b}
30	core	0.75 ± 0.96	1.50 ± 1.29	2.50 ± 1.73	2.67 ± 1.53	3.33 ± 2.08
	edge	1.00 ± 1.41^{a}	1.25 ± 0.96^{a}	4.75 ± 0.96^{b}	5.00 ± 1.87^{b}	5.20 ± 3.56^{ab}
90	core	0.25 ± 0.50	0.25 ± 0.50	0.25 ± 0.50	1.00 ± 1.00	0.00 ± 0.00
	edge	0.75 ± 0.96	0.50 ± 0.58	1.25 ± 1.26	0.40 ± 0.55	1.20 ± 0.84

B.

Source	df	SS	MS	Pseudo-F	p-value
Density	4	10.26	2.57	3.11	< 0.05
Time	2	6.75	3.38	4.10	< 0.05
Position	1	6.02	6.02	7.31	≤ 0.01
Density x Time	8	0.82	1.10	1.34	ns
Density x Position	4	1.71	0.43	0.52	ns
Time x Position	2	0.74	0.37	0.45	ns
Density x Time x Position	8	3.47	0.43	0.53	ns
Residual	90	74.13	0.82		
Total	119	119			

Pairwise comparison

Time	Position	Density 0	100	500	1000	2000
7	core	3.83 ± 7.65	0.23 ± 0.45	2.60 ± 3.31	32.00 ± 54.48	21.17 ± 22.72
	edge	0.00 ± 0.00	24.65 ± 31.15	2.28 ± 2.89	83.26 ± 128.58	92.88 ± 92.17

Table S4. Continued.

30	core	0.28 ± 0.49	0.68 ± 0.76	31.95 ± 36. 80	27.90 ± 44.55	42.47 ± 40.68
	edge	16.90 ± 29.75	16.43 ± 29.15	45.85 ± 46.33	85.78 ± 55.98	63.42 ± 60.18
90	core	0.75 ± 1.50	0.18 ± 0.35	0.80 ± 1.60	1.50 ± 1.30	0.00 ± 0.00
	edge	24.40 ± 28.45	1.50 ± 2.27	18.83 ± 30.88	0.68 ± 1.36	13.42 ± 24.02

Table S5. Three-way PERMANOVA results comparing the omnivores' (A) relative abundance and (B) biomass among treatments with increasing *C. fluminea* density levels (0, 100, 500, 1000 and 2000 ind.m^{-2}) in the river bank of the Minho River (NW Iberian Peninsula) at different positions (core and edge) throughout sampling time (7, 30 and 90 days). ns = non-significant p-value. Different letters indicate significant differences among treatments.

A.

Source	df	SS	MS	Pseudo-F	p-value
Density	4	9.91	2.48	5.24	< 0.001
Time	2	43.12	21.56	45.61	< 0.001
Position	1	0.45	0.45	0.96	ns
Density x Time	8	12.36	1.54	3.27	≤ 0.01
Density x Position	4	4.62	1.15	2.44	ns
Time x Position	2	2.81	1.41	2.98	ns
Density x Time x Position	8	14.86	1.86	3.93	< 0.001
Residual	90	42.54	0.47		
Total	119	119			

Pairwise comparison

Time	Position	Density 0	100	500	1000	2000
7	core	0.00 ± 0.00	0.75 ± 0.96	0.25 ± 0.50	1.33 ± 1.53	0.33 ± 0.58
	edge	0.25 ± 0.50	0.50 ± 0.58	0.75 ± 0.96	0.60 ± 0.89	1.60 ± 1.14
30	core	2.75 ± 2.87	2.25 ± 0.50	1.75 ± 1.50	6.00 ± 4.58	11.00 ± 7.94
	edge	1.00 ± 0.82^{a}	2.00 ± 0.82^{ac}	6.00 ± 2.45^{bc}	3.40 ± 2.70^{abc}	3.80 ± 1.79^{c}
90	core	0.50 ± 1.00	0.25 ± 0.50	0.75 ± 0.96	0.00 ± 0.00	0.00 ± 0.00
	edge	0.25 ± 0.50^{a}	0.00 ± 0.00^{ab}	0.75 ± 0.50^{ac}	1.00 ± 1.73^{ad}	1.00 ± 0.00^{bcd}

B.

Source	df	SS	MS	Pseudo-F	p-value
Density	4	2.85	0.71	0.72	ns
Time	2	3.63	1.81	1.83	ns
Position	1	1.86	1.86	1.87	ns
Density x Time	8	5.25	0.66	0.66	ns
Density x Position	4	2.68	0.67	0.68	ns
Time x Position	2	2.15	1.07	1.09	ns
Density x Time x Position	8	5.28	0.66	0.67	ns
Residual	90	89.11	0.99		
Total	119	119			

Pairwise comparison

Time	Position	Density 0	100	500	1000	2000
7	core	0.00 ± 0.00	5.08 ± 5.90	0.08 ± 0.15	4.83 ± 5.24	0.30 ± 0.52
	edge	4.68 ± 9.35	2.55 ± 4.97	2.68 ± 4.83	2.36 ± 5.00	6.90 ± 9.39

Table S5. Continued.

30	core	1.90 ± 2.43	0.98 ± 0.71	3.93 ± 7.19	3.07 ± 2.65	2.40 ± 1.47
	edge	0.20 ± 0.14	0.40 ± 0.29	0.96 ± 0.48	34.52 ± 70.09	39.86 ± 61.15
90	core	0.10 ± 0.20	0.03 ± 0.05	0.03 ± 0.05	0.00 ± 0.00	0.00 ± 0.00
	edge	0.10 ± 0.20	0.00 ± 0.00	3.00 ± 5.87	0.08 ± 0.13	0.32 ± 0.55

Table S6. Three-way PERMANOVA results comparing the herbivores' (A) relative abundance and (B) biomass among treatments with increasing *C. fluminea* density levels (0, 100, 500, 1000 and 2000 ind.m^{-2}) in the river bank of the Minho River (NW Iberian Peninsula) at different positions (core and edge) throughout sampling time (7, 30 and 90 days). ns = non-significant p-value. Different letters indicate significant differences among treatments.

A.

Source	df	SS	MS	Pseudo-F	p-value
Density	4	9.96	2.49	3.49	≤ 0.01
Time	2	28.20	14.10	19.78	< 0.001
Position	1	2.1963E-3	2.1963E-3	3.0813E-3	ns
Density x Time	8	7.81	0.98	1.37	ns
Density x Position	4	0.23	5.7817E-2	8.1114E-2	ns
Time x Position	2	0.97	0.49	0.68	ns
Density x Time x Position	8	7.77	0.97	1.36	ns
Residual	90	64.15	0.71		
Total	119	119			

Pairwise comparison

Time	Position	Density 0	100	500	1000	2000
7	core	0.25 ± 0.50	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	edge	0.00 ± 0.00	0.00 ± 0.00	0.50 ± 0.58	0.20 ± 0.45	0.40 ± 0.55
30	core	0.25 ± 0.50^{a}	0.75 ± 0.96^{ab}	1.00 ± 0.82^{ab}	1.33 ± 0.58^{b}	2.00 ± 1.00^{b}
	edge	0.50 ± 0.58^{a}	1.25 ± 0.96^{ab}	0.50 ± 0.58^{a}	1.80 ± 0.84^{b}	1.20 ± 1.10^{ab}
90	core	0.50 ± 0.58	0.75 ± 0.96	0.75 ± 0.50	1.00 ± 0.00	0.67 ± 0.58
	edge	0.25 ± 0.50	0.25 ± 0.50	0.50 ± 0.58	0.60 ± 0.89	1.20 ± 0.84

B.

Source	df	SS	MS	Pseudo-F	p-value
Density	4	2.62	0.66	0.68	ns
Time	2	6.73	3.36	3.50	< 0.05
Position	1	1.23	1.23	1.28	ns
Density x Time	8	4.57	0.57	0.59	ns
Density x Position	4	3.10	0.78	0.81	ns
Time x Position	2	1.67	0.83	0.87	ns
Density x Time x Position	8	5.99	0.75	0.78	ns
Residual	90	86.62	0.96		
Total	119	119			

Pairwise comparison

Time	Position	Density 0	100	500	1000	2000
7	core	0.10 ± 0.20	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	edge	0.00 ± 0.00	0.00 ± 0.00	3.58 ± 6.89	0.60 ± 1.34	0.04 ± 0.05

Table S6. Continued.

30	core	0.03 ± 0.05[a]	0.10 ± 0.14[ab]	0.20 ± 0.18[ab]	0.23 ± 0.15[b]	1.17 ± 1.08[ab]
	edge	0.05 ± 0.06	0.45 ± 0.54	0.10 ± 0.14	0.90 ± 1.08	2.82 ± 6.08
90	core	0.70 ± 1.21	11.2 ± 13.32	4.80 ± 9.00	6.57 ± 8.41	1.37 ± 1.52
	edge	0.38 ± 0.75	0.20 ± 0.40	4.48 ± 5.70	31.28 ± 67.79	38.60 ± 50.19

Table S7. Three-way PERMANOVA results comparing the detritivores' (A) relative abundance and (B) biomass among treatments with increasing *C. fluminea* density levels (0, 100, 500, 1000 and 2000 ind.m^{-2}) in the river bank of the Minho River (NW Iberian Peninsula) at different positions (core and edge) throughout sampling time (7, 30 and 90 days). ns = non-significant p-value. Different letters indicate significant differences among treatments.

A.

Source	df	SS	MS	Pseudo-F	p-value
Density	4	12.08	3.02	3.49	≤ 0.01
Time	2	17.83	8.92	10.29	< 0.001
Position	1	0.52	0.52	0.60	ns
Density x Time	8	6.37	0.78	0.92	ns
Density x Position	4	1.34	0.33	0.39	ns
Time x Position	2	0.24	0.12	0.14	ns
Density x Time x Position	8	2.98	0.37	0.43	ns
Residual	90	77.97	0.87		
Total	119	119			

Pairwise comparison

Time	Position	Density 0	100	500	1000	2000
7	core	0.00 ± 0.00	0.25 ± 0.50	0.50 ± 1.00	0.00 ± 0.00	1.00 ± 1.00
	edge	0.25 ± 0.50^{a}	0.00 ± 0.00^{ab}	0.75 ± 0.50^{ac}	0.60 ± 0.89^{abc}	1.60 ± 1.52^{abc}
30	core	0.25 ± 0.50^{a}	1.75 ± 1.50^{ab}	1.75 ± 1.26^{ab}	2.00 ± 1.00^{b}	2.00 ± 1.00^{b}
	edge	0.75 ± 0.96	2.25 ± 3.20	2.75 ± 2.06	1.80 ± 1.30	1.20 ± 1.65
90	core	0.25 ± 0.50^{a}	0.50 ± 1.00^{ab}	0.75 ± 0.96^{ab}	0.67 ± 0.58^{ab}	1.67 ± 0.58^{b}
	edge	0.00 ± 0.00^{a}	0.50 ± 0.58^{ab}	1.00 ± 0.82^{b}	1.40 ± 1.67^{ab}	1.00 ± 0.71^{b}

B.

Source	df	SS	MS	Pseudo-F	p-value
Density	4	7.60	1.90	2.00	ns
Time	2	3.79	1.90	2.00	ns
Position	1	0.93	0.93	0.98	ns
Density x Time	8	7.00	0.87	0.92	ns
Density x Position	4	2.12	0.53	0.56	ns
Time x Position	2	2.69	1.34	1.42	ns
Density x Time x Position	8	6.60	0.83	0.87	ns
Residual	90	85.30	0.95		
Total	119	119			

Pairwise comparison

Time	Position	Density 0	100	500	1000	2000
7	core	0.00 ± 0.00	0.08 ± 0.15	5.63 ± 11.25	0.00 ± 0.00	0.57 ± 0.51
	edge	0.003 ± 0.01	0.00 ± 0.00	7.23 ± 14.32	4.12 ± 6.81	9.50 ± 9.79

Table S7. Continued.

30	core	0.003 ± 0.01	0.10 ± 0.14	0.13 ± 0.10	5.84 ± 10.02	0.10 ± 0.10
	edge	0.03 ± 0.05	0.18 ± 0.21	4.08 ± 7.75	0.08 ± 0.05	0.10 ± 0.17
90	core	0.03 ± 0.05	0.53 ± 1.05	0.80 ± 1.47	0.53 ± 0.84	1.73 ± 1.40
	edge	0.00 ± 0.00	0.35 ± 0.64	1.20 ± 1.15	0.76 ± 1.04	1.34 ± 1.33

Table S8. Mean (±SEM) of organic C and total N (%), NH_4^+, NO_2^-, NO_3^-, PO_4^{3-}, Ca and K (mg kg^{-1}) and two-way PERMANOVA results at different densities of *C. fluminea* (0, 100, 500, 1000 and 2000 ind. m^{-2}) and sampling times (7, 30 and 90 days) and their interaction term. * = $p < 0.05$; ns = non-significant..

	Corbicula fluminea densities					Two-way PERMANOVA		
Nutrients	0	100	500	1000	2000	Density	Time	Interaction
Carbon								
7	4.20 ± 1.20	4.73 ± 0.14[a]	3.44 ± 0.51	4.95 ± 2.17	4.23 ± 0.85			
30	2.47 ± 0.65	3.86 ± 0.17[b]	3.36 ± 0.11	2.10 ± 0.73	2.57 ± 0.93	Pseudo-F = 0.21[ns]	Pseudo-F = 3.95*	Pseudo-F = 0.52[ns]
90	3.61 ± 1.58	2.30 ± 0.76[c]	2.11 ± 0.48	2.14 ± 1.24	3.02 ± 1.29			
Nitrogen								
7	0.28 ± 0.08	0.30 ± 0.01	0.24 ± 0.03	0.40 ± 0.12	0.34 ± 0.07			
30	0.21 ± 0.03	0.28 ± 0.01	0.25 ± 0.01	0.21 ± 0.03	0.26 ± 0.05	Pseudo-F = 0.58[ns]	Pseudo-F = 2.32[ns]	Pseudo-F = 0.66[ns]
90	0.29 ± 0.11	0.21 ± 0.03	0.19 ± 0.01	0.24 ± 0.06	0.28 ± 0.08			
Ammonium								
7	15.66 ± 7.44	20.81 ± 5.20	36.72 ± 4.46	87.64 ± 42.57	106.43 ± 21.59			
30	8.82 ± 5.84	6.14 ± 0.80	31.39 ± 3.36	26.80 ± 0.27	61.93 ± 15.83	Pseudo-F = 15.51*	Pseudo-F = 6.31*	Pseudo-F = 0.93[ns]
90	8.09 ± 4.00	6.79 ± 1.99	25.16 ± 6.77	39.88 ± 16.30	67.27 ± 14.14			
Nitrite								
7	0.44 ± 0.04	6.92 ± 8.06	0.59 ± 0.08	0.57 ± 0.05	0.43 ± 0.02			
30	1.58 ± 0.39	5.14 ± 0.94	11.95 ± 4.25	12.03 ± 1.23	11.96 ± 6.80	Pseudo-F = 2.43[ns]	Pseudo-F = 4.74*	Pseudo-F = 1.60[ns]
90	1.37 ± 0.06	5.38 ± 2.92	18.53 ± 8.33	8.16 ± 2.93	3.31 ± 0.43			
Nitrate								
7	0.18 ± 0.09	0.13 ± 0.00	0.17 ± 0.11	0.13 ± 0.00	0.13 ± 0.00			
30	1.97 ± 0.60	6.47 ± 0.89	13.41 ± 4.44	12.67 ± 1.16	12.97 ± 8.73	Pseudo-F = 4.99*	Pseudo-F = 42.87*	Pseudo-F = 1.75[ns]
90	2.06 ± 1.27	4.40 ± 3.52	15.59 ± 3.37	8.80 ± 3.36	2.16 ± 0.82			
Phosphate								
7	7.24 ± 1.03	7.22 ± 0.81	4.68 ± 0.95	8.23 ± 2.60	12.50 ± 3.72			
30	7.68 ± 1.51	7.35 ± 1.23	7.20 ± 0.73	5.51 ± 0.70	15.90 ± 6.35	Pseudo-F = 5.66*	Pseudo-F = 1.14[ns]	Pseudo-F = 0.48[ns]
90	5.64 ± 1.33	4.50 ± 0.40	4.39 ± 0.62	6.62 ± 2.56	14.63 ± 3.38			
Calcium								
7	15.14 ± 7.51	29.72 ± 14.51	28.07 ± 10.76	51.76 ± 24.04	31.61 ± 17.97			
30	19.85 ± 5.18	27.43 ± 5.93	32.54 ± 6.28	18.41 ± 9.22	16.40 ± 5.89	Pseudo-F = 0.35[ns]	Pseudo-F = 1.00[ns]	Pseudo-F = 0.96[ns]
90	34.20 ± 9.06	23.88 ± 3.54	31.57 ± 6.07	25.20 ± 14.13	53.90 ± 23.91			
Potassium								
7	23.90 ± 6.83	34.15 ± 2.10	36.42 ± 1.93	61.84 ± 22.18	58.26 ± 19.32			
30	21.70 ± 5.51	26.62 ± 4.12	32.56 ± 2.05	22.09 ± 5.81	33.33 ± 10.23	Pseudo-F = 2.42[ns]	Pseudo-F = 3.02[ns]	Pseudo-F = 0.72[ns]
90	24.25 ± 4.67	21.55 ± 3.58	30.26 ± 5.04	31.72 ± 14.43	49.72 ± 15.85			

Table S9. Three-way PERMANOVA results comparing the fungal biomass in the five treatments (control, live, open, soft and total), two sampling times (11 and 33 days) in the presence or absence of invertebrates (with and without). ns = non-significant p-value. Different letters indicate significant differences among treatments.

Source	df	SS	MS	Pseudo-F	p-value
Treatment	4	302.81	75.70	12.39	≤ 0.001
Time	1	394.10	394.10	64.51	≤ 0.001
Presence of invertebrates	1	700.77	700.77	114.71	≤ 0.001
Treatment × Time	4	264.31	66.08	10.82	≤ 0.001
Treatment × Presence of invert.	4	44.20	11.05	1.81	ns
Time × Presence of invert.	1	88.54	88.54	14.50	≤ 0.001
Treatment × Time × Presence of invert.	4	48.64	12.16	1.99	ns
Residual	60	366.58	6.11		
Total	79	2209.90			

Pairwise comparison

Presence of invert.	Time	Treatment				
		Control	Live	Open	Soft	Total
With	11	119.79 ± 51.45^{a}	213.56 ± 52.06^{b}	279.92 ± 40.92^{bc}	144.12 ± 34.84^{ab}	206.66 ± 54.11^{abc}
	33	287.11 ± 2.75^{ab}	419.62 ± 112.76^{a}	280.99 ± 156.49^{ab}	206.70 ± 128.40^{b}	180.94 ± 60.65^{b}
Without	11	32.26 ± 8.86^{a}	31.12 ± 11.06^{a}	61.85 ± 6.40^{b}	20.22 ± 8.16^{a}	21.85 ± 4.76^{a}
	33	125.73 ± 11.43^{a}	435.80 ± 103.66^{b}	96.21 ± 12.48^{c}	141.61 ± 31.09^{ad}	53.68 ± 22.74^{a}

Table S10. Three-way PERMANOVA results comparing the leaf mass loss in the five treatments (control, live, open, soft and total), two sampling times (11 and 33 days) in the presence or absence of invertebrates (with and without). ns = non-significant p-value. Different letters indicate significant differences among treatments.

Source	df	SS	MS	Pseudo-F	p-value
Treatment	4	2.45	0.61	4.80	≤ 0.01
Time	1	58.81	58.81	460.48	≤ 0.001
Presence of invertebrates	1	7.01	7.01	54.89	≤ 0.001
Treatment × Time	4	0.82	0.21	1.61	ns
Treatment × Presence of invert.	4	0.64	0.16	1.26	ns
Time × Presence of invert.	1	0.02	0.02	0.13	ns
Treatment × Time × Presence of invert.	4	0.56	0.14	1.09	ns
Residual	58	7.41	0.13		
Total	77	77			

Pairwise comparison

Presence of invert.	Time	Treatment				
		Control	Live	Open	Soft	Total
With	11	38.80 ± 2.28	41.65 ± 2.26	40.38 ± 1.43	38.53 ± 4.03	38.47 ± 1.98
	33	53.34 ± 2.75[a]	62.68 ± 2.81[b]	56.97 ± 3.37[a]	52.29 ± 5.06[a]	56.80 ± 1.85[a]
Without	11	34.04 ± 3.09	34.61 ± 2.26	33.85 ± 4.15	33.40 ± 0.38	34.71 ± 1.67
	33	50.58 ± 2.52[ab]	54.32 ± 5.22[a]	49.99 ± 2.83[ab]	50.40 ± 7.27[ab]	46.81 ± 2.62[b]