Daniel de Jesus Andrade e Crespo

Interactive effects of global ecological changes in the ecosystem functioning of estuarine systems: invasive species and climate change

Tese de doutoramento em Biociências, ramo de especialização em Ecologia Marinha, orientada pelo Professor Doutor Miguel Ângelo do Carmo Pardal e pela Doutora Marina Paula Dolbeth Henriques da Silva, apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

Outubro de 2016

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UNIVERSIDADE DE COIMBRA

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FCTUC FACULDADE DE CIÊNCIAS
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UNIVERSIDADE DE COIMBRA

Doctoral thesis in Biosciences, scientific area of Marine Ecology, supervised by Prof. Dr. Miguel Ângelo do Carmo Pardal e Dr. Marina Paula Dolbeth Henriques da Silva, presented to the Faculty of Sciences and Technology of the University of Coimbra

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Department of Life Sciences | University of Coimbra
Coimbra | 2016

This thesis was supported by:

The Portuguese Foundation for Science and Technology (FCT) through a Ph.D. grant attributed to Daniel de Jesus Andrade e Crespo (SFRH/BD/80252/2011), subsidized by the European Social Fund and MCTES (Portuguese Ministry of Science, Technology and Higher Education), through the POPH (Human Potential Operational Programme), QREN (National Strategic Reference Framework) and COMPETE (Programa Operacional Factores de Competitividade).

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PORTUGAL 2007.2013

GOVERNO DA REPÚBLICA PORTUGUESA

UNIÃO EUROPEIA
Fundo Social Europeu

This thesis is based on the following manuscripts:

This thesis includes four manuscripts listed below, published or submitted for publication in scientific journals in the areas of ecology and marine biology:

Chapter I

Crespo D, Dolbeth M, Leston S, Sousa R, Pardal, MA (2015) Distribution of *Corbicula fluminea* (Müller, 1774) in the invaded range: a geographic approach with notes on species traits variability. Biol Invasions 17:2087–2101. doi: 10.1007/s10530-015-0862-y

Chapter II

Crespo D, Leston S, Martinho F, Pardal, MA, Dolbeth M. (2016) Survival of *Corbicula fluminea* (Müller, 1774) in a natural salinity and temperature gradient: a field experiment in a temperate estuary. Hydrobiologia (in press). doi: 10.1007/s10750-016-2887-3

Chapter III

Crespo D, Solan M, Leston S, Pardal MA, Dolbeth M. Ecological consequences of invasion across the freshwater-marine transition in a warming world. Submitted for publication in Biological Invasions

Chapter IV

Crespo D, Grilo TF, Baptista J, Lillebø AI, Coelho JP, Cássio F, Fernandes I, Pascoal C, Pardal MP, Dolbeth M. New climatic targets for climate change: does a 2ºC temperature rise affect estuarine benthic communities? Manuscript

This Thesis should be cited as:

Crespo D. (2016) Interactive effects of global ecological changes in the ecosystem functioning of estuarine systems: invasive species and climate change. PhD Thesis, University of Coimbra, Coimbra, Portugal, 196pp**.**

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Acknowledgements/Agradecimentos

É através das nossas histórias partilhadas que construímos as nossas narrativas pessoais e é sobre estas narrativas que nos debruçamos antes de seguir rumo ao futuro. Somos seres sociais, gregários, em que tudo o que faz de nós indivíduos é um reflexo das ligações interpessoais, amizades, colaborações e afetos pelos quais viajamos, absorvendo experiências, conhecimento, para dar forma às nossas próprias experiências e conhecimento.

Quero com isto dizer que um exercício de natureza científica, como a elaboração de uma tese, é mais que o debruçar reflexivamente sobre determinada temática: é sim o produto do trabalho coletivo, da paciência infindável e da abnegação de um conjunto de pessoas às quais a devida retribuição será sempre utópica, restando, portanto, deixar a minha humilde homenagem nas breves palavras que se seguem.

Começando pelas pessoas que mais condições criaram para que esta tese pudesse tornar-se realidade, os meus orientadores: Doutor Miguel Pardal e Doutora Marina Dolbeth.

Obrigado Miguel, por me teres dado a oportunidade de ser aquilo que sempre sonhei ser, “cientista”. Obrigado por teres acreditado e me teres encaminhado, logo a partir do momento em que bati à porta do gabinete, à procura de um estágio. Ainda cá estou, e mais perto de ser um desses “cientistas”! A tua visão abrangente sobre os assuntos aqui discutidos, bem como os teus conselhos, foram sempre uma mais-valia para este trabalho.

Obrigado Marina! Sem a tua orientação e a tua dedicação, bem como a tua permanente disponibilidade, esta tese não teria sido possível. Obrigado pelas palavras motivadoras, pelas horas perdidas (ou ganhas, conforme a perspetiva) de

volta dos textos, dos dados e da estatística. Foi uma honra ser o teu 1º aluno de Doutoramento, e é um privilégio ter a tua amizade!

Obrigado a ambos pelos ensinamentos e pela inspiração que foram nesta fase do meu percurso pessoal. A minha dívida para convosco será eterna.

Um sentido agradecimento a todos os co-autores dos artigos que deram origem a este trabalho, que também é vosso: Marina Dolbeth, Sara Leston, Ronaldo Sousa, Miguel A. Pardal, Filipe Martinho, Martin Solan, Tiago F. Grilo, Joana Baptista, Ana Isabel Lillebø, João Pedro Coelho, Cláudia Pascoal, Fernanda Cássio, Isabel Fernandes.

Um agradecimento especial à Sara, pelas horas partilhadas de laboratório e de viagens ao estuário. Ganhámos memórias de dores nas costas e nos braços, bonitas fotos de areia fluorescente e amizade!

Também tenho de estender este agradecimento à Joana Baptista e ao Tiago Grilo, cujas contribuições para este trabalho foram fundamentais.

To Martin Solan, for being such a wonderful host at Southampton, and for all the scientific and technical advice. To Matthias, Christina, Marla and Louise, because you made my stay in Southampton an even better experience!

À Joana Oliveira e ao João Rito, porque irei sempre preservar as memórias das nossas aventuras conjuntas com amostragens, análises, triagens e identificações! Foram períodos agitados nas nossas vidas, mas sobrevivemos para contar. Fomos uma excelente equipa!

Filipe, Sónia, Ligia, Patricia, obrigado pela amizade, e pelo exemplo de cientistas que pretendo ser! Quando for grande quero ser como vocês! Joana Baptista, Joana

Falcão, João Rosa, João Neves, Marielena, companhia de laboratório e de tantos almoços e outras tantas conversas “non-sense”, obrigado por aturarem o meu “mau feitio” e terem sentido de humor. Pieter, José Xavier, obrigado pelas reflexões, disfarçadas de voltas de bicicleta, sobre a forma de estar na ciência.

A todos os amigos envolvidos nas minhas “outras atividades”: os meus companheiros das voltas de bicicleta, os ensaios e concertos com a Barca. A vossa companhia foi imprescindível para reduzir o stress e manter a sanidade nestes últimos tempos.

À família, pai, mãe e irmão. Foram pilares da minha construção pessoal e sei que sentem o orgulho na conclusão de mais este capítulo da minha vida. Só vos tenho a agradecer por estarem sempre presentes na minha vida, por terem a porta aberta para mim sempre com uma frase de incentivo. Obrigado pelo vosso apoio incondicional.

Por fim, e nunca menos importante, à minha querida Milene. Dizias “Eu acredito em ti, tu vais conseguir”: a tua palavra foi o maior incentivo de todos, o teu acreditar impulsionou-me para conseguir terminar este manuscrito, mesmo quando não via as coisas a avançar. Obrigado por estares ao meu lado durante todo este tempo, por teres abdicado de realizar tantas coisas para me fazer companhia enquanto escrevia, por todo o amor e carinho que me dedicaste. Obrigado por me entenderes tão bem.

Content

Summary and Keywords

The human species is responsible for changes in the environment since its appearance. The rate of those changes increased as civilizational advances required progressively more resources, which led to the globalization of pressures and impacts on biosphere and the planet. Among those global trends, we may find global warming and the increase in the number of extreme climatic events. These are responsible for a decline in biodiversity. Global changes are threatening biodiversity-based stability, with highly unpredictable outcomes. Current changes have a global manifestation, but its effects should be studied locally, where they may be more notorious.

Anthropogenic pressures are felt across all ecosystems, and estuarine systems are no exception. Estuaries are under large cyclic environmental parameters oscillations, beside strong gradients from freshwater to marine conditions, shaping communities within estuaries. Nevertheless, estuaries are responsible for a large amount of biological processes and functions that are fundamental for human populations. Due to the cyclic environmental conditions of estuaries, the effects of global changes may be concealed. However, some of the global changes' effects are already noticeable, such as declines in biodiversity. The biodiversity reduction due to anthropogenic pressures is impairing ecosystem stability and functioning. As such, the Biodiversity-Ecosystem Functioning is one of the most important debates in Ecology and more recently in Marine Ecology. Ecosystem processes and functions are influenced by biodiversity modifications induced by global changes, affecting ecosystem stability. The analysis of natural processes such as bioturbation allows us a better knowledge on the relationships between biodiversity and ecosystem functioning.

One of the most recognized sources of biodiversity reduction is the transport and introduction of non-native species, with invasive potential, and which effects are

enhanced by the interactions with other global changes pressures. Estuaries are often close to large human settlements and are areas where several activities linked with biological invasion are carried out, thus becoming highly susceptible areas to the introduction of non-native invasive species. The bivalve *Corbicula fluminea* is one of the most invasive freshwater organisms and is able to invade the oligohaline sections of estuaries. The species, with its origin in Southeast Asia, has currently an almost global distribution.

In order to understand how global changes interact with biodiversity shifts and how these impair estuarine ecosystems' functioning, we tried to assess which ecosystem functions are affected by the introduction of non-native species and by global climatic changes. The chapter I describes the paths of the invasion and worldwide dispersion of *C. fluminea*, as well as the chronology of the invasion, based in scientific literature and public reports. Some biogeographic tendencies were observed. The species is limited by altitude and low winter temperature. Nevertheless, global warming and local changes events may be responsible for an increase in the current range of the species. Chapter II describes a field experience where the survival ability of *C. fluminea* along the gradient of the Mondego river estuary was tested. The current limits of the species in this system were defined, as well as its population structure along the estuarine gradient. Simultaneously the estuarine resistance to the invasion by *C. fluminea* was evaluated. In the winter there were almost no mortality among *C. fluminea*, while in summer we found a LT_{50} (median lethal time) of 9 days in the most downstream site. 100% mortality was never achieved during the testing period, adding concern to the species invasion potential in estuarine systems. Chapter III complements the information of the former chapter, as it allowed the assessment of *C. fluminea*'s contribution to the ecosystem processes (bioturbation) and functions (nutrients release). The interaction of temperature increase and salinity variation, as consequence of climate change, with the *C. fluminea* individuals' size/age, was tested.

Bioturbation was mostly influenced by size, and less by temperature and salinity, while the nutrients in the water column were influenced by different interactions among the studied variables. This chapter showed that invasion consequences are context dependent. Also, the potential for the invasive species to simultaneously occupy multiple components of a system was highlighted. The chapter IV describes the results of a field experiment where the effects of a mild temperature rise on the estuarine benthic communities in a seagrass bed and a sandflat were tested. This temperature increase was within the values declared as acceptable by recent international agreements. Different habitats responded differentially with the warmness, as the seagrass bed showed some temperature buffering ability. The seagrass bed offered thermal protection, ensuring higher stability when facing external disturbances. However, the effect of the habitat itself on the benthic communities was greater than the effect of the temperature increase. Once again we verified that context defines local ecological responses to global changes.

The Mondego estuary has been a proficient data source on anthropogenic disturbances for nearly three decades, supported by a substantial scientific literature. This database allowed us to clarify the ecological mechanisms underlying the effects of anthropogenic induced changes in Biodiversity-Ecosystem Functioning relationships in estuaries.

Biodiversity-ecosystem functioning; Environmental gradients; Anthropogenic changes; Temperature increase; Salinity variation; Biological invasions; *Corbicula fluminea*; Benthic communities.

Resumo e Palavras-Chave

A espécie humana tem sido responsável, desde o seu surgimento, por alterações ambientais. À medida que os avanços civilizacionais exigiram uma maior utilização de recursos, globalizaram-se um conjunto considerável de pressões sobre a biosfera e o planeta, entre as quais encontramos o aquecimento global e o aumento do número de eventos climáticos extremos, contribuindo para uma redução na biodiversidade. A estabilidade garantida pela biodiversidade encontra-se ameaçada sob o efeito das alterações globais, cujas consequências apresentam um elevada imprevisibilidade. Além de estas alterações se manifestarem globalmente, é a nível local que os seus efeitos são detetados de forma mais óbvia.

As pressões antropogénicas fazem-se sentir em todos os ecossistemas, e os sistemas estuarinos não são exceção. Os estuários encontram-se sujeitos a grandes variações cíclicas nos seus parâmetros físico-químicos e fortes gradientes entre água doce e marinha, condicionando as suas comunidades biológicas. No entanto, os estuários são responsáveis por um grande número de processos e funções biológicas, importantes para as populações humanas. A natureza cíclica dos estuários pode mascarar os efeitos das alterações globais, apesar de serem já discerníveis algumas das suas consequências. Um dos efeitos mais prementes das pressões antropogénicas prende-se com a redução da biodiversidade, pondo em causa a estabilidade e funcionamento dos ecossistemas. Assim a Biodiversidade e Funcionamento do Ecossistema destaca-se como um dos mais importantes debates em Ecologia, e mais recentemente na área da Ecologia Marinha. As alterações na biodiversidade como consequência das alterações globais influenciam processos e funções do ecossistema e põem em causa a sua estabilidade. A avaliação de processos naturais como a bioturbação permite-nos perceber melhor as relações entre a biodiversidade e o funcionamento do ecossistema.

Uma das principais causas reconhecidas para a redução global da biodiversidade é a introdução de espécies não-nativas, potencialmente invasoras, cujos efeitos são magnificados pelas interações com outras alterações globais. Os estuários são particularmente suscetíveis, pela sua proximidade às populações humanas, sendo áreas onde são realizadas inúmeras atividades responsáveis pela introdução de espécies invasoras. O bivalve *Corbicula fluminea* é um dos principais invasores em água doce, podendo invadir as áreas oligohalinas dos estuários. Originária do Sudeste Asiático, a espécie tem atualmente uma distribuição quase global.

Na presente dissertação, procurou-se responder às questões sobre que funções do ecossistema são mais afetadas pela introdução de espécies invasoras e como as funções são alteradas num cenário de alterações climáticas. No capítulo I traçaram-se as vias pelo qual a *C. fluminea* se dispersou globalmente, bem como a cronologia dessa invasão, baseando-se em literatura científica e relatórios técnicos. Observaram-se algumas tendências biogeográficas. A espécie encontra-se limitada entre pela altitude e baixas temperaturas invernais. No entanto, o aquecimento global e modificações a nível local poderão ser responsáveis pelo alargamento da área invadida. O capítulo II descreve uma experiência de campo onde se determinou a capacidade de sobrevivência da espécie *C. fluminea* ao longo do gradiente do estuário do rio Mondego. Definiram-se os limites atuais da espécie e a sua estrutura populacional neste sistema. Além disso, avaliou-se de que forma o gradiente estuarino limita a invasão da *C. fluminea*. Durante o inverno praticamente não houve mortalidade, enquanto no verão se verificaram valores de LT_{50} (tempo letal mediano) de 9 dias para o local mais a jusante. Em nenhuma das condições experimentais se verificou 100% mortalidade, aumentando a preocupação em relação à espécie, enquanto invasora de sistemas estuarinos. O capítulo III permitiu quantificar a contribuição da espécie para os processos (bioturbação) e funções (libertação de nutrientes) do ecossistema, com base numa experiência laboratorial. Testou-se a interação de variáveis que refletem alterações climáticas (temperatura e salinidade) com o tamanho/idade de indivíduos de *C. fluminea.* Os valores de bioturbação foram influenciados pelo tamanho dos indivíduos, sendo que a temperatura e salinidade tiveram uma influência menor. A concentração de nutrientes na coluna de água foi influenciada por diferentes interações entre as variáveis em estudo. Demonstrou-se que as consequências das invasões dependem do contexto em que ocorrem. Foi ainda destacado o potencial da espécie para ocupar simultaneamente diferentes componentes de um sistema. No capítulo IV testaram-se os efeitos de uma ligeira elevação de temperatura sobre as comunidades bentónicas estuarinas dos bancos de macrófitas e de zona arenosa. O ligeiro aumento da temperatura corresponde ao, preconizado como objetivo global segundo recentes acordos internacionais. Os dois habitats responderam diferencialmente a este incremento de temperatura, pois os bancos de macrófitas apresentaram uma proteção térmica garantindo assim uma maior estabilidade contra perturbações externas. O efeito do tipo de habitat foi mais significativo sobre as comunidades estudadas do que o aumento de temperatura. Mais uma vez se verificou que o contexto condiciona as repostas ecológicas locais às alterações globais.

O estuário do Mondego tem sido uma profícua fonte de dados sobre perturbações antropogénicas, suportada numa substancial literatura científica. Esta base de dados permitiu-nos clarificar alguns dos mecanismos subjacentes aos efeitos das alterações antropogénicas nas relações entre a Biodiversidade e Funcionamento do Ecossistema em estuários.

Biodiversidade e funcionamento do ecossistema; Gradientes ambientais; Alterações antropogénicas; Aumento de temperatura; Alteração de salinidade; Invasões biológicas; *Corbicula fluminea*; Comunidades bentónicas.

General introduction

> *"Ecology can be defined as the study of relationships between organisms and the environment. (…) Our survival has depended upon how well we could observe variations in the environment and predict the responses of organisms to those variations"*
>
> Manuel C. Molles Jr.
>
> Ecology – Concepts and Applications

1 GLOBAL CHANGES

Every living species is responsible for some level of impact on Earth. Whether large or small, there is always a "footprint" left behind by any organism (Jones et al., 1997; Vitousek et al., 1997). Unfortunately some species have higher impacts than others and, currently, the fiercest impacts are due to the Human species (Vitousek et al., 1997; Millennium Ecosystem Assessment, 2005; Barnosky et al., 2012). Since the rise of *Homo sapiens* as a species, humans are responsible for changes in their surrounding ecosystem (Boivin et al., 2016), a trend that culminated in present-day. Contemporary civilizational achievements have been possible on the expenses of large ecosystem manipulation and exploitation (Millennium Ecosystem Assessment, 2005). In the last six decades human activities were responsible for a number of changes on ecosystems and biodiversity in a rate with no parallel in any other period of mankind history (Parmesan & Yohe, 2003; IPCC, 2014).

When discussing global changes, the most mentioned issue nowadays is global warming, which is the most visible face on a large set of global anthropogenic shifts (Levitus et al., 2000; Millennium Ecosystem Assessment, 2005; IPCC, 2014). Global warming is a consequence of greenhouse gas emissions to the atmosphere (CO_2 with the largest share), which in turn derives from the burn of fossil fuels due to increasing industrial production (36% of carbon dioxide emissions are attributable to manufacturing industries (IEA, 2007)), transport of persons and goods (25% according to IEA (2009)) and domestic energetic necessities. Simultaneously, as human population grows, so it does agricultural and livestock production, which is also an important source of atmospheric CO_2 (which can account for 20-30% of total greenhouse gas emissions (IPCC, 2014)). The increase of average atmospheric temperature often translates in an increased complexity on weather events. The number of extreme climatic events has increased notoriously since the 1950's decade and included a decrease in cold temperature extremes, an increase in episodic warm temperature extremes, glacier ice melting, sea level rise, and severe changes in precipitation dynamics, with floods and droughts becoming more and more frequent (IPCC, 2014). But climate warming is not the only global anthropogenic threat to biodiversity and ecosystems: this includes land-transformation, habitat fragmentation, degradation and loss, urban/industrial construction, discharge of pollutants, changes in biogeochemical cycles, land-use for food production and forestry, overexploitation and the introduction of invasive species (Vitousek et al., 1997; Millennium Ecosystem Assessment, 2005; Barnosky et al., 2012), which are consequences of the growing demands in land occupation, food, raw materials and fuel for an increasingly consuming society.

Global anthropogenic changes are in fact one of the most complex multidimensional challenges that society and particularly scientist and decision-makers are currently facing. The already intricate natural balance of ecosystems is now under an additional set of pressures and disturbances that can disrupt this balance. Is therefore of uttermost importance to be aware of the ecosystems threshold to the disturbance impacts and how the natural stability of the system is affected by such disturbances (Barnosky et al., 2012; Donohue et al., 2016). In the past, natural systems changed in such a pace that enabled species to evolve and habitats to cope with global changes. Nowadays, the rate of global change is so fast that biodiversity is being reduced because the extinction values are higher than speciation rates (Millennium Ecosystem Assessment, 2005; Butchart et al., 2010; Pimm et al., 2014). The sheer magnitude in the complexity of cycles, interactions and feedbacks in biota is greatly enhanced by current global trends, therefore increasing unpredictability. The scientific efforts must now contribute to disentangle the effects of global changes on those cycles (biotic and abiotic (Donohue et al., 2016)), by improving the knowledge on the current structural status of ecosystems and the predictive power of models. Simultaneously, scientists should take part in the increasing global awareness of the human population on the consequences of such global changes.

1.1 LOCAL EFFECTS

The global natural biotic and abiotic parameters are shifting from previous reference values into new and unpredictable ones. The rate of those variations, most of times, has globally negative outcomes to ecosystems and biodiversity. Nevertheless, local systems are more frequently affected by changes in land-use or management, or by natural fluctuations in biodiversity, with short term effects, than by the effects of global climate changes (Parmesan & Yohe, 2003). Also, local systems are more likely to be affected by the effects of threshold crossing regarding stability, with abrupt shifts in ecosystem structure (Barnosky et al., 2012). The global climatic changes have locally and regionally very specific effects. For instance, the effects of heavy precipitation could be enhanced or mitigated by the type of land-use (Millennium Ecosystem Assessment, 2005) or changes of hydrological regime could bring notorious modification on local and regional climate (Vitousek et al., 1997; IPCC, 2014). It is fundamental to study locally the effects of the global trends, as there is a process of local evolution and adaptation of species that can be lost and may affect local ecosystem resilience and stability (Vitousek et al., 1997; Millennium Ecosystem Assessment, 2005; Hillebrand et al., 2008; Donohue et al., 2016). Still, local and regional shifts can be forced either by local stress events or by the pressure of global-level drivers. This is true at smaller scales and can be expanded for the global scale (Barnosky et al., 2012).

The assessment of most anthropogenic impacts can only be measured locally and then extrapolated or modelled at regional or global scales (Vitousek et al., 1997; Hillebrand et al., 2008; Donohue et al., 2016). The ranges of smaller species and its alterations can only be detected when looking at smaller scales (Parmesan & Yohe, 2003) and then integrated in a broader perspective. Therefore local studies must be on a par with global ones because ecological responses to climate change manifest themselves with high local specificity.

1.2 OCEANS AND GLOBAL CHANGE

In the global changes context, oceans represent a particular challenge for scientists. Human population concentrates near oceans. The first 100km strip of land after the coast shelter more than half of the human population (Vitousek et al., 1997; Crossland et al., 2005; Keiser, 2015; Neumann et al., 2015) and as human population is predicted to grow, so will the pressure over coastal ecosystems (IPCC, 2014). Oceans are responsible for a significant amount of global primary production, roughly equivalent to terrestrial ecosystems (Field et al., 1998; Sigman & Hain, 2012), mostly due to phytoplankton growth (Behrenfeld et al., 2006; Sigman & Hain, 2012). Also, marine secondary production is the source of food for millions of people (Holmlund & Hammer, 1999; Worm et al., 2006; Keiser, 2015). Nevertheless, these resources are under high stress due to pollution, overexploitation, and inadequate fishing methods and targets

(Vitousek et al., 1997; Millennium Ecosystem Assessment, 2005; Halpern et al., 2008; Keiser, 2015).

Oceans concentrate a huge amount of climate processes, and together with atmosphere, regulate the distribution of heat across the planet. Water is able to store and transport an incredible quantity of thermal energy because of its high density and specific heat (Levitus et al., 2000; IPCC, 2014). The oceans account for more than 90% on heat accumulation (IPCC, 2014). As expected, the upper layers of the ocean are the ones that accumulate more energy (Lyman et al., 2010; Trenberth, 2010), and data show that the top 75m of the oceans warmed 0.09 – 0.11ºC.decade^{-1} from 1971 to 2010 (IPCC, 2014). Simultaneously, as consequence of CO_2 increase in the atmosphere, dissolved CO_2 is also increasing, leading to changes in carbonate chemistry and pH drop (Caldeira & Wickett, 2003; Kroeker et al., 2010; Barnosky et al., 2012; IPCC, 2014), which is proving to be harmful for organism, with negative effects on survival, calcification, growth and reproduction (Kroeker et al., 2010; Queirós et al., 2015). Oceans are CO_2 sinks and the biological effects of this CO_2 increase are already in process.

Oceans are the final destiny for most terrestrial discharges. Pollutants from human activities are arriving to oceans, altering cycles of elements and nutrients, with consequential effects on biodiversity (Millennium Ecosystem Assessment, 2005; Doney, 2010; Barnosky et al., 2012; Cabral-Oliveira et al., 2013). Pollutants may suffer biomagnification and bioacumulation processes, with consequential ecotoxicological consequences and reduction in ecological fit of the species (Pörtner, 2012; Bozinovic & Pörtner, 2015). Another consequence of the anthropogenic release of substances is the nutrient enrichment specially in coastal areas, that could lead to harmful algal blooms (Rabalais et al., 2009; Sousa et al., 2012a; Dolbeth et al., 2015) or oxygen depletion (Whitney et al., 2013).

Anthropogenic pressures on ocean are now ubiquitous. As 41% of ocean area is under the strong effect of multiple stressors (Halpern et al., 2008) and oceans cover most of the planet, it becomes of highest importance to quantify in detail the ecosystem state (Barnosky et al., 2012). Within the ocean, a great part of the anthropogenic pressures occur in the coastal areas, which provide several ecosystems services essential to mankind.

1.3 TRANSITIONAL SYSTEMS UNDER GLOBAL CHANGES

The European Water Framework Directive (WFD) defines transitional systems as ‘‘bodies of surface water in the vicinity of river mouths which are partially saline in character as a result of their proximity to coastal waters but which are substantially influenced by freshwater flows’’ (European Community, 2000). Estuaries are included in these and despite the wide range that this definition covers (Elliott & McLusky, 2002; McLusky & Elliott, 2007), estuaries are notoriously characterized by strong gradients (McLusky, 1993), covering the full spectra from freshwater to marine features and are under cycles of variation with different lengths that superimpose themselves (tidal, circadian, monthly, seasonal, or even larger cycles) (McLusky, 1993; Pratt et al., 2014). These characteristics shape biological variables, such as community structure, as they respond to shifts in physicochemical parameters. Nevertheless, the estuarine biota have evolved to adapt to environments with such large range variation in the physicochemical parameters (Bamber & Henderson, 1988; Bilton et al., 2002; Féral, 2002; Bozinovic & Pörtner, 2015). Species richness in estuaries is usually reduced, as few taxa evolved towards the required broad tolerance (Attrill, 2002; Whitfield et al., 2012). Despite low richness, estuaries are able to deliver high production rates (Underwood & Kromkamp, 1999; Kennish, 2002; Dolbeth et al., 2011; Hicks et al., 2011), due to continuous organic matter input from river basins, nutrient dynamics through decomposition and organic matter mineralization and habitat heterogeneity (Underwood & Kromkamp, 1999; McLusky & Elliott, 2007; Godbold & Solan, 2009; Hicks et al., 2011; Dolbeth et al., 2012; Stief, 2013).

As mentioned before, human populations are essentially established near coastlines. Estuaries are especially attractive, for social and economic reasons, as they provide several ecosystem services, such as shelter for harbours, food sources and recreational areas (Kennish, 2002; McLusky & Elliott, 2004; Barbier et al., 2011; Costanza et al., 2014). Several of the most important cities developed within estuarine areas and became important commercial centres, which has been the main cause of high pressures over their communities, natural functions and services (McLusky & Elliott, 2004; Borja et al., 2011; Sekovski et al., 2012). These pressures are known to affect important ecosystem services (Worm et al., 2006; Barbier et al., 2011; Dolbeth et al., 2016), and as interface areas, are particularly vulnerable to global ecological changes (Millennium Ecosystem Assessment, 2005; Rabalais et al., 2009; Grilo et al., 2011; IPCC, 2013). Global changes are affecting transitional ecosystems, but because of their natural variability, the detection of anthropogenic pressures is more difficult than for naturally more stable environments (Martinho et al., 2015). However, several global anthropogenic effects in estuaries are discernible (Vitousek et al., 1997; Kennish, 2002; Fonseca et al., 2013).

Pollution has multiple sources, with more diffuse or point source origins, and introduces substances or energy in the systems that are harmful to organisms. The consequences include eutrophication by excessive nutrient load, anoxia and hypoxia derived from oxygen consuming wastes as sewage, pathogens, thermal loading or petroleum derivatives and heavy metals contaminations (Kennish, 2002). Over fishing is responsible for the decline of fish and shellfish stocks, with consequences on reproductive cycles of the species that use estuaries as nursery, for instance (Dolbeth et al., 2008b), or alteration in size and age structure of the populations (Crespo et al., 2010). Freshwater diversions such as channelling or upstream dams are responsible for changes in natural flow regimes, with consequential damage in brackish wetlands (Kennish, 2002; Ahmadian et al., 2014) and change in the hydrological cycle. Changes in the natural precipitation regime, as droughts or floods also impair the health of estuarine ecosystems (Dolbeth et al., 2011; Grilo et al., 2011). Dredging and shoreline constructions are also important stressors as they are responsible for direct disturbance of the physical habitats of organisms (Kennish, 2002; Hiddink, 2003; Simpson et al., 2015) and are also responsible for the resuspension of pollutants otherwise adsorbed to sediments (Latimer et al., 1999). Adding to this, habitat loss and alteration is a relevant anthropogenic pressure that affects estuarine populations by physical modification of the ecosystem, as for example, the reclamation of wetlands for agriculture and other human needs (Kennish, 2002). Sea level rise is not yet a major concern, but it is predictable that in the future it will be responsible for the retreat of estuarine shoreline with increasing flooding and erosion and loss of wetland habitats (Kennish, 2002; Barbier et al., 2011; Ahmadian et al., 2014; Chua & Xu, 2014). Temperature increase is already affecting some biological communities, by changing predator-prey relationships (Sanchez-Salazar et al., 1987; Freitas et al., 2007), by altering reproductive cycles and abundances (Honkoop & van der Meer, 1998; Power et al., 2002; Hicks et al., 2011), or by changing processes rates (Godbold & Solan, 2013). Also, populations of species with some level of mobility that live near the limits of their geographic range are now moving their boundaries in order to cope with climate changes (Martinho et al., 2010). This is also true for invasive species: as consequence of high human activity related with estuaries, these areas are particularly susceptible to biological invasions (Kennish, 2002; Williams & Grosholz, 2008; Simberloff et al., 2013).

2 BIODIVERSITY DECLINE AS CONSEQUENCE OF GLOBAL CHANGES

Biodiversity encompasses several organizational levels, which can go from genetic variability among species populations to the macroscopic level of landscapes and biomes (Chapin et al., 2000; Hooper et al., 2005; Magurran & McGill, 2011). Biodiversity can be characterized by richness (number of species), evenness (proportional abundance between species), composition (the particular species present), by functional diversity measurements, interactions between species and with species and abiotic factors and the variation of those components along time and space, among other components (Chapin et al., 2000; Magurran & McGill, 2011). Changes in the values of those components may modify the ecosystem ability to cope with environmental changes, altering either resilience and resistance of the systems (Chapin et al., 2000; Hooper et al., 2005; Cardinale et al., 2012). The decline in biodiversity may severely impair the ability of ecosystems to provide goods and services, thus affecting human populations that depend on those resources (Worm et al., 2006; Cardinale et al., 2012). This knowledge has boosted research on the relationships between biodiversity and ecosystem functioning (*Biodiversity-Ecosystem Functioning* debate), which has intensified in latest years (Bulling et al., 2006; Raffaelli, 2006; Naeem et al., 2009; Hale et al., 2014; Piot et al., 2014; Strong et al., 2015).

Despite the unknown number of living species, scientists are aware of rate of extinction on the biosphere (Naeem et al., 2012). Estimates refer that current species extinction is 1000 times higher than the background (before human domination of Earth) extinction rates (Vitousek et al., 1997; Pimm et al., 2014). This is relevant because it is not expected that speciation rates have had a notorious departing from its background rates and therefore the net gain is clearly negative with depletion of biological diversity. Larger species are major targets of extinction, primarily because of hunting and fishing pressures (Vitousek et al., 1997; Cardillo et al., 2005; Olden et al., 2007; Séguin et al., 2014), and their loss brought changes in many ecosystems functions which favour further extinctions, in complex feedback cycles. Land transformation is, presently, the primary biodiversity pressure (Vitousek et al., 1997; Parmesan & Yohe, 2003; Boivin et al., 2016), but the effects of the remaining global change components, as global warming or changes in biogeochemical cycles and pollutants emission are now becoming perceptible (Parmesan & Yohe, 2003; Mooney et al., 2009; Lillebø et al., 2010b; Costa et al., 2012; Cabral-Oliveira et al., 2013).

Diversity relates with stability, but the terminology can be misleading. While one can mention stability as the maintenance of community composition, stability can also stand for the preservation of ecosystem processes rates (Hooper et al., 2005). The reduction in biodiversity affects stability whatever concept we are using. Nevertheless, the latter is much harder to quantify and to identify its occurrence, because of redundancy in functional traits (Hooper et al., 2005). That means that even if loosing species, a system can still perform in a proper way, if the functions of lost species are kept by some of the remaining species. But that is only possible if the system originally possess enough species with high functional redundancy, i.e. close enough traits. Functional redundancy acts as an ecological insurance for natural processes (Balvanera et al., 2006; Mooney et al., 2009; Mouillot et al., 2014). However, the threshold for the maintenance of such processes is very site specific and, with increasing global changes, the amount of disturbance that a system can cope with in order to keep a proper performance is being reduced.

2.1 BIOLOGICAL INVASIONS

Adding to extinction, one of the most important sources of compositional changes in biodiversity may arrive due to the introduction of invasive alien species. Mankind is the main responsible for the introduction of invasive alien species, by means of the transportation of several species across the planet, a trend that, with the globalization of economy, is now larger than ever (Vitousek et al., 1997; Caldwell et al., 2007; Galil et al., 2007; Richardson, 2013). Changes in land-use, or altered hydrology, associated to introductions of species (which can be either deliberated or unintentional), set the perfect ground for highly efficient invasions (Vitousek et al., 1997; Caldwell et al., 2007; Richardson, 2013). Also, climate changes are responsible for an increase in the number of invasions: previous local conditions were acting as barriers to invasions (McDowell et al., 2014), but shifts in those conditions may now favour the non-native species while harming native ones (Rahel & Olden, 2008). Without their natural predators, parasites, competitors, or even their own habitat carrying capacity, non-indigenous invasive species (NIS) find in their relocation areas perfect niches to occupy with success, causing several disruptions in the native communities, and ultimately, a global biodiversity reduction (Sousa et al., 2008b; Butchart et al., 2010). As extinction, biological invasions occur naturally, but the rate of this processes is greatly enhanced by human activities (Vitousek et al., 1997; Richardson, 2013; Simberloff et al., 2013). Invasions are often associate with already disturbed ecosystems, but it is not clear if invasions are consequence or a contribution for those disturbed status (Karatayev et al., 2009; Simberloff et al., 2013).

The number of alien species in Europe is one of the indicators of pressures that shows increasing trends over recent decades (Butchart et al., 2010; Katsanevakis et al., 2014). The subject raises several questions closely linked to a wide range of sciences, from ecology to economics and social sciences, through engineering and resources management (Simberloff et al., 2013). The impacts should be analysed taking into account the full range of those sciences, as they may be hazardous or beneficial depending on the point of view. Populations of NIS are not dangerous *per se*, nevertheless, when compared to native populations, they may became a source of ecological hazard (Simberloff et al., 2013). There is often a perception bias associated with the biological invasion concept (Strayer & Hillebrand, 2012). Invasions are most of times responsible for changes in biodiversity, yet there could be positive effects of invasion (Katsanevakis et al., 2014; Novais et al., 2015). For example, invasive filter feeders may have positive effects in water beds with high loads of organic matter, or bioturbation can be improved by some invasive species, with benefits in aeration or solutes distribution (Hakenkamp & Palmer, 1999; Queirós et al., 2011). Nevertheless, among the most negative effects, we may find public health issues, economic losses, as for example biofouling of industrial and urban facilities, disruption of ecosystem processes, structure, functioning and biodiversity losses in native biota (Vitousek et al., 1997; Caldwell et al., 2007; Grosholz & Ruiz, 2009; Strayer & Hillebrand, 2012; Simberloff et al., 2013; Nakano & Strayer, 2014). NIS, as source of shifts in ecological compositions, are thus an important issue to be considered when talking about Biodiversity-Ecosystem Functioning, coupling with other factors such as climate change, nutrient loading, land use alterations and several others anthropogenic changes (Strayer & Hillebrand, 2012).

Due to the transitional nature of estuaries, NIS have several invasion opportunities along the estuarine gradients, i.e., in estuaries the sources of invasions could be either freshwater or marine. Simultaneously, estuaries are particularly prone to invasion by non-indigenous species because of their proximity to human populations (Cohen et al., 1997; Caldwell et al., 2007; Preisler et al., 2009; Ruiz et al., 2013) that are sources of major vectors for introductions, such as fisheries, ballast water, general human occupation or aquaculture (Cohen & Carlton, 1998; Williams & Grosholz, 2008; Crespo et al., 2015; Gallardo et al., 2016). The particular physicochemical conditions of estuaries are responsible for generally low diversity (Kennish, 2002; Dolbeth et al., 2011; Hicks et al., 2011) and invasive species, with high spreading and outcompeting potential, may force severe changes on ecosystem functioning due to disruptions on native community structures (Stachowicz & Byrnes, 2006; Sousa et al., 2008b).

2.1.1 *Corbicula fluminea* (Müller, 1774) as a model species for testing the impact of biological invasions across the freshwater-marine transition

The Asian Clam *Corbicula fluminea* (Müller, 1774) (Fig. 1) is one of the most invasive species of freshwater systems (Sousa et al., 2008a; Pigneur et al., 2011; Bódis et al., 2012). Despite appearing in the fossil record wide across several geographic areas, until the first decades of the 20th century the genus *Corbicula* were confined to Asian, African and Australian territories (McMahon, 1999). The first records outside their original source were documented in the British Colombia of the United States of America, probably brought there by Chinese emigrants as a food resource (Sousa et al., 2008a). It rapidly dispersed through all North-American territory and it was found in Europe in the early 1980's (Mouthon, 1981; Araujo et al., 1993). Despite being described as a freshwater bivalve, *Corbicula fluminea* tolerates salinities up to 10-14 (McMahon, 1983, 1999) and colonizes the upstream areas of estuaries (Sousa et al., 2008b; Franco et al., 2012; Ilarri et al., 2014), where the tidal influence is felt (Ilarri et al., 2012), but with small saline variation (Sousa et al., 2008a). In the Mondego Estuary, this species is present since 2001 (Franco et al., 2012). The species is a recognized ecosystem engineer (Sousa et al., 2009), and is implicated in natural processes that, ultimately, could alter the ecosystem functioning (Sousa et al., 2008b, 2012b). The efficiency of *C. fluminea* as an invader seems to depend more on its natural features, such as rapid growth and sexual maturity, high fecundity and efficient dispersal mechanism than on its physiological tolerance to environmental pressures (McMahon, 2002).

Fig. 1 The Asian clam *Corbicula fluminea* (Müller, 1774)

Nowadays, *Corbicula fluminea*'s spatial distribution is almost worldwide (Crespo et al., 2015), which raised global awareness on the impacts of the species in the invaded ecosystems, with an increasing number of scientific publications on the species (Bódis et al., 2012; Ilarri & Sousa, 2012; Kamburska et al., 2013; Sousa et al., 2014). This ubiquity makes the species a suitable candidate to be a model in a number of areas of biological sciences. The most scrutinized area is ecotoxicology, followed by ecology and distribution. For instance, the species could be useful in the detection on environmental contamination, as an efficient biomonitor (Doherty, 1990; Chen et al., 2012). Physiology and management are the research fields with less references (Ilarri & Sousa, 2012). When looking at biodiversity and ecology, as any invasive species, *C. fluminea* is described to outcompete and replace the native fauna (Sousa et al., 2008a). Also, it influences several other species that relates directly with the benthic strata (Ilarri et al., 2014). Distribution and biogeography of the species is also well described, since the first moment that the species was detected outside its natural range (e.g.: Martín and Estebenet 2002; Schmidlin and Baur 2007; Ayres 2008; Pérez-Bote and Fernández 2008; Munjiu and Shubernetski 2010; Clavero et al. 2012; Crespo et al. 2015). Population features such as growth, reproduction and production are discussed in an significant amount of literature (Ishibashi et al., 2003; Vohmann et al., 2010; Pigneur et al., 2011; Bódis et al., 2012; Modesto et al., 2013), as well as several aspects of its physiology (McCorkle & Dietz, 1980; Xiao et al., 2014). The interaction of species with some abiotic ecosystem components have also been evaluated (Hakenkamp & Palmer, 1999; Majdi et al., 2014; Xiao et al., 2014). The ecological and predictive models for the invasion success of the species across other regions have also been developed (Gama et al., 2016). Finally, the economic impact of *C. fluminea* invasions have also been accounted in several works (Mattice, 1977; Rosa et al., 2011; Wittmann et al., 2012; Nakano & Strayer, 2014).

3 BIODIVERSITY-ECOSYSTEM FUNCTIONING

Classical ecology has been focused in studying the abiotic drivers that modify ecosystems composition and structure (Bulling et al., 2006). In the last decades, the Biodiversity-Ecosystem Functioning (BEF) debate has emerged as one of the most important research areas in ecology, focusing on the evaluation of functions performed by an ecosystem and placing biodiversity as an explanatory variable, rather than as response variable (Loreau et al., 2001; Solan et al., 2006; Castillo-Monroy et al., 2011; Dossena et al., 2012). It emerged from terrestrial and plant ecology, driven by the need to study the consequences of biodiversity loss on the ecosystem functioning (Loreau et al., 2001; Worm et al., 2006; Bulling et al., 2010; Cardinale et al., 2012). Only in the last two decades the BEF debate has increased among marine ecologists (Emmerson et al., 2001; Covich et al., 2004; Solan et al., 2004a, 2012; Raffaelli, 2006). Although rapid changes in biodiversity were becoming evident or have been documented from geological records, the ecological implications of these changes are still not well understood, despite recent relevant advances on the topic (Covich et al., 2004; Solan et al., 2004a, 2006, 2008, Bulling et al., 2006, 2008; Raffaelli, 2006; Lefcheck et al., 2015; Strong et al., 2015). The debate appears to be focused on two main issues (adapted from Loreau et al., 2001):

- The effects of biodiversity on the ecosystem processes and functions, such as nutrient cycling and the rate of biological flow through the system (e.g. Solan et al., 2008; Dolbeth et al., 2015);

- How ecosystem stability (resilience and resistance) could be affected by species diversity against a background of global ecological changes (e.g. Emmerson et al. 2005).

In the BEF context, mesocosm experiments have been applied with success (Loreau et al., 2001; Solan et al., 2004a; Ieno et al., 2006; Godbold et al., 2011; Hale et al., 2014), using benthic invertebrate species, which mediate several processes in the marine ecosystem (Solan et al., 2008). Thus, what happens at the benthic compartment may provide important insights on the impacts for goods and services provided by the ecosystem, presenting environmental and socio-economic implications (Millennium Ecosystem Assessment, 2005). Several of these experimental approaches have placed efforts in the study of species identity or density (e.g. Bulling et al. 2008) and to a lower extent on the redistribution of species abundance (i.e. evenness, Hillebrand et al., 2008). However, evenness often responds more rapidly to human activities or altered environmental constraints than species richness (Hillebrand et al., 2008), especially in estuarine systems, which often have lower levels of diversity (Elliott & Whitfield, 2011).

There are some ecosystem processes that could be measured and analysed in order to understand relationships between biodiversity and ecosystem functioning. Bioturbation in benthic sediments is an example of ecological engineering, which is related with fair amount of other ecosystem processes and functions (Schiffers et al., 2011; Queirós et al., 2013). Therefore, the sediment mixing depth is assumed to be a significant proxy for benthic function and habitat quality (Teal et al., 2010; Hale et al., 2014, and references within). Nevertheless, this relationship is highly context-dependent, i.e., one cannot withdraw conclusions on dynamics and ecosystem process solely based in the mixing depth estimations (Teal et al., 2010). Some methods were developed in order to monitor the dynamics of bioturbation. One of the most easy and efficient is the time-lapse fluorescent sediment profile imaging (f-SPI) described in Solan et al. (2004b), which is based in the detection of the movements of luminophores along a time period detected with fluorescence in the presence of Ultra-Violet light. This technique provides multiple undisturbed cross-sectional images of the benthos movements in the sediment. It allows identifying species and simultaneously to track and quantify the infaunal activity. On the other hand, the bioturbation has a close relationship with the nutrients release from the sediments (Raffaelli, 2006; Godbold & Solan, 2009; Godbold et al., 2011), which is an important measure of the ecosystem function.

3.1 THE MONDEGO ESTUARY AS A TESTING AREA

During the last three decades several sources of disturbance occurred in the Mondego estuary, either anthropogenic or climatic ones. As consequence of those disturbances, changing patterns of invertebrate biodiversity have occurred (Cardoso et al., 2008; Dolbeth et al., 2011, 2014), with potential consequences for other trophic levels (Martinho et al., 2010) and ultimately for the overall goods and services provided by the estuary (Dolbeth et al., 2008a; Pinto et al., 2014). The Mondego estuary (Fig. 2) is a well described system (e.g. Flindt et al., 1997; Cardoso et al., 2005; Dolbeth et al., 2015), and it is also depicted in the following chapters. Studies in the estuary have started since the 90's (Marques et al., 1993; Flindt et al., 1997), covering several aspects of the ecological condition of the system (Martinho et al., 2010; Dolbeth et al., 2011, 2014) and it was also used as an experimental site (Martins et al., 2005; Cardoso et al., 2007). Data is available regarding issues such as nutrient dynamics, primary and benthic production, flora and fauna population structure and reproduction

(Lillebø et al., 1999; Pardal et al., 2000; Dolbeth et al., 2003, 2005, 2014; Viegas et al., 2007; Crespo et al., 2010; Verdelhos et al., 2011; Grilo et al., 2012; Primo et al., 2012; Marques et al., 2014), eutrophication (Lillebø et al., 1999; Pardal et al., 2000; Dolbeth et al., 2003; Cardoso et al., 2004; Coelho et al., 2004; Verdelhos et al., 2005; Leston et al., 2008), impact of weather extremes and hydrological changes in local fauna populations and communities (Martinho et al., 2007, 2008; Marques et al., 2008; Grilo et al., 2011; Gonçalves et al., 2012; Primo et al., 2012), fisheries (Vasconcelos et al., 2007; Crespo et al., 2010; Martinho et al., 2012), impacts of contaminants (Costa et al., 2010; Cardoso et al., 2011, 2012, Leston et al., 2011, 2013; Baptista et al., 2013), parasitology (Costa et al., 2013; Bordalo et al., 2014), among others. In particular, the long dataset on Mondego estuary macrobenthic communities enabled us to better understand the ecological reasons behind the observed results in the experiments. The small size of the estuary as well as the contrasting features between the north and south arms (the north arm is very artificially modified, as consequence of the urban and harbour implementation, while the south arm shows more natural features, with large intertidal areas, high sediment deposition and lower water circulation) offers researchers interesting opportunities for studying BEF along estuarine gradients. It is also possible to test contrasting environmental features, within the same geographic area, with advantages in data interpretation. In the Mondego estuary, the changing nutrient dynamics patterns are related to changes in the ecosystem functioning (Cardoso et al., 2010) with potential important consequences for the economy: a shift in these dynamics may alter a fundamental natural service (Sousa et al., 2012a) and the fauna that depend on those resources (e.g. Martinho et al., 2010; Dolbeth et al., 2011; Rodrigues & Pardal, 2014).

For the purposes of the current dissertation, the Mondego estuary was used either for *in situ* experiments, described in Chapters II and IV, or as the source of the *ex situ* tested organisms, described in Chapter III (Fig. 2).

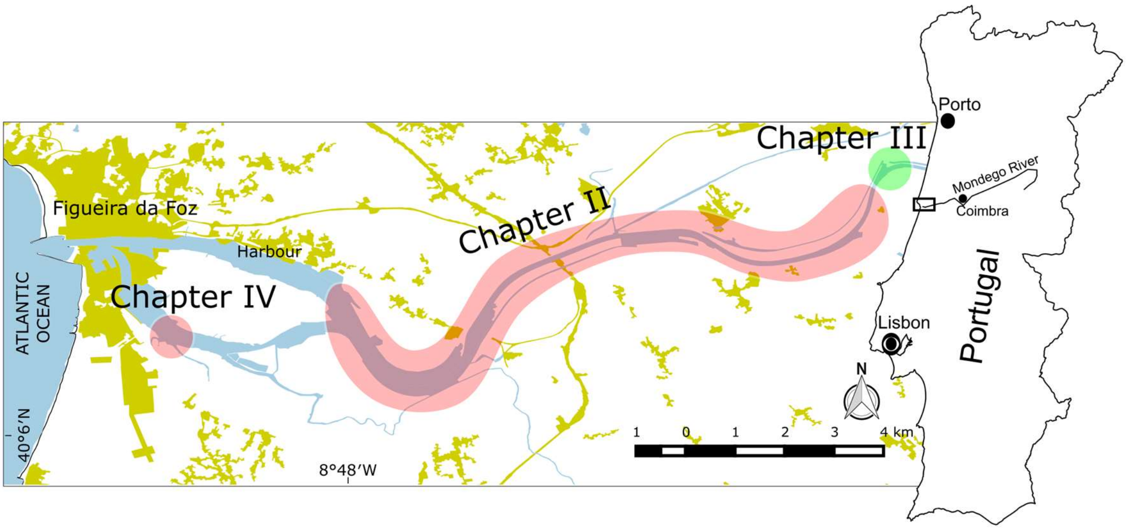

Fig. 2 The Mondego estuary as an appropriate experimental area. The red coloured areas represent in situ experiments (Chapter II and Chapter IV) and the green coloured area represent the source of organisms for laboratorial experiment (Chapter III)

4 OBJECTIVES

The objective of the present dissertation is to contribute to a better understanding of how pressures from global changes interact with biodiversity compositional changes, in order to evaluate impacts in the functioning of the estuarine ecosystem, that overall will affect goods and services that underpin human well-being. Particular effort was dedicated on the effects of a non-native invasive species (NIS), which creates monotonous communities and therefore is a relevant source of compositional diversity change. An overview of the global current status of species was included (Chapter I), as well as the assessment of its ability to invade the system and the effects in terms of ecosystem processes and functioning (Chapters II and III). Simultaneously, an assessment of the effects of temperature increase under natural estuarine conditions on naturally assembled communities was performed (Chapter IV). The procedures were based on mesocosm and *in situ* field experiments, and benefited from the good field background on several disturbances sources and impacts on the composition, structure and functioning of macrobenthic communities from the Mondego estuary, provided by extensive literature of more than 30 years of studies (e.g. Cardoso et al., 2010, Lillebø et al., 2010, Grilo et al., 2011, Dolbeth et al., 2011). The approach focused on the biodiversity compositional changes representative of global ecological changes, due to the occurrence of non-indigenous invasive species and due to the global ecological changes that have been less exploited as extreme droughts, floods and heat waves that will increase in frequency and intensity (IPCC, 2014) and how this interaction affects the functioning of estuarine ecosystems. Among the scientific research questions of the present thesis are:

- Which ecosystem functions are affected most often or most severely by invasive species?

- How is the ecosystem function altered under realistic scenarios of climate change?

Based on the above questions, a more specific objective of this dissertation is to disentangle how much of the estuarine ecosystem functioning will be impaired by the interactive effects of global changes. Those global changes could be either ecological as the introduction of a NIS, or climatic as temperature and salinity variations, as often observed during extreme weather events, in cycles of altered precipitation levels or as preconized as future climate change effects. The current status of the invasive *Corbicula fluminea* worldwide was assessed, as well as its ability to survive and perform along the freshwater – marine continuum of the Mondego estuary, under a context of global climate change. Simultaneously, a more holistic perspective of the functioning among the estuarine benthic communities under warming was achieved.

4.1 THESIS OUTLINE

The present document is a contribution for a better understanding of the effects of three important global ecological changes (temperature increase, precipitation dynamics, which is reflected in salinity changes, and NIS) on the biodiversity-ecosystem functioning of a temperate west-European estuary. Chapters were organized taking into account two major components:

- Chapters I, II and III address to the invasive species *C. fluminea*, current global status and its effects on the estuarine ecosystem;

- Chapter IV describes the effects of a mild warming under natural estuarine conditions on the local community.

These themes are linked by being consequences of anthropogenic global tendencies of shifts that impair the functioning in local ecosystems. Also, abiotic variation impacts are consistently evaluated throughout the thesis. Next, each chapter is described with more detail, as well as the respective objectives.

4.1.1 Chapter I - Distribution of *Corbicula fluminea* (Müller, 1774) in the invaded range: a geographic approach with notes on species traits variability

Despite the existence of a large amount of literature on the invasive species *Corbicula fluminea*, including several reviews (Mattice & Dye, 1976; McMahon, 1983, 1999; Belanger et al., 1985; Doherty, 1990; Araujo et al., 1993; Lee et al., 2005; Pérez-Quintero, 2008; Sousa et al., 2008a), the patterns involved in the dispersion of the species outside its natural range are not completely well described. There are clear physical gradients which follow up geographic differences. Latitudinal differences, as well as distance to large water bodies (as large lakes or oceans) and altitude may account for gradients in temperature, precipitation, sun exposure (which regulates primary production) and winter duration. In turn, species react in accordance to these gradients, altering their population and individual features proportionally to these gradients, either with positive or negative correlations.

In this chapter is developed an extensive review on the *Corbicula fluminea* population characteristics across the world, analysing the parameters that could be related to the environmental gradients mentioned above. Using Geographic Information System tools, the aim was to produce figures (world maps) with the chronology of the invasion worldwide, based on geostatistical extrapolation methods as kriging (Tonkin & Larson, 2002). Simultaneously, global limits and barriers for the species were assessed, as well as the relation of some traits of *C. fluminea* along geographic gradients.

The objective is to contribute to a better understanding of the current global status of the invasive *C. fluminea* as well as to describe some of the most common features of the habitats around the world that are invaded by this species, based on the species' population characteristics.

4.1.2 Chapter II - Survival of *Corbicula fluminea* (Müller, 1774) in a natural salinity and temperature gradient: a field experiment in a temperate estuary

A field experiment was performed with *Corbicula fluminea* individuals in order to determine its survival along the estuarine gradient of the Mondego River, regarding changes in salinity and temperature. This experimental set was performed twice, in summer and in winter, in order to evaluate seasonal differences in the survival ability of the species

The results of this experiment allowed us to define natural limits for the species with regard to its salinity and temperature tolerance (e.g. mortality rates and survivorship curves). There is some information on those vital thresholds on literature for freshwater systems and/or laboratory essays. However, to our knowledge, this sort of testing has not been performed in natural estuarine systems. This acquires special importance because, although *C. fluminea* is a freshwater species, it has been occurring more often in estuarine areas. Its dominance in estuarine waters is surely contributing for changes in the functioning of the system, either as inducing the decline of the native species or as for its own functional role in the community (Sousa et al., 2008b).

This chapter has two main objectives. The first is to assess the current distribution of *C. fluminea* and its population structure along the Mondego estuary gradient. The second objective is to understand how much the estuarine physicochemical gradient acts as an effective barrier to *C. fluminea* invasion and distribution spread. Ultimately, the goal is to understand how much threat do this clam represents for the estuarine ecosystem.

4.1.3 Chapter III - Ecological consequences of invasion across the freshwater-marine transition in a warming world

A laboratory mesocosm experience was performed in order to assess the contribution of different size classes of the invasive species *C. fluminea* for the ecosystem functioning in response to different salinities and temperature regimes. Three size classes of the bivalve was used, summing up to same total biomass in each mesocosm, according to: 1) Without fauna, serving as control; 2) small sized individuals; 3) medium sized individuals; 4) large sized individuals. Each one of these scenarios were tested at two different temperatures and 2 salinities. The measured responses were: 1) nutrient dynamics (ecosystem function); and 2) bioturbation (f-SPI method) (ecosystem process).

Two different drivers of global ecological alterations and their interactions (invasions and climate) were analysed in this experiment. Therefore the experiment contributed for a more holistic perspective on the effects of those global changes, and how eventual synergistic effects could alter the ecosystem functioning.

The objective of this chapter is to understand how the invasive *C. fluminea* performs along a freshwater/marine gradient, under a scenario of global climatic changes, expressed by changes in salinity and temperature. It complements the information gathered in Chapter II, because it allowed to quantify some aspects impossible to measure in the field experiment: e.g. the contribution of different size classes to bioturbation and nutrients release.

4.1.4 Chapter IV - New climatic targets for climate change: does a 2ºC temperature rise affect estuarine benthic communities?

In order to evaluate the effect of warmness in a real field situation, an experiment took place in the intertidal areas of the Mondego estuary, where previous heat wave impacts were notorious. The experiment was performed during summer. Transparent plastic boxes were placed in the field on a bare mudflat and on a seagrass area. The boxes acted as greenhouses, i.e., they retained the heat from solar radiation and increased the mean temperature inside the boxes. The estuarine hydrological regime was maintained by small perforations in the side of the boxes that allowed the water circulation during tidal cycles. In the first and in the final moment of the experiment samples of benthic communities were collected and the infauna was assessed. Bioturbation was measured *in situ* using the f-SPI method (Solan et al., 2004b). The same procedures were used in the surrounding areas, which served as control for the experiment. Data on the microfauna and water and sediment nutrients were also collected.

This experiment provided information on the ecosystem responses to a temperature increase scenario under the predictions of recent global agreements, i.e. 1.5 to 2ºC increase (United Nations, 2015). As a field experiment, despite the stochastic effects, a more realistic and complete environmental response was expected, reflecting a larger number of interactions that cannot be emulated in the laboratory.

The objective for this chapter is to understand the response of the benthic communities as a unit, under the mild warmness that are the new climatic targets for climate change. The chapter is a complement to the remaining thesis, as it provides an assessment of BEF in a real scenario under a manipulated temperature increase, within a complex biological assemblage.

Chapter I

Distribution of *Corbicula fluminea* (Müller, 1774) in the invaded range: a geographic approach with notes on species traits variability

1 ABSTRACT

Corbicula fluminea is considered one of the most important non-native invasive species (NIS) in aquatic systems mainly due to its widespread distribution and ecological and economic impacts. This species is known to negatively affect native bivalves, also with severe effects on biodiversity and ecosystem functioning. Throughout an exhaustive bibliographic survey and with the aid of Geographic Information Systems tools, this study tracks the species dispersion from its native range, including the description of important physical and environmental barriers. Additional analyses were conducted to examine possible influences of latitudinal/temperature gradients on important traits (e.g. life span, maximum and mean body length, growth at the end of first year). Altitude and winter minimum temperature appear to be delaying the invasion worldwide, but it seems inevitable that the species will spread across the globe. Latitude and summer temperature show a relationship with growth and life span. Overall, the information gathered in this review may be relevant to forecast future distribution patterns of this NIS, and to anticipate the possible implementation of effective management measures. Moreover, it may constitute a valuable tool in the prediction of population responses to an increasingly changing environment.

2 KEYWORDS

Corbicula fluminea; Invasive species; Vectors of dispersion; Geographic gradients; Invasion history; Species traits

3 INTRODUCTION

In the last decades, globalization has been dissolving natural biogeographic barriers and allowing the transport (either inadvertently or not) of many species (Simberloff et al., 2013; Gutiérrez et al., 2014). These transported species, usually defined as non-indigenous, non-native or alien, could become invasive. By definition, a non-native invasive species (NIS) needs to expand its geographical range into an area not previously occupied, marked by successive stages of arrival, establishment and integration (Vermeij, 1996). In introduced areas some of these NIS find niches with similar conditions to those available in the native range, but without their natural predators, parasites or competitors (Sakai et al., 2001; Torchin et al., 2003). Not surprisingly, NIS have been causing disruptions in the native communities, and ultimately, a global biodiversity reduction due to, for example, predation, introduction of diseases and parasites, competition and altered abiotic conditions (Ruiz et al., 1997; Dukes & Mooney, 1999; Revenga & Kura, 2003; Sousa et al., 2008b; Butchart et al., 2010).

The traits that define the invading potential of a species have not yet been consistently tested, especially in animals. Anyway, high reproductive rate, single parent or vegetative reproduction, eurytopy, polyphagy, early maturation and small body size have been considered important (Kolar & Lodge, 2001). Usually, these species show r-strategies (opportunist behaviour), with several generations per year, short periods between spawning activity and spend a great effort in reproduction and recruitment (Den Hartog et al., 1992; Lavoie et al., 1999). However, the characteristics of the NIS *per se* may be a highly reductionist view to define the invading potential because the characteristics of the invaded habitat (Byers, 2002) and propagule pressure (Simberloff, 2009), at least, may also play a fundamental role.

NIS are amongst the most serious threats that aquatic systems are currently facing, and their impacts are not always easy to follow (Strayer, 2010). One faunal group responsible for high ecological and economic impacts in aquatic ecosystems comprises bivalves (Sousa et al., 2014). Within this group, *Corbicula* has become a threat during the last century due to its invasive potential (Nguyen and De Pauw 2002; Elliott 2008; Sousa et al. 2008a, 2008b; Pigneur et al. 2011). With its origins in Asia, Australia and Africa, species belonging to this genus have spread worldwide during the 20th century (Araujo et al. 1993; Sousa et al. 2007, 2008a) with *Corbicula fluminea* (Müller, 1774) being considered one of the most important NIS in aquatic systems (Sousa et al., 2008a; Morais et al., 2009; Ilarri & Sousa, 2012). This species first established in the United States of America (USA) during the first half of the 20th century, entered the South American systems in the late 1960s/beginning of the 1970s and reached Europe probably during the 1980s (Araujo et al., 1993; Mouthon, 2001; Darrigran, 2002; Morais et al., 2009). Human activities (e.g. ballast water transport, use as bait or as food resource, aquarium hobbyists releases, tourist curiosity) are considered the primary introducing vectors for this species (McMahon, 1983, 1999; Vermeij, 1996; Ruiz et al., 1997; Lavoie et al., 1999; Rigby et al., 1999; Bódis, 2007; Sousa et al., 2008a). Also, pediveligers or juveniles can be dispersed by natural means, that include fluvial or tidal currents (Den Hartog et al., 1992; Rosa et al., 2012), or adhered with a byssal attachment to mobile animals such as birds and mammals (Prezant & Chalermwat, 1984; McMahon, 1999; Sousa et al., 2008a). As an opportunistic species, it is well adapted to colonize lotic and lentic ecosystems, with a very plastic feeding behaviour that includes both filter- and deposit-feeding (McMahon, 1983; Sousa et al., 2008a; Dias et al., 2014).

Populations of *C. fluminea* seem to be negatively affected by the combination of hypoxia and high temperatures. The species show a clear preference for well oxygenated sandy substrates (Belanger et al., 1985). Matice and Dye (1976) determined the lower and upper lethal temperatures as 2ºC and 34.8ºC, respectively. In the USA, the populations further north have been associated with the water outlets of steam electric power plants, which increase the temperature in adjacent areas and allow the survival during colder winters (McMahon, 1983; Den Hartog et al., 1992). In addition, despite being usually described as a freshwater bivalve, it can tolerate salinities of 10-14 (McMahon, 1983, 1999), allowing the species to colonize the upstream areas of estuaries (Sousa et al., 2006; Franco et al., 2012; Modesto et al., 2013).

As for reproduction, it usually presents two major spawning periods per year (McMahon, 1983; Rajagopal et al., 2000; Sousa et al., 2008a). *Corbicula fluminea* is usually described as a simultaneous hermaphrodite, which incubates fertilised eggs within its inner demibranchs and releases pediveligers with reduced mobility to the water column that rapidly settle into the sediments (McMahon, 1983; Rajagopal et al., 2000; Sousa et al., 2008a). This species is capable of androgenetic self-fertilization, which may increase its invasive potential: a single individual in the proper environmental conditions has enough potential to start a new population (Pigneur et al., 2011).

Corbicula fluminea can affect hydrology, biogeochemical cycling and biotic interactions via two general mechanisms: assimilatory-dissimilatory (uptake and release of energy and materials) and physical ecosystem engineering (physical environmental modification by organisms), with impacts ranging from individuals to ecosystems (Sousa et al. 2008a, 2009). This species alters community features, especially evenness, as it creates large monoculture patches on the invaded systems. Those changes often affect other natural processes such as productivity, nutrient cycles and community stability, by using resources in a different way (Ilarri & Sousa, 2012). Several impacts of the species are reported in the literature: - *C. fluminea* is responsible for the creation of a different habitat matrix for benthic fauna via bioturbation and shell production (Ilarri et al., 2012); - it has high filtration rates (Sousa et al., 2008a); - it is a highly productive species, which may cause disruption on food chains, either as food source for higher trophic levels mainly when massive die-offs occur (Sousa et al., 2008b, 2012b) and as a consumer reducing phytoplankton availability for other species and; - it may be responsible for alterations in biogeochemical cycles due to their pedal feeding, with consequential alterations in the abiotic characteristics of the top layer of the sediments and high rates of nitrogen excretion (Sousa et al., 2008a, and references therein).

Taking into account the ecological importance of this species and its high invasive potential, the objective of this work is to evaluate, through an exhaustive bibliographic survey, the effect of geographic gradients relating to environmental variables on the most descriptive population traits, such as the life span, maximum and mean body length and growth at first year. Simultaneously, we look upon those gradients in order to detect possible pathways of dispersion and areas of potential future introduction and establishment of the species. This information may help us anticipate future distribution patterns of this NIS and also to aid in the implementation of management measures. Moreover, this information may be used in the prediction of population responses to an increasingly changing environment.

4 MATERIALS AND METHODS

For this review, a wide bibliographic survey was conducted on scientific publications, technical reports, theses and web sites devoted to the NIS *C. fluminea*. The present work focused on population traits such as the number of reproductive periods, life cycle duration, first year growth, number of simultaneous cohorts, density, biomass, mean and maximum body length. Nevertheless, literature was very sparse with respect to some of those data and therefore it was not possible to use all of those population traits. The traits with more extensive and useful data were life span, maximum body length, mean body length and length at the end of the first year. Another metric that was looked at was the year of invasion. For each population, the geographic position was identified, either using the precise coordinates, whenever they were reported, or searching by their toponym with the Google Earth ® search engine. In the case of wide areas and/or unspecified geographic coordinates, the central point of the system was chosen. The geographic data were loaded in the open-source software Quantum GIS (QGIS) Desktop 2.2.0-Valmiera together with all population traits associated with their respective coordinates. Data were transformed to raster layer using the data interpolation tool. The method used was the Inverse Distance Weight (IDW). The river basin shape files were obtained in the Water Base project site (http://www.waterbase.org/home.html) (George & Leon, 2007). The invaded basins were selected and for better visualization, a fixed distance (1 map units) buffer was created. These shapes were used to cut the interpolation surfaces. The grids for temperature and altitude were obtained in the WorldClim site (http://www.worldclim.org/) (Hijmans et al., 2005), with a 2.5 arc-minutes resolution.

Information on the population traits was plotted against temperature and latitude classes (boxplots). These data were tested with an ANOVA being each population trait the dependent variable and temperature and latitude the explanatory variables. Prior to the analyses, we checked for collinearity among explanatory variables with variation inflations factors (VIF, Zuur et al. 2009). The assumptions of the linear regression were verified with graphical validation tools (Zuur et al., 2009). For the non-parametric data, Kruskal-Wallis tests were used. Also, the sighting sites were grouped and characterized according to geographic and temperature classes and their relation assessed. All analyses were performed with R software (R Development Core team 2012).

5 RESULTS

In the bibliographic survey we retrieved 1058 sites with sightings, based in 96 studies with data about *C. fluminea*: 74 scientific papers, 8 conference proceedings, 5 reports, 2 bulletins, 2 books, 2 book chapters and 1 MSc thesis.

The first occurrence of *C. fluminea* outside its native range was recorded in North America in 1924 at Vancouver Island, British Columbia (Fig. 1). That occurrence refers to an empty shell found in a natural system. From the Vancouver Island, the species spread across the USA, apparently from Northwest along the coastal states, then crossing the southern states. The south-eastern states were reached during the 1950s. The most recent invasions of water basins by *C. fluminea* occurred in the 21st century, on the North and Northeast USA. Besides the 1924 event in the Vancouver Island, the species only appeared again in Canada in 2006 (Simard et al., 2012). Nowadays a high percentage of North and Northeast USA has been invaded, especially in low altitude regions (Fig. 2A) and above -10ºC winter air temperature (Fig. 2B).

Central America shows a more recent invasion that started around 1998 (Fig. 1). The number of sites for Central America is low (Fig. 2). Nevertheless, is possible to track an invasion pathway that crosses Mexico and reaches some Caribbean islands, in particular Puerto Rico (Williams et al., 2001).

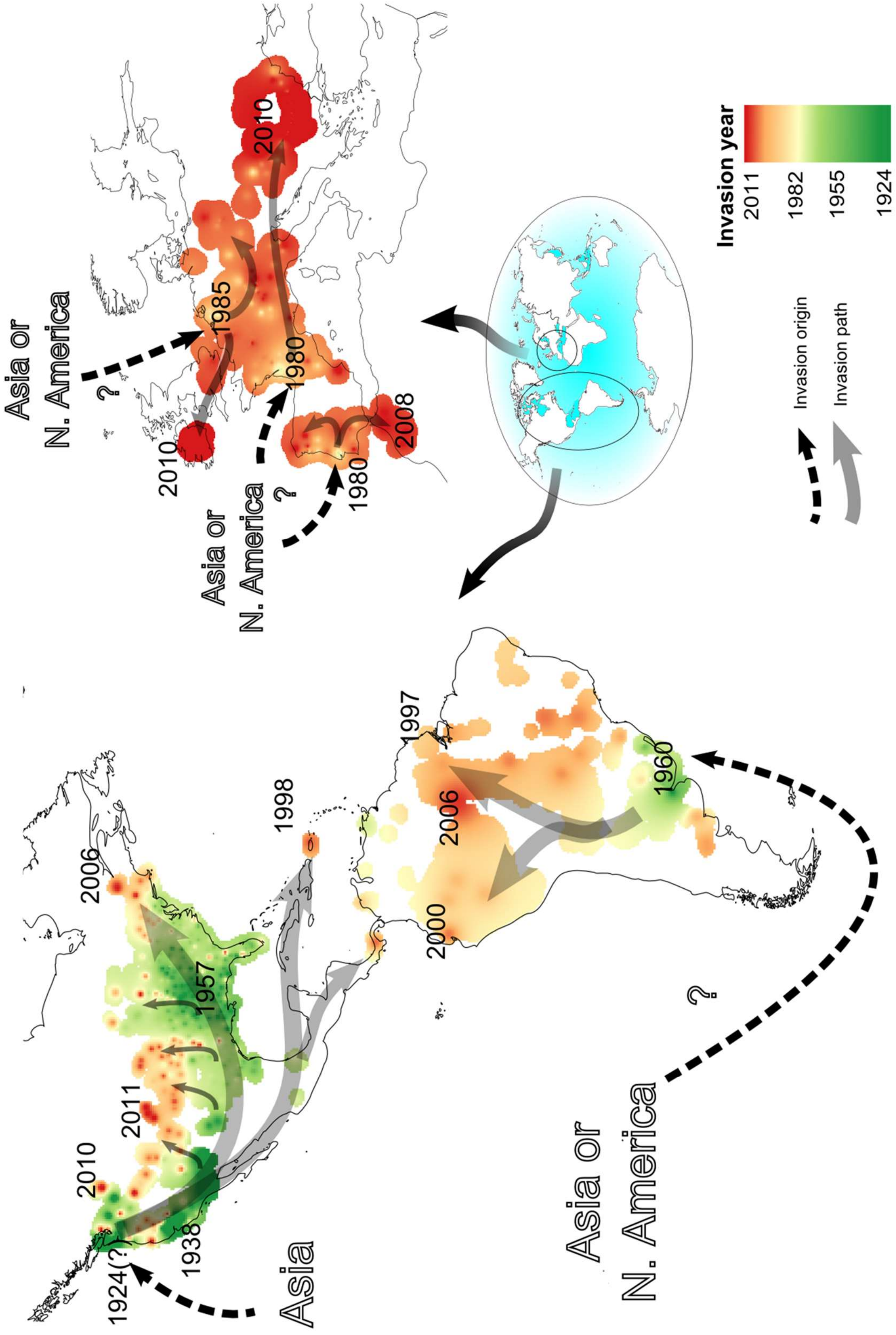

Fig. 1 Dates and main paths for *Corbicula fluminea* in the invaded range

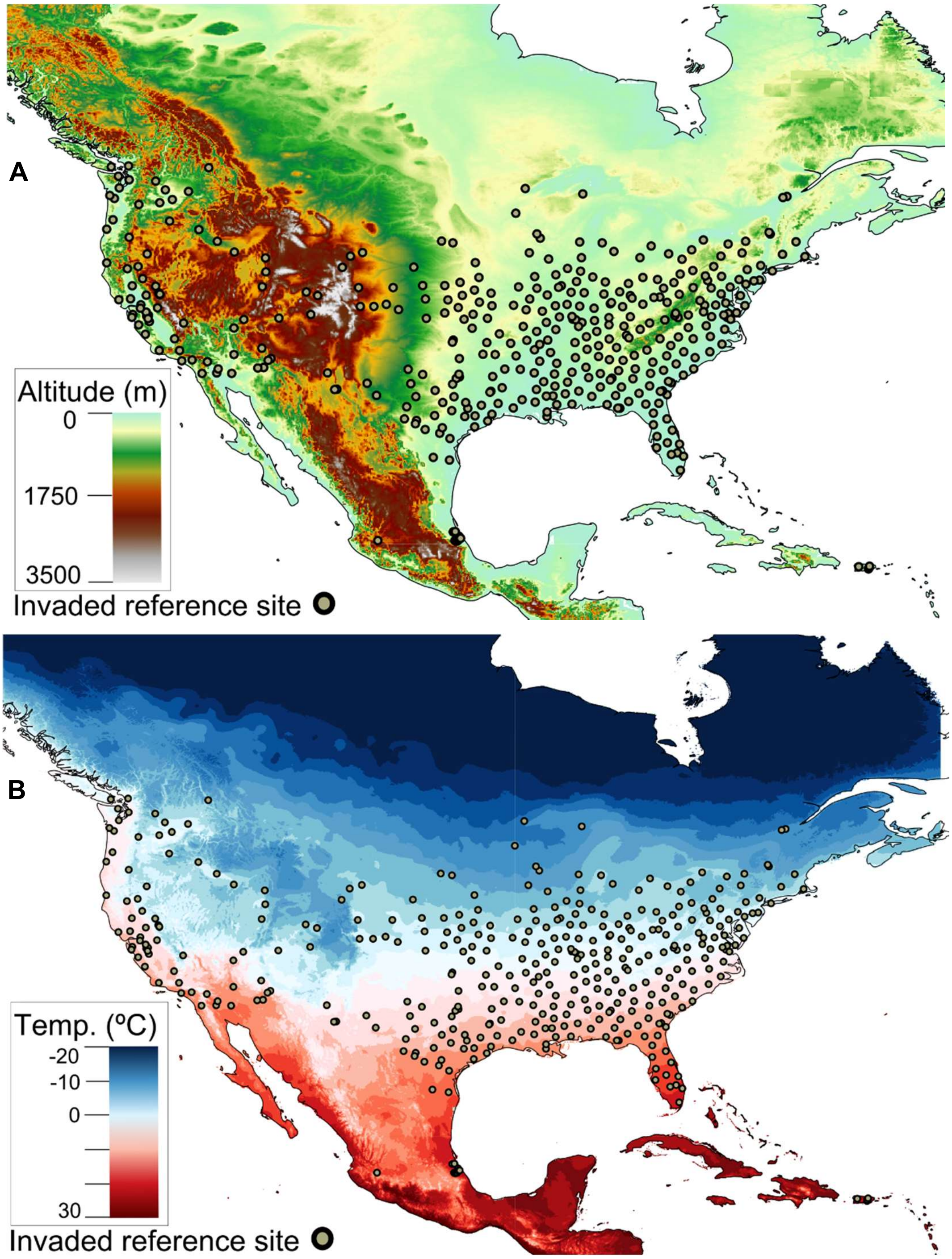

Fig. 2 North and Central America digital elevation model (A), and average air temperature for January (1950-2000) (B). The invaded sites found in the bibliography are represented by dots

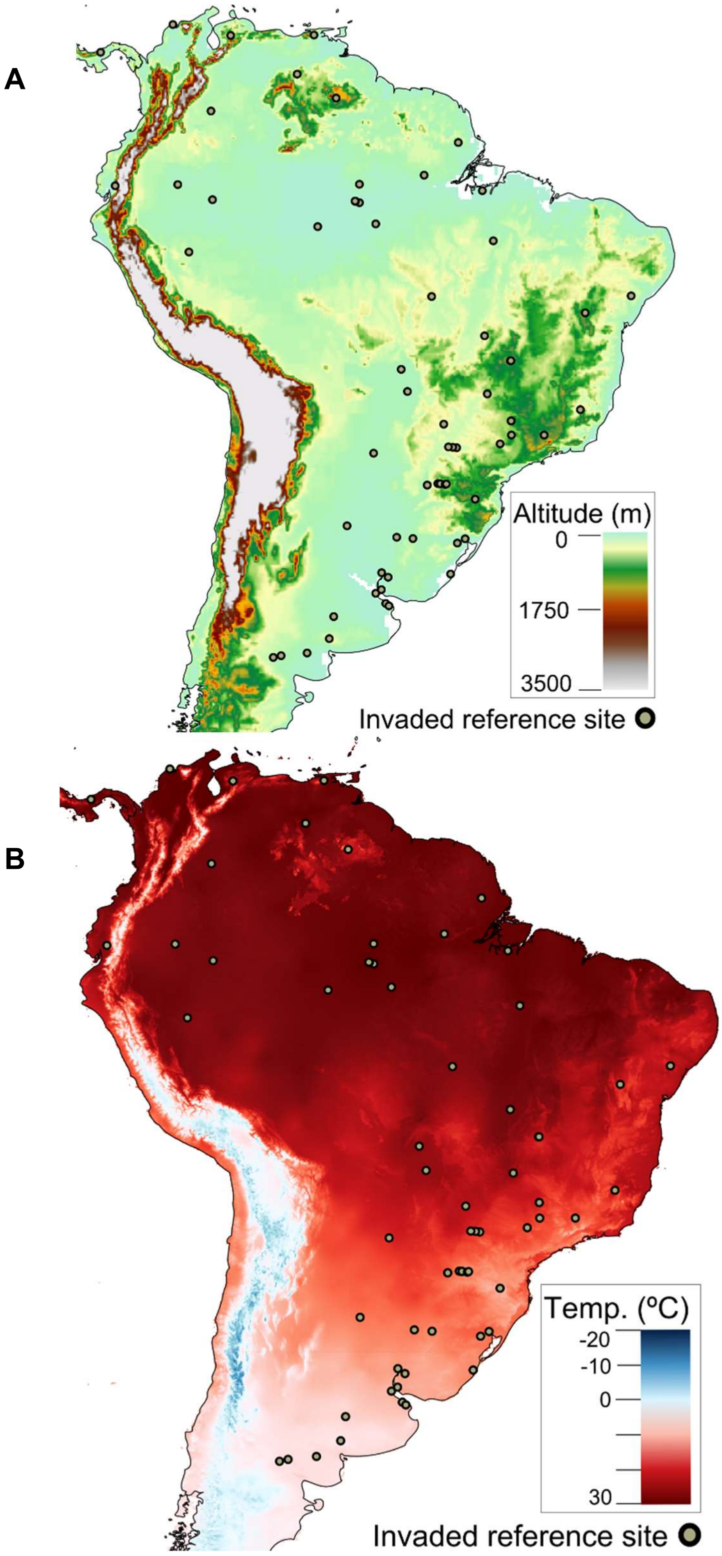

Fig. 3 South America digital elevation model (A), and average air temperature for June (1950-2000) (B). The invaded sites found in the bibliography are represented by dots

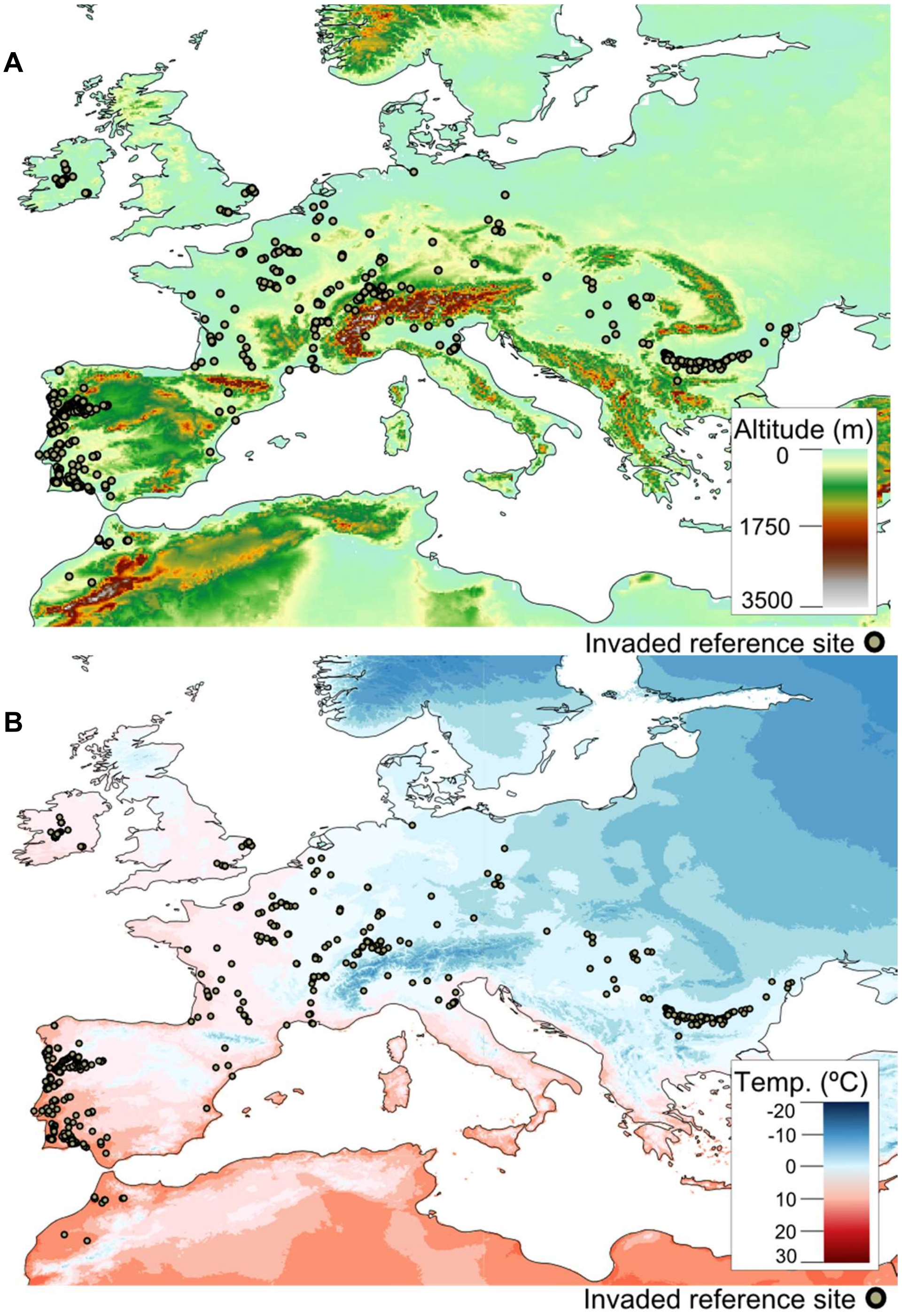

Fig. 4 Europe and North Africa digital elevation model (A), and average air temperature for January (1950-2000) (B). The invaded sites found in the bibliography are represented by dots

The first reference in South America is in Rio de la Plata estuary (Ituarte, 1981) by the end of the 1960s (Fig. 1). The number of invaded sites is lower than in North America (Fig. 2-3). Still, invaded sites are mostly at low altitude, although the species can also be present at high altitude (Fig. 3A), and in considerably high temperature regions (around 30ºC, Fig. 3B). The progression from Argentina towards the Northeast of Brazil took nearly 40 years.

In Europe, the first records were described in the Tagus Estuary in Portugal and the Garrone Estuary in France in 1980 (Mouthon, 1981), followed by the River Rhine, near Rotterdam (Holland) in 1985 (Fig. 1). The most recent invasions in Europe were recorded in Ireland (Caffrey et al., 2011), in some central European systems (Schmidlin et al., 2012) and in an Italian lake on the Alps (Kamburska et al., 2013). Almost all rivers and oligohaline estuarine areas in Portugal have been invaded by the species (Fig. 4). In remaining European countries, *C. fluminea* distribution appears sparser, with exception of France. Again, its distribution seems preferentially associated to low-altitude areas (Fig. 4A) and to regions with winter temperature higher than -5ºC (Fig. 4B).

Africa recorded its first invasion in Morocco in 2008 (Fig. 1) (Clavero et al., 2012).

The invaded sites exhibited some similar characteristics in terms of geography and temperature (Fig. 5). 88% of invaded sites were below 500 m altitude (Fig. 5A) and more than 90% were between latitudes 30° and 55° (Fig. 5B). In fact, no records or only recent records were reported in high altitudes such as Rocky Mountains (USA, Fig. 2A), the Andes (South America, Fig. 3A) or Alps and Pyrenees (Europe, Fig. 4A), although the species appeared in surrounding areas. More than 75% of the sites were characterized by winter air temperatures between -5 to 10ºC (Fig. 5C). In fact, for all continents only one record was found below -10ºC, and only occasional occurrences were registered around -5ºC (Fig. 2-4A). For summer temperatures, almost 97% of the sites fall in the 15 to 30ºC range (Fig. 5D). For annual average temperature, almost 90% of the invaded sites were within 5 to 20ºC, and from this range 40% were located in the 10 to 15ºC interval (Fig. 5E).

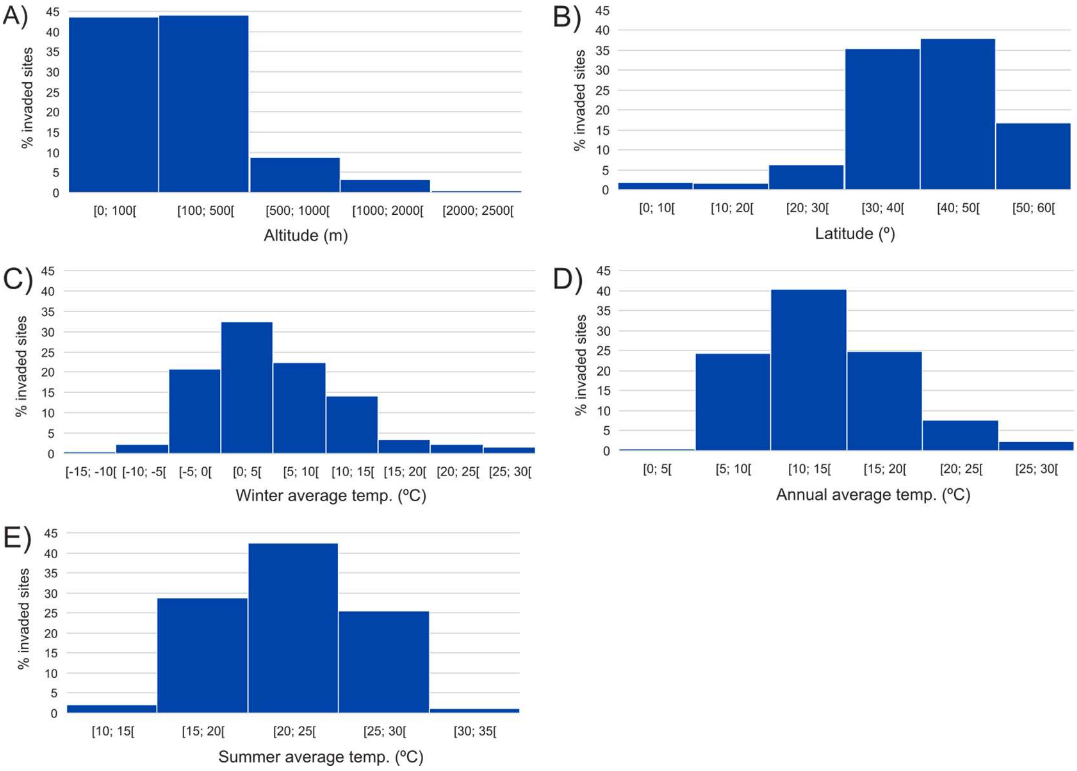

Fig. 5 Percentage of invasion sightings for (A) altitude; (B) latitude; (C) winter average temperature; (D) summer average temperature; (E) annual average temperature (n=1058)

Corbicula fluminea population traits were plotted against geographic and environmental gradients to identify trends in those relationships (Fig. 6 to 9). The data used included all available information related to the traits more frequently found in the bibliographic survey.

No statistical differences were found between the classes of life span, growth at the 1st year and mean body mass (ANOVA Life span: F-statistic= 1.528, p= 0.2168; 1st year growth: F-statistic= 1.251, p> 0.05; mean body length F-statistic= 2.487, p= 0.07927). Yet, some trends could be identified: longer life span and higher mean body mass were found in higher altitude regions (apparent positive relation), while the growth in the first year generally decreased with increasing altitude (Fig. 6). In addition, a negative relationship between latitude and growth at the end of the 1st year and mean body mass seemed to occur. Temperature (summer and winter) showed a positive effect on 1st year growth and mean body length (Fig. 8 and 9).

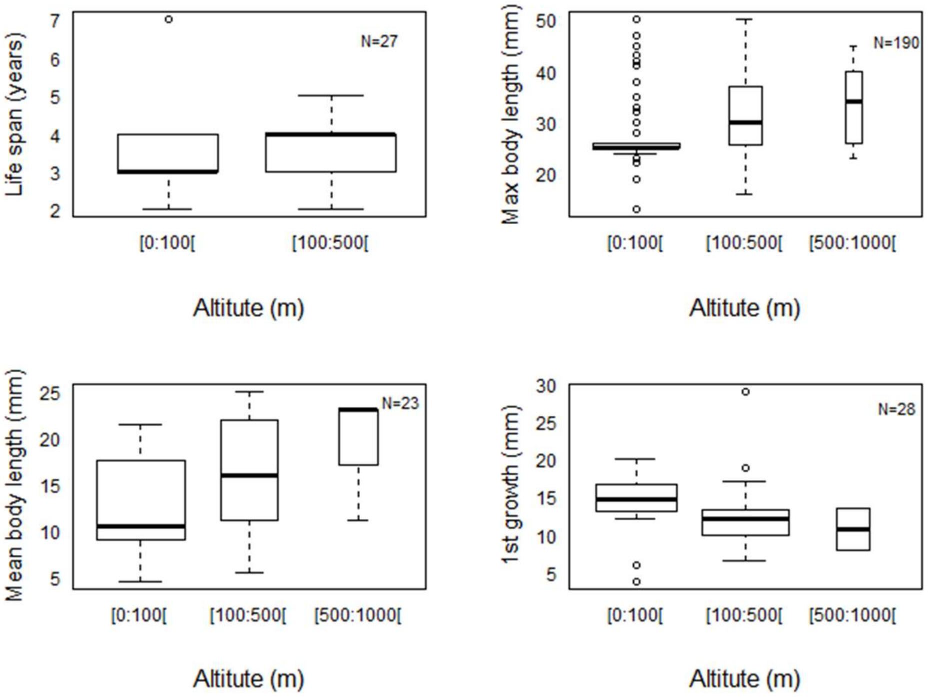

Fig. 6 Boxplots of the variation of life span, maximum and mean body length and length at the 1st year of growth per altitude. The middle line and extremities of the box are at the 25th, 50th, and 75th percentiles; the maximum length of each whisker represents the 5th and 95th percentiles

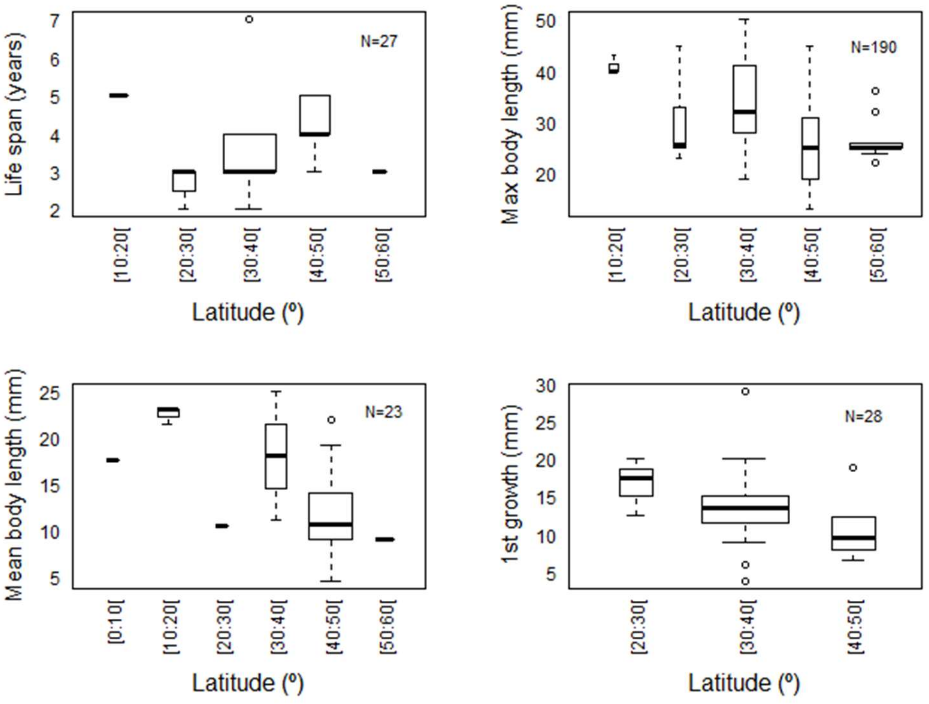

Fig. 7 Boxplots of the variation of life span, maximum and mean body length and length at the 1st year of growth per latitude. The middle line and extremities of the box are at the 25th, 50th, and 75th percentiles; the maximum length of each whisker represents the 5th and 95th percentiles

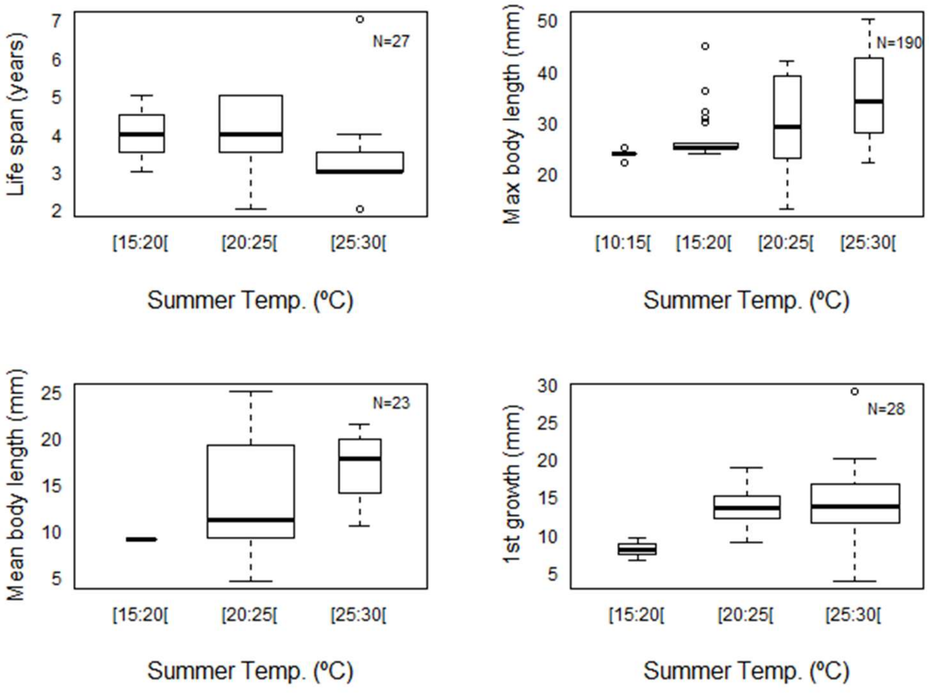

Fig. 8 Boxplots of the variation of life span, maximum and mean body length and length at the 1st year of growth per classes of summer average temperature. The middle line and extremities of the box are at the 25th, 50th, and 75th percentiles; the maximum length of each whisker represents the 5th and 95th percentiles

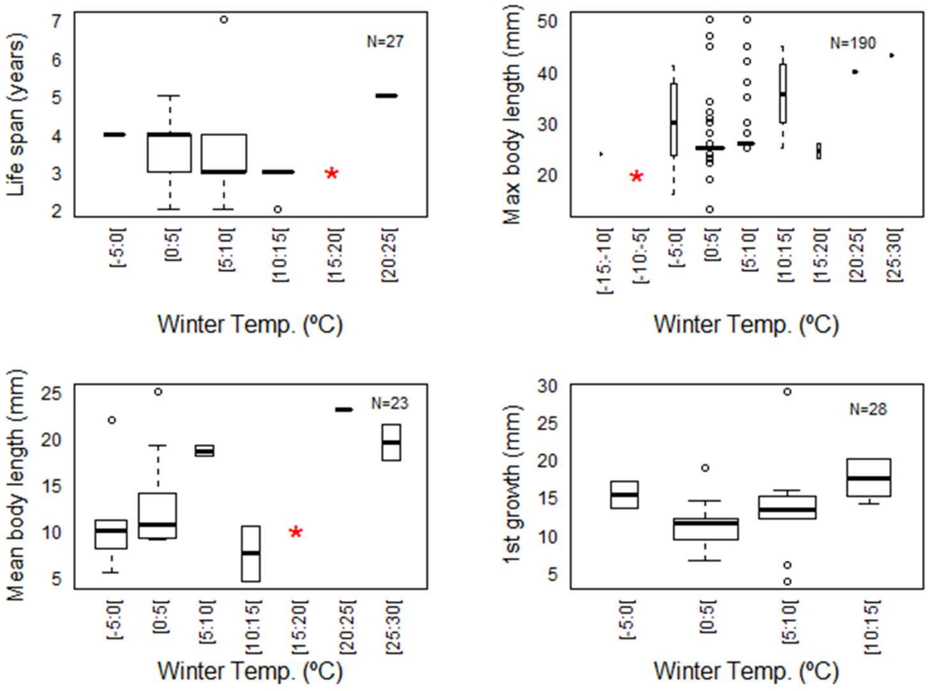

Fig. 9 Boxplots of the variation of life span, maximum and mean body length and length at 1st year of growth per classes of winter average temperature. The middle line and extremities of the box are at the 25th, 50th, and 75th percentiles; the maximum length of each whisker represents the 5th and 95th percentiles. (*): classes with no occurrences

With regard to maximum body length alone, higher maximum body mass was associated with higher altitude (Kruskal-Wallis chi-squared – KW= 17.9156, p-value< 0.001), with higher temperatures (Summer temperature KW= 59.6941, p-value< 0.001; Winter temperature KW= 66.4444, p-value< 0.001) and lower latitude (KW= 52.8702, p-value< 0.001).

6 DISCUSSION

6.1 INVASION HISTORY, PATHS AND CURRENT RECORD OF *C. FLUMINEA*

The dispersion in North America could be described as an arch, avoiding the central states of the USA until very recent years (Fig. 1). This may be related with the Rocky Mountains, crossing the continent from north to south, which acts as a barrier to the invasion of eastern areas. Indeed, many examples confirm the Rocky Mountains as a biogeographic barrier for fishes (e.g. Kruse et al. 1997; Adams et al. 2001; Rahel 2007; Smith et al. 2010) and invertebrates (e.g. Finn et al. 2006). The natural dispersion of *C. fluminea* seems to be limited by high slopes, characterized by low order streams. In the north, the limit is also defined by winter temperatures that fall below the lower lethal temperatures (2ºC, Mattice and Dye, 1976). Apparently, the invasion spread into new areas decreased with time for North America, taking about 82 years to spread from the Northwest coast to the Northeast coast. Still, there were some areas close to the oldest invasion sites with very recent introductions, probably related to human activities: a) the building of new channels for water diversion, or channel widening can create new available niches for the species; b) new industrial facilities that use water as cooling medium, create preferable conditions for new introductions, and therefore allowing for the species to appear in systems that, until that time, were protected against invasion; c) the opening of water courses to recreational fisheries or other recreational activities could also be responsible for the introduction of the species in previously uninvaded areas.

The invasion in Central America probably resulted from a branch of the North American main invasive path. Some Caribbean isles were also invaded, probably following the main land invasion. This insular invasion was quite recent (1998) and, like similar island introductions, it was due to human activities since, as a freshwater species, *C. fluminea* is unable to survive an oceanic crossing (Verbrugge et al., 2011). Similarly to North America, Central America mountain ranges seemed untouched by this NIS.

In South America, the origin of the introduction is still uncertain, although genetic studies suggest that south American populations shared some genetic similarities with North American populations (Lee et al., 2005). Therefore, hypotheses of a North American or Asian source are both viable. Apparently, the species has been kept away from the Amazonian Basin until recently. The high diversity that characterizes tropical habitats may be responsible for higher biological resistance (i.e. higher predation pressure, more parasites and diseases) and higher competition for resources, and therefore less susceptible to NIS establishment (Stachowicz & Byrnes, 2006). Simultaneously, many of the water bodies in the Amazonian Basin are known to have anoxic waters (Scarpelli, 2005; Bracho-Nunez et al., 2012; Junk et al., 2012), impairing the establishment of this species.

The species invasion did not occur in the higher mountain range of the Andes, where the high altitude probably acted as a barrier for invasion success. The winter temperatures, on average higher than those recorded in the North American continent, might have also acted as a restrictive factor to the invasion, at least in the southernmost areas of the continent, where the average temperatures for June can be close to or below 0ºC. In addition, the oligotrophic conditions that characterize high mountain ranges act as an important limiting factor for the survival of many freshwater bivalves (Dillon, 2004).

For Europe it was very hard to track a defined invasion path. The first records of the species were almost simultaneous and scattered across different geographic locations. This fact supports the theory that the introduction of the species across large scale distances is human mediated and connected with maritime transportation (ballast water). Those introductions were responsible for the dispersal across Europe towards the Mediterranean and Eastern part of the continent. Britain and Ireland were recently invaded, probably, from already established populations in central and northern Europe. The Pyrenees range might have acted as a physical barrier to the progression of *C. fluminea*, therefore, the central European populations could have origin on invasion episodes different of the Iberian ones (Figs. 1). In general, the invasion success was somewhat limited in the higher mountain ranges, low temperature regions and oligotrophic conditions.

For Africa, there are only recent records of the species in Morocco (Clavero et al., 2012). However, the species invasion in other African areas is possible, but going unnoticed due to the lack of environmental management and scientific awareness.

The data records presented in this study reflected the first invasive reference found in the literature, and the dates might present a slight delay relative to the real introduction dates. Nevertheless, Fig. 1 represents an accurate illustration of the main paths history of *C. fluminea* invasion*.* Even so, many uncertainties still exist about the global dispersion of this NIS and further biogeographic studies encompassing genetic data may be highly informative.

The USA showed a large record of invasion when compared with other countries. As the first country to be invaded by *C. fluminea* outside its native range, the awareness of the risk associated with the species has been raised very early on, as reflected, for example, on the U.S. Geological Survey web site (http://nas2.er.usgs.gov/viewer/omap.aspx?SpeciesID=92), which displays a vast number of records of the species, with different levels of accuracy. Some regions across the globe may be less aware (e.g. South and Central America, Eastern Europe or Africa), and therefore the number of sightings might be underestimated. This could justify the difference on the number of sightings when comparing USA and the rest of the world.

In the worldwide invaded range, the southernmost reference is Negro River at Chimpay (Patagonia - Argentina) (Cazzaniga & Pérez, 1999), with the latitude of -39.2034º and longitude -66.159203º. In the North Hemisphere, the most northern reference is the Shannon River at Carrick on Shannon (Ireland), with the latitude of 53.9422º and longitude of -8.087545542º (Minchin, 2014). The average winter air temperature in Chimpay falls to 7.3ºC whereas at Carrick on Shannon it drops to 4.3ºC. Nevertheless, there were other latitudes with more extreme negative winter temperatures: the coldest site in our database, with a winter average temperature of -13.8ºC is at St. Louis, USA (N 47.02257876º; W 92.21607517º). The survival of the species in those locations was probably related to site-specificities: e.g. areas near industrial and residential water discharges that allow Asian clam populations to receive a constant input of warm water. These artificial conditions, in which the water temperature never goes under the vital limits of the species, allow the survival of *C. fluminea* in those geographic locations. Interestingly, in the northern hemisphere *C. fluminea* invaded further higher latitudes probably due to human activities that in one hand increased their dispersion rates and on the other hand enhanced the probabilities of establishment due to the thermal pollution associated with larger human agglomerates. In the southern hemisphere we have a less dense human population and thermal pollution is probably much less severe. Therefore, the thermal boundary is more effective in South America, only allowing the survival of *C. fluminea* at higher atmospheric temperature and lower absolute latitude.

According to this study, the preferable habitat for *C. fluminea* seems to be under 500m of altitude, between the latitudes of 30 to 50º and annual mean temperature between 5 to 20ºC, preferably 10 to 15ºC. Higher altitudes and the oligotrophic conditions associated with the higher mountain slopes seem to limit the natural upstream dispersion of *C. fluminea*. A temperature of 10 to 15ºC allows all vital biological activities of the species, including reproduction. In a scenario of predicted global warming, this may represent an increased challenge for management, because it widens an already extensive susceptible range for invasion (McDowell et al., 2014).

In the present study, the distinct distribution patterns across the invaded areas could be justified by three main reasons, gradually more relevant: 1) a differential biological resistance to invasion; and 2) distinct amounts of human activities in the studied areas; 3) an uneven research effort on the species.

6.2 GEOGRAPHIC AND ENVIRONMENTAL GRADIENTS *VS.* POPULATION TRAITS RESPONSES

Despite the trends of lower 1st year growth and larger maximum and mean body mass with increasing latitude, winter temperatures do not seem to have such a clear influence on the body mass. This situation might be related to the thermal pollution in some of these low winter temperature areas (e.g. in -1ºC air temperature, Gollasch, 2006). Summer temperature seems to have a larger influence in the population traits, namely in the growth at the end of the 1st year and in the maximum body length. This could be related to more efficient metabolism during the growing season (Mattice & Dye, 1976; Vidal et al., 2002).

Life span showed a positive relationship with the latitudinal gradient. Populations from higher latitudes have a slower growth, reflecting in general a slower metabolism and a longer life span. This type of relationship has been described for other mollusk species, including freshwater bivalves (e.g. Cardoso et al. 2002; Verdelhos et al. 2011; Varandas et al. 2013). On the contrary, populations at lower latitudes, with higher water temperatures, have higher growth rates and, consequently, shorter life spans. Also, the availability of food may be impaired at higher latitudes due to shorter sunlight periods. Colder environments reduce phytoplankton standing stocks on which *C. fluminea* mainly rely (although the species can also pedal feed and consume organic matter available in the sediments; Hakenkamp and Palmer 1999). Altitude may also play a role in this, because at higher altitudes nutrients availability is usually reduced.

We expected to have clearer patterns regarding the population traits and environmental gradient. Nevertheless, the species shows a high ecophenotypic plasticity (McMahon, 1999) and local conditions may have a decisive role on the populations traits, which could confound the general latitudinal and geographic gradients.

The availability of calcium is also a fundamental issue for bivalves, as the shell is built on it. Organic matter is also fundamental for the growth of soft tissues as well as for the shell. Despite the lack of data on the hardness and nutrients for most of the sites where *C. fluminea* is present, it is expected that higher altitudes present lower calcium concentrations and oligotrophic conditions, thus contributing to the limiting of the species progression and populations growth in a synergistic effect with temperature.

7 CONCLUSIONS

Corbicula fluminea invaded many ecosystems worldwide, being present from 39º South to 53º North latitude. In less than 100 years, it has invaded all continents, except Antarctica, making this clam one of the most successful invasive species in aquatic ecosystems. This progression is human mediated and, apparently, only delayed by orographic constraints (e.g. high slope and oligotrophic conditions) and low temperature. However, the thermal pollution may help the species to establish beyond their current latitudinal and temperature limits.

It was possible to identify trends between latitude (and summer temperature) and some population traits such as growth in the first year and life span. Still, the highly ecophenotypic plasticity of the species makes those relationships less clear than expected. Simultaneously, the lack of a standard protocol for the study of the species population traits makes it harder to provide worldwide generalizations on the relationships between traits and invasion success.

Nevertheless, with the predicted climate change, one could expect that the species will go further beyond its current limits, invading freshwater and estuarine systems further north and south. Some of the high altitude systems have been already invaded. With the data gathered in this work, we hope to contribute to future models, where the susceptibility of certain geographical areas to invasion by *C. fluminea* may be predicted more accurately. Those models could be a precious help in order to detect the invasion in its earlier phases, eventually allowing successful eradication at lower ecologic and economic costs.

8 ACKNOWLEDGEMENTS

This research was supported by FCT (Portuguese Foundation for Science and Technology), through a PhD grant attributed to D. Crespo (SFRH/BD/80252/2011), a post-doc grant attributed to S. Leston (SFRH/BPD/91828/2012) and M Dolbeth (SFRH/BPD/41117/2007) and BIOCHANGED project (PTDC/MAR/111901/2009), subsidized by the European Social Fund and MCTES (Ministério da Ciência, Tecnologia e Ensino Superior) National Funds, through the POPH (Human Potential Operational Programme), QREN (National Strategic Reference Framework) and COMPETE (Programa Operacional Factores de Competitividade). We also acknowledge two anonymous reviewers for their helpful and constructive comments which improved the manuscript.

Chapter II

Survival of *Corbicula fluminea* (Müller, 1774) in a natural salinity and temperature gradient: a field experiment in a temperate estuary

1 ABSTRACT

The invasive bivalve *Corbicula fluminea* is described as a freshwater species, but can colonize estuarine upper reaches. In a 46-day field experiment, the survival ability of the species along an estuarine gradient was tested. Our goal was to understand its tolerance limits in a transitional area and in which conditions a potential invasion in the estuary could thrive, in order to predict the potential threat of the species distribution and settlement along the freshwater-estuarine continuum. The Lethal Time (LT) method was adapted to this field test with success. The population held an adult stock in the most upstream areas of the estuary (salinity< 0.5). Dispersal mechanisms allowed the species to be present in the downstream areas, however, with a different population structure as consequence of the interaction of the diverse physicochemical gradients of the estuary, in particular different salinity and temperature combinations. During winter, the species was able to survive in all test sites, with practically no mortality, while in summer LT_{50} (median lethal time) changed considerably when comparing the upstream and the most downstream sites (82 days (predicted value) versus 9 days, respectively). Still, 100% mortality was never achieved, adding concern to the species invasion potential in this estuary.

2 KEYWORDS

Tolerance limits; Climate change; Invasive species; Dispersal

3 INTRODUCTION

Despite some unpredictability regarding ecological impacts, human mediated global changes will impair biodiversity and ecosystems functions, including physiological and metabolic processes (Hicks et al., 2011), species populations and communities (Sorte et al., 2010; Dolbeth et al., 2011; Eggers et al., 2012) and ultimately, large ecosystems and the biosphere (Schröter et al., 2005; Barnosky et al., 2012). Biological invasions by non-native species often take place due to human intervention and represent one of the biological/ecological alterations occurring on a worldwide scale as a consequence of globalization (e.g. Crespo et al. 2015).

In the context of current global changes, biological invasions arise as an event of their own (Simberloff et al., 2013). Transitional ecosystems, particularly estuaries, are highly susceptible to events of biological invasion, as several human activities that are major vectors for introductions concentrate in those areas (e.g. fisheries, ballast water, general human occupation, aquaculture) (Cohen & Carlton, 1998; Williams & Grosholz, 2008). Known as fundamental biological areas, estuaries are highly productive habitats (Kennish, 2002; Dolbeth et al., 2011; Hicks et al., 2011) and functionally important (e.g. Sousa et al. 2010; Sousa et al. 2012a). Since estuaries are generally characterized by low diversity due to particular physicochemical conditions (Loo & Rosenberg, 1996; Kennish, 2002; Dolbeth et al., 2011; Hicks et al., 2011), the introduction of novel species with high spreading and outcompeting potential could have a disproportionate effect on ecosystem functioning (Stachowicz & Byrnes, 2006; Sousa et al., 2008b).

The Asian Clam, *Corbicula fluminea* (Müller, 1774), is among the most invasive species of freshwater systems (McMahon, 2002; DAISIE, 2008; Sousa et al., 2008a), presently with a global widespread distribution and high invasive efficiency (Crespo et al., 2015). This species can affect the hydrology, biogeochemical cycling and biotic interactions via two general mechanisms: assimilatory-dissimilatory processes (uptake and release of energy and materials) and physical ecosystem engineering (physical environmental modification by organisms), with impacts ranging from individuals to ecosystems (Hakenkamp et al., 2001; Sousa et al., 2009; Atkinson et al., 2011). This species can create a different habitat matrix for benthic fauna via bioturbation and shell production (Ilarri et al., 2012, 2014), and has high filtration rates, reducing phytoplankton availability for other species (Foe & Knight, 1985; Phelps, 1994; Hakenkamp et al., 2001; Sousa et al., 2008a). It is a highly productive bivalve, which may cause disruption of the food chain, either as a consumer or as food source for higher trophic levels, especially when massive die-offs occur (McMahon, 2002; Sousa et al., 2008b, 2012b). The species is able to dominate the benthic fauna of several lenthic and lothic habitats (McMahon, 2002; Crespo et al., 2015). Changes in the biogeochemical cycles have been reported as a consequence of the species pedal feeding, with subsequent alterations in the abiotic characteristics of the top layer of sediments and high rates of nitrogen excretion (Sousa et al. 2008a, and references therein).

Despite being a freshwater species, *Corbicula fluminea* can tolerate salinities up to 10-14 (in PSU, unitless) (McMahon, 1983, 1999), allowing it to colonize the upstream areas of estuaries (Sousa et al., 2008b; Franco et al., 2012; Ilarri et al., 2014). Its life cycle favors the ability to invade other systems, due to fast growth rates, early maturation (McMahon, 1999, 2002; Sousa et al., 2008a) and capability of androgenesis and self-fertilization (Pigneur et al., 2011). Juveniles are released as pediveligers and their dispersal is aided by a mucilaginous drogue line (Prezant & Chalermwat, 1984; Rosa et al., 2012). Also, the species is known to have a great phenotypical plasticity, indicated by a wide range in shell characteristics and body mass for geographically close systems (Sousa et al., 2007; Vohmann et al., 2010; Rosa et al., 2012), which is another feature contributing to its overall invasiveness potential.

The awareness on the species' impacts has been increasing, since it has led to major economic impacts in North America, due to biofouling (Mattice, 1977; Rosa et al., 2011). The species has been present in North America since the mid-1930's, in South America since the 1960's (Darrigran, 2002) and in Europe since the 1980´s (Ilarri & Sousa, 2012; Crespo et al., 2015). Despite the large availability of information on its vital limits, genetics, physiology and metabolism, there is still an information gap on how the species can deal with adverse cyclic conditions such as those found in estuaries. The physicochemical parameters (e.g. salinity, temperature, water depth, turbidity) change accordingly to cycles of different lengths (tidal, lunar, seasonal, annual and even larger global cycles, McLusky, 1993; Teixeira et al., 2008; Pratt et al., 2014) in estuaries, so it becomes important to evaluate if *C. fluminea* populations are able to develop in such instable environments. Adding to this, as transitional habitats, estuaries show complex gradients (e.g. in a neotropical reservoir, Paschoal et al., 2015), with horizontal, vertical, cross-sectional, or other patterns, when referring to the above abiotic factors (McLusky, 1993). As an invasive species responsible for high secondary production levels (Phelps, 1994; McMahon, 2002; Sousa et al., 2008b), it may capitalize on a large amount of the available energy found in estuaries (Vannote et al., 1980). This could represent an additional threat for native biodiversity, with disruptive effects on functions and processes of native organisms.

This paper reports the results of a field experiment where the invasive species *C. fluminea* was placed along a temperate European estuary following the natural estuarine gradients, in summer and winter. Some of the experimental sites were set in areas where adults of *C. fluminea* were not previously reported (Franco et al., 2012; Modesto et al., 2013). This will allow us to predict if the species represents a threat for the downstream reaches of the estuaries or if the natural gradients and cycles are sufficient to avoid further advance.

4 MATERIAL AND METHODS

4.1 EXPERIMENTAL DESIGN

This study took place in the Mondego estuary (Portugal, 40°80' N, 8°50' W), a well-described system with temperate coastal climate of Mediterranean and Atlantic influence. The lower reaches of the estuary comprise two arms, north and south, separated by an alluvium-formed island with a total length of 7 km. This estuary extends for approximately 22km, and a large portion of its margins are artificially maintained with stones and concrete structures for flood management. The tides are responsible for two large daily salinity oscillations (~3 m tidal range), that in the estuary mouth can reach 30-34 (euhaline) and in the uppermost areas 0.5-5 (oligohaline) (for further information refer to Teixeira et al. 2008; Franco et al. 2012; Modesto et al. 2013).

The north arm of the estuary was surveyed to establish the downstream occurrence limits for *C. fluminea*. Seven sites, designated as survey sites (S1 to S7, Fig. 1), were selected after Franco et al (2012) and Modesto et al (2013). At each site, samples were collected with a Van Veen grab, and we measured *in situ* the following physicochemical parameters: temperature, salinity, Secchi depth and pH. Sediment samples were also collected for granulometry and organic matter determination. The median grain size was estimated with GRADISTAT 8.0 (Blott S.J. & Pye K., 2001).

Sampling sites were selected to account for the downstream distribution limit of *Corbicula fluminea* in this estuary, the population structure and density levels to include in each experimental unit and finally salinity. Three sites were selected (A to C) in summer and four sites in winter (A to D) (Fig. 1) to test the tolerance limits of *C. fluminea*. Site D was added to the experimental set for winter conditions to account for the increase in freshwater discharge that intensifies during this season. *Corbicula fluminea* individuals were handpicked in site A (coincident with S1) in the upstream limit of the estuary where the saline plume can seldom reach, with oligohaline characteristics. This area is still under tidal influence, despite the distance from the estuary mouth (22 km).

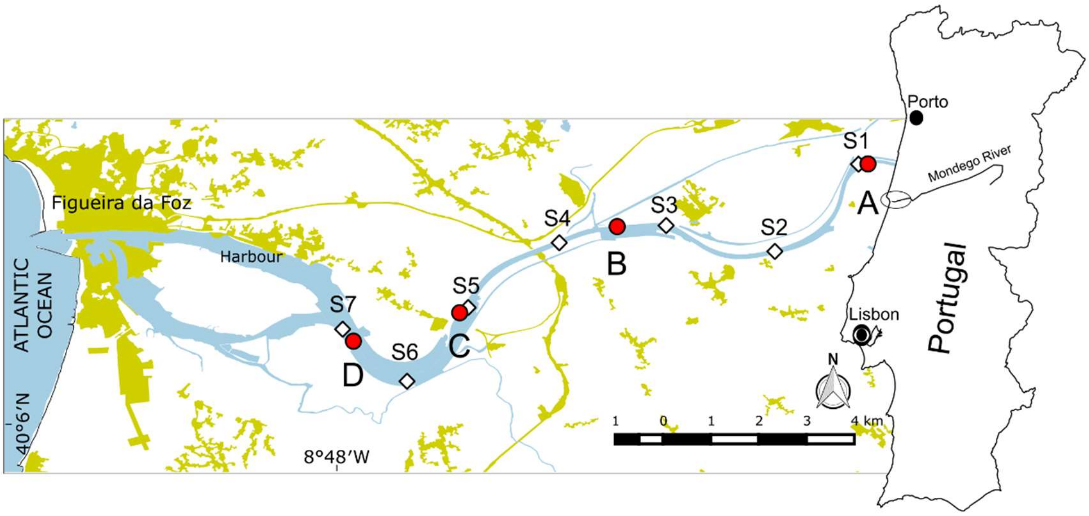

Fig. 1 Location of the field survey (S1 to S7 (◇)) and experimental sites (A, B, C and D (●)) in the Mondego estuary, in the western coast of Portugal

At each site, four mesh bags (20x30 cm, 1 mm mesh) with individuals were placed in the water and tied to a fixed prop, to guarantee full submersion during low tide. In summer, a total of 13 individuals were placed in each bag, with the following size classes that mimic the population structure found *in situ* for each season: 3

organisms under 20 mm; 8 organisms between 20 and 25 mm; 2 organisms between 25 and 35 mm. In the winter a total of 14 individuals were placed in each bag, with the following size classes: 3 organisms under 20 mm; 10 organisms between 20 and 30 mm; 1 organism larger than 30 mm.

The experiment ran for a total of 46 days, in each season. At T0, T1 (1 day), T2 (2 days), T3 (4 days), T4 (6 days), T5 (8 days), T6 (14 days), T7 (22 days), T8 (30 days) and T9 (46 days), mortality levels were verified and temperature, salinity, oxygen (% and mg/L) and pH were measured in low tide conditions.

4.2 STATISTICAL ANALYSIS

The species density levels per size class of the field survey data were analysed with a 2-way crossed ANOSIM for the ‘sampling stations’ and ‘season’ factors, after square root transformation of the density data and upon a zero-adjusted Bray-Curtis matrix (Clarke & Gorley, 2015). The data was further analysed with distance based linear modelling (DISTLM), where we explored the relationship between species density and physicochemical parameters, using AICc as selection criteria for a best model selection procedure (Anderson et al., 2008). These results were also explored with a dissimilarity based redundancy analyses (dbRDA), to visualize the significant model (Anderson et al., 2008). Prior to the analysis, the physicochemical variables were checked for collinearity with Draftsman plot, and collinear variables removed.

A similar procedure was done for the survivorship percentage data from the experimental set (2-way crossed ANOSIM + DISTLM), but upon a Euclidean Distance matrix on non-transformed data.

In addition, with the survival experiment data, samples from each site were characterized with lethal time 25% (LT_{25}) and lethal time 50% (LT_{50}), which represents the moment when mortality reaches 25% and 50%, respectively

(Environmental Technology Center Canada, 2007). This method is usually used for ecotoxicological tests, under controlled conditions (Abel & Garner, 1986; Newman & Aplin, 1992; Environmental Technology Center Canada, 2007; Costa et al., 2012), and was adapted for this field experiment. For this study, LT_{25} and LT_{50} provide us with a proxy of the species tolerance to the ecological features of each experimental site, therefore allowing us to compare this tolerance along the estuarine gradient and eventually with other estuarine systems. The LT's were calculated with software package Statistica ® 7.0 (http://www.statsoft.com/). Site survival data, in each case, were fitted to the best model among:

- Logistic model ($surviving = a / (1 + (p/1-p) * (time/LT_p)\hat{}b)$);
- Exponential model ($surviving = a * exp\ (log\ ((a-a*p-b*(1-p))/a) * (time/ LT_p)) + b$);
- Gompertz model ($surviving = a * exp\ ((log(1-p))) * (time/ LT_p)\hat{}b$)

These formulas were used to estimate both lethal times (LT_{25} and LT_{50}), where: a= the y-intercept (the control response); LT_p= the lethal time for p dead individuals for the data set; p= proportion of dead individuals (0 to 1) on the LT of interest; *time*= exposure time; b= a scale parameter (estimated between 1 and 4) (Environmental Technology Center Canada, 2007).

5 RESULTS

5.1 FIELD SURVEY DATA

According to the field survey data, the downstream limits for *C. fluminea* along the estuarine gradient changed with season, as individuals were found in site S4 during winter and only as far as site S3 (further upstream) for summer conditions (Fig. 2). Different patterns in the density levels and population structure were observed within each site (Fig. 2, ANOSIM for differences between sites across all seasons: global R: 0.64, p= 0.001). During summer, adults (> 20mm) were found in the three uppermost sites (S1 to S3). Their highest density occurred in S1, yet the largest individuals (> 35mm) were found in S3. Juveniles under 10mm were dominant at S3, which presented the highest density levels over the whole study period (Fig. 2).

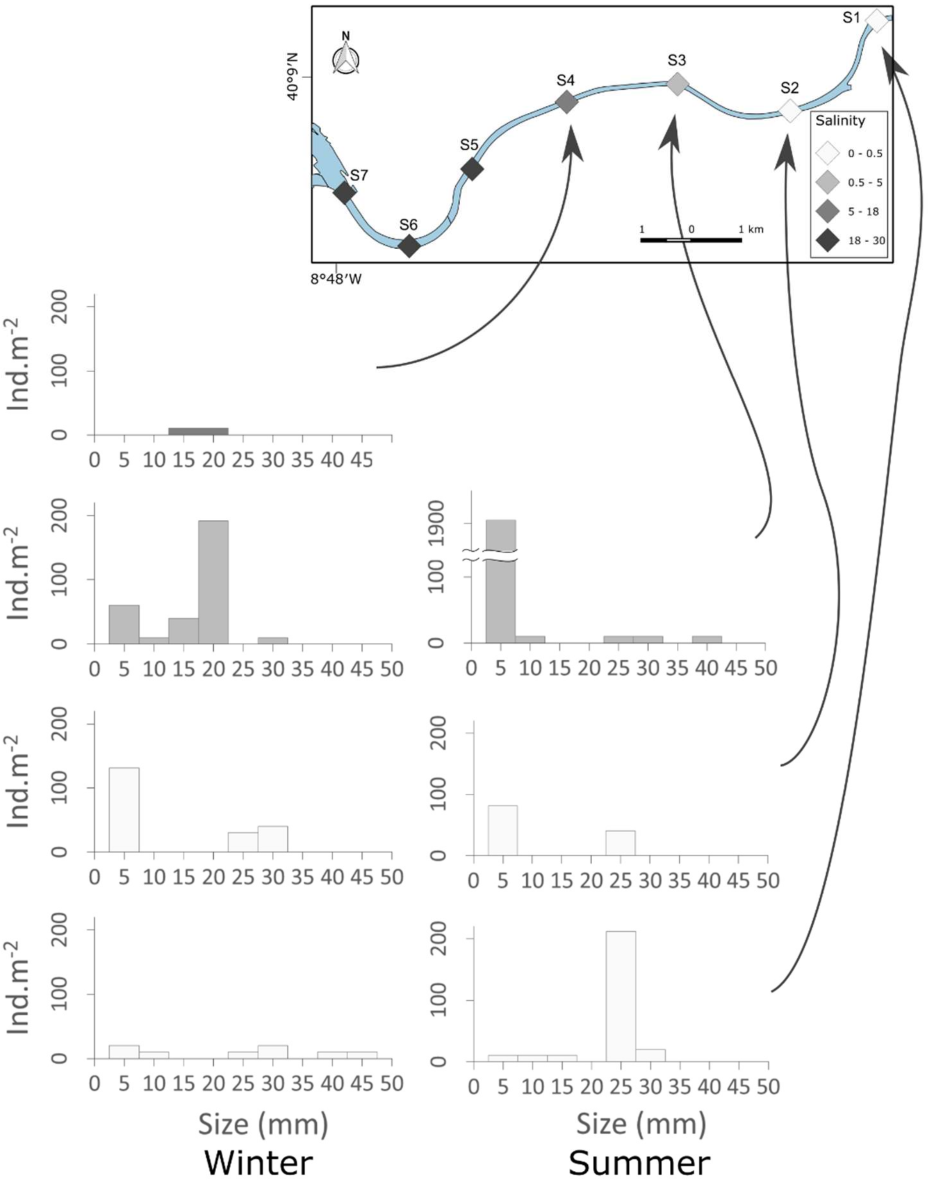

Fig. 2 Field survey results. *Corbicula fluminea* was found in S1, S2, S3 and S4. The population structure in each prospection site in summer and winter is shown in the histograms. The histogram colors refer to the salinity range described for each site. It is clear the downstream shift on the population structure, with adults' strong presence in S1 and a clear dominance of juveniles in S3

During winter, spatial variation of the population structure was not so clear, as both juveniles and adults (> 20mm) occurred at the three uppermost sites. Juveniles' density was considerably lower when compared to S3 in the summer. In addition, the largest (= oldest) individuals were found in S1.

The best significant model relating population structure density and the environmental data was composed by salinity, temperature and median grain size (MGS), which together accounted for 96% of explained variation. However, only 31% of the total variation was explained by the best fitted model. The downstream site had higher salinity, while higher temperature was associated to the upstream areas, particularly in the summer. MGS was more important in differentiating between the upstream sites during the winter (dbRDA results, plot not presented).

5.2 SURVIVAL EXPERIMENT ANALYSIS

Practically all individuals survived in the 46-days of the winter experiment, while in summer there were different mortality levels dependent on the site (with different temperature/salinity combinations) (Fig. 3). Nevertheless, 100% mortality was never reached at any site, in the same period length. The survival data and curves are clearly different between winter and summer (Fig. 3, ANOSIM for the difference between seasons, across all sites: global R: 0.972, p= 0.001), but not within stations (p> 0.05). DistLM showed that the variables most contributing to these differences within season were the maximum water temperature, which explained 91.1% of variation in the survivorship percentage data. High temperature was associated to the summer conditions and to the sites at the uppermost areas (dbRDA results, plot not presented).

Lethal times were only calculated for summer, as mortality in winter was negligible. By the end of the experimental run, more than 30% of the individuals died in the upstream area (site A) and more than 50% in the downstream areas (sites B and C). The lethal times for 50% survivorship followed the natural estuarine gradient, being shorter at site C (9 days) (with higher salinity values) and longer at site A (82 days, predicted value, considering that the test ran for 46 days) (Fig. 4).

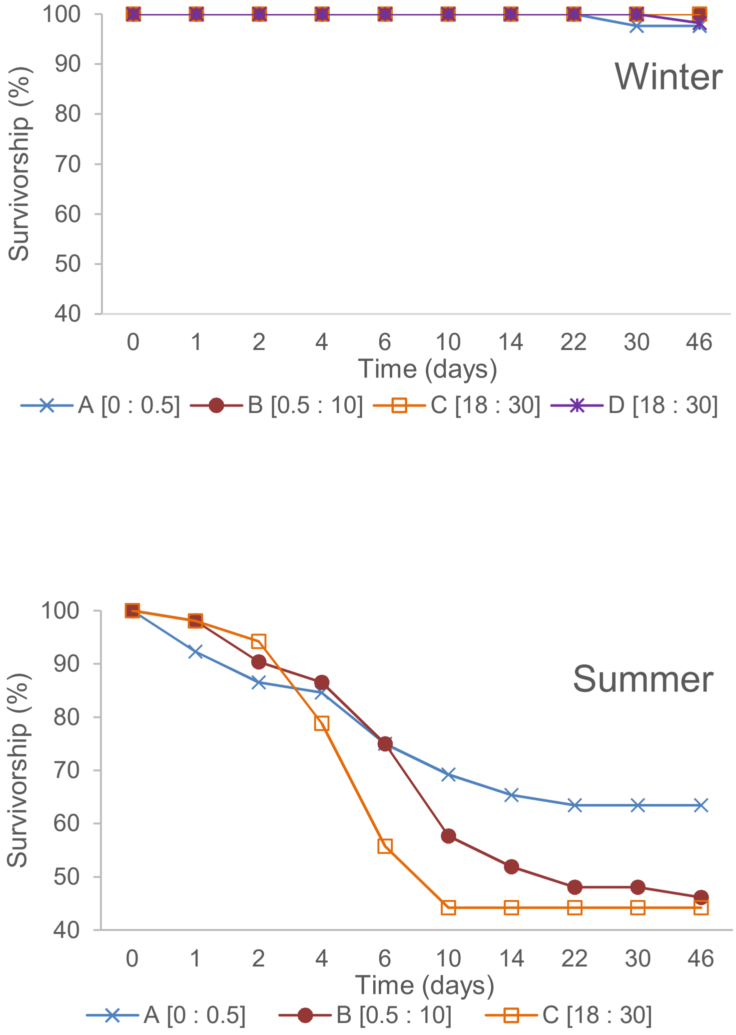

Fig. 3 Average survivorship of *Corbicula fluminea* along the estuarine salinity gradient. Each site shows the values for the Venice classification system of salinity inside the square brackets as an indication. The winter experiment showed very small mortality, while in summer mortality was higher and influenced by salinity. It should be noticed that 100% mortality was never achieved during the 46 days experiments

The confidence intervals decreased as well as the LT_{50} in downstream areas of the estuary. The LT_{25} pattern along the estuary is less clear. These values were very close between 6 and 7 days, but the confidence interval for site C is larger than for LT_{50} (Fig. 4). The average salinity measured in low tide was 0, 0.14, and 2.4 for sites A, B and C respectively (Fig. 4). The average salinity in high tide was not recorded but previous studies in the same area (Dolbeth et al., 2008b) recorded values close to 20, in site C, during the summer.

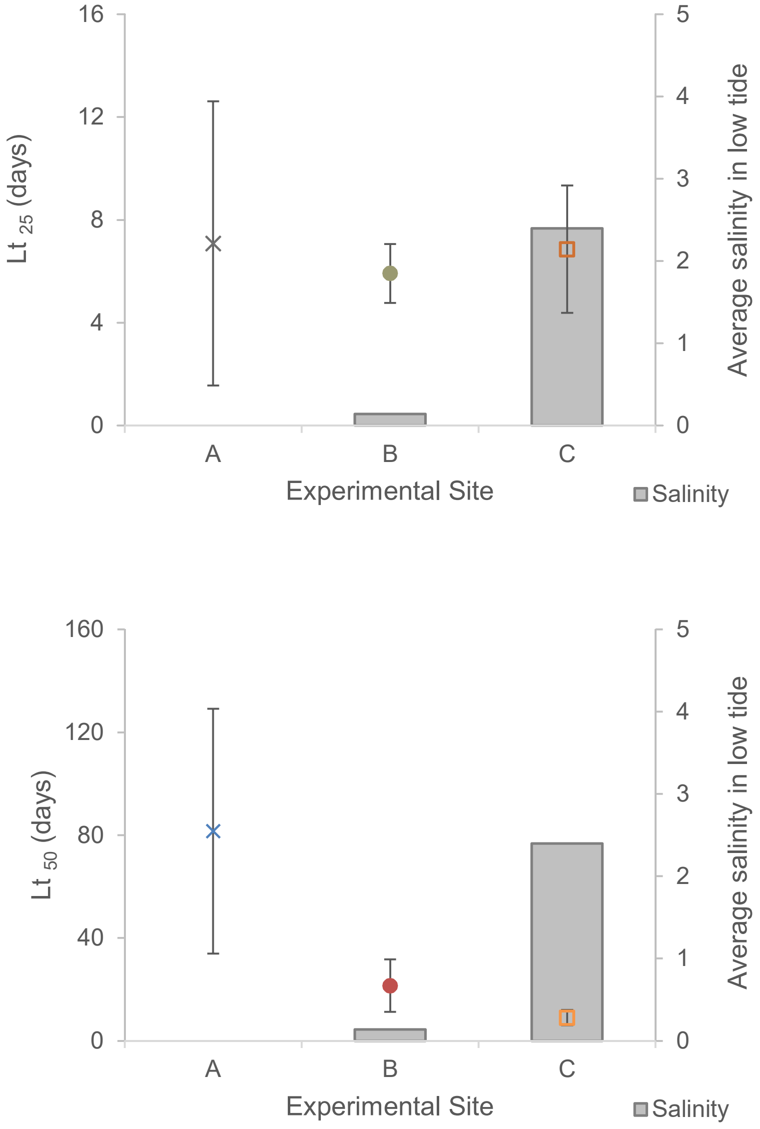

Fig. 4 Lethal time (25 and 50% mortality, and respective confidence intervals) and average salinity recorded during low tide, in summer. LT_{50} showed a larger difference between sites

6 DISCUSSION

6.1 *CORBICULA FLUMINEA* IN ITS NATURAL BORDERS

Corbicula fluminea is a freshwater species that is able to osmoregulate under salinity 13 (Morton & Tong, 1985). As such, it has the potential to spread to middle sections of estuaries, using to its advantage the daily low tides and hence temporary low salinity, when it can perform fundamental tasks for surviving. Therefore, *C. fluminea* can establish in the upstream areas of estuarine systems, as previously recorded in the Mondego estuary. The population distribution and structure along this system responds to the estuarine gradient that includes, among several parameters, salinity as an important driver. The largest densities were found in the oligohaline areas - S2 and S3 (salinity between 0.5-5, according to the Venice classification system), with adults between 15 and 20 mm most abundant in S3. Density levels were within the average range reported for other Portuguese estuarine systems (Sousa et al., 2012b), with exception of S3 in summer, which registered higher density (mainly constituted of juveniles, almost 2000 ind.m^{-2}).

The population structure at each surveyed site resulted from the interaction of specific physicochemical conditions and the strong dispersal abilities of the species. In the summer, when the salinity influence is higher, the adult stock is kept in the uppermost reaches of the estuary (*i.e.*, S1). The population structure shifts when moving downstream, with both young adults and juveniles balanced in the S2, and clearly dominated by juveniles in S3. During winter, *C. fluminea* also occurred in a station further downstream in a mesohaline area – S4, which can be explained by the larger freshwater runoff, able to carry individuals from the upper reaches to downstream areas. McMahon (2002) states that *r*-selected traits such as high reproductive effort and secondary production efficiency could be more important in the invasive potential of a species than the physiological tolerance for a broad environmental range. The field survey data reflect this hypothesis. The shift in the population structure between sites and among seasons may be a consequence of a large investment of *C. fluminea* in the reproduction and dispersal of juveniles, by releasing pediveligers from the stable population at the uppermost area (S1) and drifting along the estuarine gradient. S1 has freshwater characteristics, more suitable for the adult stock population, which is the source of *C. fluminea*'s individuals downstream.

However, high salinity may impair the settlement of the species in the areas closer to the sea, due to physiological constrains, acting as a natural barrier to the invasion. While adults can survive in high salinities during long exposures, juveniles are more sensitive to high salinity and may not have the same energy resources to protect themselves as adults do (McMahon, 1983, 1999; Ilarri & Sousa, 2012). Therefore, even though dispersal mechanisms may be enabling pediveligers of benthic settlement, they cannot handle the estuarine natural change in the physicochemical conditions and die before the first year. This justifies the absence of large proportions of adults downstream S1 (as shown by Franco et al (2012) and found in the present field survey campaign), despite the presence of young juveniles in our survey sites 1 to 4. Juveniles may be able to cope with the higher salinity of S3 and S4 until energetic requirements imply a larger period with the valves open for feeding purposes, therefore becoming more exposed to salinity.

Regarding temporal variability, the shift from winter to summer conditions implies a reduction in freshwater runoff, which might have reduced the survival of the 20mm class during summer. The reason for the high density of the 5mm class in the most saline S3 during summer can only be speculated, because no special local characteristics were found in this site. The spring recruitment occurs in the S1 area, where the adult stock is held. The highly effective dispersal mechanism of *C. fluminea*, with the mucilaginous drogue line (Prezant & Chalermwat, 1984), allows the juvenile specimens to drift along the estuary until S3. The water flow in S3 could behave in such a way that these juveniles can no longer drift downstream, accumulating in the site S3. Some of these 5mm summer individuals can survive until winter, as evidenced by the field experiment (approximately 50% survivorship), growing up to 20mm. However, to confirm this pattern, the annual cycle of the species in this system should be monitored in more depth, to understand the recurrence of these phenomena.

6.2 SURVIVING BEYOND THE EDGE

The survival experiment showed that *C. fluminea* adult population was able to survive the estuarine high physicochemical variability, particularly regarding salinity and temperature conditions. Both the distribution patterns of the species in the estuary (*i.e.*, field survey data) and the survivorship experiment point towards this conclusion. During winter, practically all individuals were able to survive for the whole experimental period in all studied areas, probably due to the decrease in the overall estuarine salinity. Therefore, the Asian clam is potentially able to survive the complete winter season in the middle sections of the Mondego estuary. During summer, mortality occurred and the LT_{50} was shorter, particularly at the downstream and most saline areas. The species faces the most adverse conditions during the summer, due to a combination of both high salinity and temperature. In addition, the species may need to feed for longer periods (*i.e.*, consequence of higher metabolic rates), and therefore, can be exposed to salinity during longer time periods than they can handle (McMahon, 1983, 1999; Ilarri et al., 2012).

LT_{25} varied within similar values for all sites in summer. Nevertheless, this sort of mortality did not occur during the winter conditions, suggesting a minimal effect of the experimental manipulation of individuals. At the uppermost site (A), source of all individuals used in the experiment, the confidence intervals both for LT_{25} and LT_{50}

were the largest. Potentially, that site has the optimal conditions for the species survival and thus, mortality might be related to an effect of density/intraspecific competition. LT_{50} values are more relevant to determine whether the species could still represent a threat to estuarine systems, even in the mesohaline areas, as a consequence of its ability to survive in conditions far above its recognized vital limits. It should be noticed that 100% mortality was never found, which adds to the concern about the species invasion potential for this estuary. The salinity barrier seems to be acting on the immature juveniles, unable to survive when submitted to typical estuarine conditions, but the adults could survive and eventually complete reproductive cycles.

With the predicted increase in the number of extreme climatic events such as extreme precipitation (IPCC, 2013), the population of *C. fluminea* in the Mondego estuary may become able to survive during critical life stages, and therefore settle in new areas within a frame of full invasion. For example, a summer with abnormal rainfall could allow the species to settle downstream and complete a full life cycle in areas previously inaccessible. Also, small modifications on the physical structure of the estuary can create shelter and context dependent opportunities for the species to thrive in new areas (Früh et al., 2012). The surveyed sites closer to the experimental sites C and D were not invaded yet, but if salinity decreases, for instance due to constant flooding or industrial freshwater runoff, the population may overcome the natural barriers that impair its settlement, and its distribution range may increase downstream. Ultimately, this can drive alterations on the overall biodiversity, by competing with species in close ecological niches and also acting on different trophic levels (Ilarri & Sousa, 2012), with consequences to the ecosystem functioning. Even if the system returns to conditions closer to its previous state and massive summer mortality occurs, the consequences of this invasion cannot be predicted. This potential for invasion could bring several economic nuisances, considering the presence of some industrial facilities along the estuary, as well as drainage systems for agriculture areas, which could be affected by biofouling events, with effective costs for their operation (Nakano & Strayer, 2014).

The use of lethal time to characterize the response of an invasive species in a specific system could be successfully adapted for other estuaries or rivers, thus providing a method for testing and comparing the susceptibility of a water body to invasion. This sort of data could be analysed with focus on species response in different contexts inside a specific water body, or the focus could be directed to the system, with lethal times indicative of ecosystem resistance to invasion. In a framework of management and protection, this method could be used together with recent detection methods for invasive species (Darrigran & Damborenea, 2015), in which molecular techniques allow to identify the presence of invasive species before it can be seen, based on plankton samples, thus providing a strong predictive tool of the invasive potential within the binary system/species. The use of *in situ* survival experiments allows incorporating natural variability to the experimental design regarding habitat and environmental conditions, in turn enabling a more realistic study of the species behavior towards those changes. This is especially relevant for an estuary, whose environmental conditions change considerably through a daily cycle (McLusky & Elliott, 2004). Nevertheless, *in situ* experiments may reduce the universality of the results, which may become system specific. This specificity makes sense in the case of this experiment and used methodology, because it was part of the objectives to understand the interaction of the species with this specific system.

There is a fine balance between the ability of species to invade and the susceptibility of the habitat to be invaded. In this case, it seems that despite the species' potential, the habitat is still presenting proper barriers to the progression of the invasion. Salinity, together with the tidal variations in several parameters (e.g.

temperature, turbidity, food availability) is likely limiting the settlement of immature adults, and therefore, despite the survival of adults for remarkably long time in these conditions, the invasion has not extended into the downstream reaches of this estuary.

7 ACKNOWLEDGEMENTS

The authors would like to thank all colleagues who helped in field and laboratory work. This research was supported by FCT (Portuguese Foundation for Science and Technology), through the grants attributed to D Crespo (SFRH/BD/80252/2011), S Leston (SFRH/BPD/91828/2012), M Dolbeth (SFRH/BPD/110441/2015), the Investigador FCT programme attributed to F Martinho (IF/01410/2012) and to BIOCHANGED project (PTDC/MAR/111901/2009), subsidized by the European Social Fund and MCTES (Portuguese Ministry of Science, Technology and Higher Education), through the POPH (Human Potential Operational Programme), QREN (National Strategic Reference Framework) and COMPETE (Programa Operacional Factores de Competitividade).

Chapter III

Ecological consequences of invasion across the freshwater-marine transition in a warming world

1 ABSTRACT

The freshwater-marine transition that characterizes an estuarine system can provide multiple options for invading species, yet the relative importance of this gradient in determining the functional contribution of invading species has received little attention. Despite the fact that many invasive species are able to adapt to a wide range of salinities, most assessments of the ecological consequences of species invasion are evaluated within a freshwater or marine context. Here, we investigate the functional consequences of different size classes of the Asian clam *Corbicula fluminea* – an invasive species able to adapt to a wide range of temperatures and salinity – across the freshwater-marine transition in the presence versus absence of warming. Specifically, we characterise how *C. fluminea* affect fluid and particle transport, important processes in mediating nutrient cycling (NH_4-N, NO_3-N, PO_4-P). We find that sediment particle reworking tends to be influenced by size class, and to a lesser extent temperature and salinity, whilst nutrient concentrations are influenced by different interactions between all variables (salinity, temperature and size class). Our findings demonstrate that the ecosystem consequences of species invasion can be highly context dependent, and highlights the potential for species to simultaneously occupy multiple components of an ecosystem. Recognising of this aspect of invasibility is fundamental to management and conservation efforts, particularly as freshwater and marine systems tend to be compartmentalised rather than be treated as a contiguous unit. More

comprehensive appreciation of the distribution of invasive species across adjacent habitats and different seasons is needed to allow the true extent of biological introductions, and their ecological consequences, to be fully realised.

2 KEYWORDS

Species introductions; Species interactions; Refugia; Bioturbation; Nutrient balance

3 INTRODUCTION

Estuaries are transitional areas that face cyclic variations in physicochemical conditions. Those interface areas are at the forefront of global ecological changes (Millennium Ecosystem Assessment, 2005; Rabalais et al., 2009; Grilo et al., 2011; IPCC, 2013) and are particularly prone to invasion by non-indigenous species because of their proximity to human populations (Cohen et al., 1997) that introduce major vectors for introductions (Williams & Grosholz, 2008; Crespo et al., 2015; Gallardo et al., 2016). Simultaneously, increasing sea-surface temperatures, rising sea levels, increasing atmospheric CO_2 concentrations and ocean acidification are already altering coastal and marine habitats (Levitus et al., 2000; Parmesan & Yohe, 2003; Kroeker et al., 2010), which themselves may stimulate the introduction of non-indigenous species (Stachowicz et al., 2002; Rahel & Olden, 2008; Williams & Grosholz, 2008; Weitere et al., 2009).

Estuaries are highly productive habitats (Kennish, 2002; Dolbeth et al., 2011; Hicks et al., 2011) and functionally important areas (e.g. Sousa et al. 2010, Sousa, Lillebø, et al. 2012). They are generally characterized by low diversity, constrained by local environmental conditions (Loo & Rosenberg, 1996; Kennish, 2002; Dolbeth et al., 2011; Hicks et al., 2011), where the introduction of new species that have different traits to the recipient community can have a disproportionate effect on the functioning of the ecosystem (Stachowicz & Byrnes, 2006; Simberloff et al., 2013). In this respect, non-indigenous invasive species (NIS) may shift the composition of native communities or otherwise propagate ecological impacts throughout the food web and generate associated effects on ecosystem functioning. These, in turn, can be alleviated or exacerbated by other factors such as climate change, nutrient loading, land use alteration and several other anthropogenic induced changes (Stachowicz et al., 2002; Strayer & Hillebrand, 2012).

Ecological modifications due to invasive events are generally described as having negative effects on the resident communities and ecosystem processes (e.g. biodiversity loss, biofouling), but positive effects have also been described (e.g. local economy, improvement of water quality) (Rosa et al., 2011; Dolbeth et al., 2012; Katsanevakis et al., 2014); although, it is recognised, and should be noted, that there is a publication bias towards negative impacts for particular species and/or habitats (Gallardo et al., 2016). Nevertheless, transitional habitats face species introductions from freshwater and/or marine sources, exacerbated by the gradient in environmental conditions that provides multiple entry points and the potential for refugia opportunities.

Most work on biological invasions focus on specific habitats and how the introduced species interact with native populations and communities (e.g. Williams & Grosholz 2008, Strayer & Hillebrand 2012, Ilarri et al. 2012, Simberloff et al. 2013). However, in most cases is difficult to establish cause-effect relationships without experimental studies (Grosholz & Ruiz, 2009). An additional challenge, particularly for coastal systems (Grosholz & Ruiz, 2009), is the difficulty of defining a causal relationship in a dynamic system (Hale et al., 2014; Murray et al., 2014) because the expression of species contributions are context dependent (Godbold & Solan, 2009) and can take a long time to emerge (Godbold & Solan, 2013). Therefore, it becomes important to address when and how the occurrence of invasive species interacts with gradients of environmental variables that often characterize transitional habitats. These are seldom studied, especially in association with other aspects of directional forcing, such as, for example, aspects of climate change (but see Schneider 2008; Weitere et al. 2009).

The Asian Clam *Corbicula fluminea* (Müller, 1774) is a prominent invader of freshwater systems (Sousa et al., 2008a; Crespo et al., 2015) and is known to affect hydrological processes, biogeochemical cycles and biotic interactions and the physical environment at an ecosystem scale (Sousa et al., 2008a, 2009). Despite being described as a freshwater bivalve, *Corbicula fluminea* is an euryhaline species (salinity, up to 10-14; McMahon 1983; McMahon 1999) and can colonize the upstream areas of estuaries (Sousa et al., 2008b; Franco et al., 2012; Ilarri et al., 2014). This is important because euryhaline species are able to invade along the freshwater-marine continuum of the estuarine environment, presenting the possibility of temporary or permanent refugia that will allow longer-term persistence (Crespo et al., 2016). Moreover, species that show phenotypic plasticity may be predisposed to establishing populations that are functionally dominant under climate change (e.g. Somero 2010)

Here, we investigate how different size classes (a proxy for age) of *Corbicula fluminea* affect important ecosystem processes (sediment fluid and particle transport) in the presence versus absence of warming across the freshwater-marine transition. By considering both the native freshwater habitat and the oligohaline waters of estuarine areas that have high invasive potential, we hypothesized that different sizes in macrobenthos could differentially mediate levels of ecological functioning and that temperature (in a simulated heat wave) could influence the intensity of the biological processes involved, with implications for long-term functioning under a changing climate.

4 MATERIALS AND METHODS

4.1 SEDIMENT AND FAUNA COLLECTION

Sediment and individuals of *Corbicula fluminea* were collected in the oligohaline upper reaches of the Mondego Estuary, Portugal (40° 9'47.91"N, 8°40'12.42"W) from a tidally influenced location. Sediment (gravel 38.7%, sand 58.9% and mud 2.5%, 0.4 ± 0.2% organic matter content, loss on ignition at 400ºC, 8h) was defaunated using CO_2 enriched water (bubbled CO_2) for ~1h to initiate upward movement of infaunal organisms to the sediment surface by lowering dissolved O_2 (adapted from Coelho et al. 2004).

4.2 EXPERIMENTAL SET-UP AND DESIGN

Our experimental design (Appendix I - Fig. 1) included all possible permutations of three different size classes of *Corbicula fluminea*, with fixed biomass achieved through density adjustment (18.79 ± 0.94 g aquaria^{-1} wet biomass: *small*, measuring <1cm, ~1 year old, 13 ind. aquaria^{-1} (= 902 ind m^{-2}); *medium* 2-2.5 cm, ~ 2 years old, 2 ind. aquaria^{-1} (= 138 ind. m^{-2}); *large* > 3 cm, > 3 years old, 1 ind. aquaria^{-1} (= 69 ind m^{-2}) at levels representative of the population at study site (Franco et al. 2012, Crespo et al. 2016)). These size classes were crossed with two levels of salinity (freshwater, 0, and oligohaline, 5) and two levels of temperature (24ºC and 30ºC) in glass aquaria (12 x 12 x 35 cm, internal dimensions). Each aquaria contained sediment ~10 cm depth overlain with water to 30 cm depth. To distinguish the role of microbial and meiofaunal components of the system we included a treatment where *C. fluminea* were absent. Temperature approximated summer water temperatures at the study site (24ºC), or extreme heat-wave conditions (30ºC, Mouthon and Daufresne 2006). Our design required a total of 48 aquaria (4 size treatments x 2 temperature x 2 salinity x 3 replicates, Appendix I - Fig. 1). All aquaria were continually aerated and maintained under natural daylight conditions for a period of 6 days. Salinity, temperature, pH and oxygen levels (O_2) were measured at the beginning and at the end of the experiment. Realised experimental conditions are presented in Appendix I - Table 1).

4.3 MEASUREMENT OF PARTICLE REWORKING (ECOSYSTEM PROCESS)

The extent of particle reworking was measured non-invasively using fluorescent sediment profile imaging (f-SPI, Solan et al. 2004). Briefly, this method allows dyed sediment particles that fluoresce under UV light (luminophores: 30 g aquaria^{-1}, 125–250 μm diameter, green colour; Brian Clegg, Ltd, UK), to be preferentially visualised (Schiffers et al., 2011) and the distribution of luminophores to be determined at high spatial resolution from images of the side of the aquaria. We used a Canon EOS 350D single lens reflex digital CMOS camera (8.0 megapixels) set for an exposure of 10 s, f= 6.3 and a film speed equivalent to ISO 200. Images were saved in red-green-blue (RGB) colour with JPEG (Joint Photographic Experts Group) compression, cropped to the full internal width of the aquaria (952 pixels, effective resolution= 12.61 μm per pixel) and analyzed using a custom-made plugin that runs within ImageJ (Version 1.48c), a java-based public domain program developed at the US National Institute of Health (available at http://imagej.nih.gov/ij/). Following Hale et al. (2014) we determined the mean ($^{f\text{-}SPI}L_{mean}$, time dependent indication of mixing), median ($^{f\text{-}SPI}L_{med}$, typical short-term depth of mixing) and maximum ($^{f\text{-}SPI}L_{max}$, maximum extent of mixing over the long-term) mixed depth of particle redistribution. In addition, we determined the maximum vertical deviation of the sediment-water interface (upper – lower limit = surface boundary roughness, SBR), which provides an indication of surficial faunal activity.

4.4 MEASUREMENT OF NUTRIENT CONCENTRATIONS (ECOSYSTEM FUNCTION)

Water samples (10 ml, pre-filtered 0.45 µm, Whatman) were taken at day 0 and day 6 to determine water column nutrient concentrations (NH_3-N, NO_3-N, PO_4-P). These were analyzed with Continuous Flow Analyzer Skalar Sanplus with Segmented Flow Analysis (SFA), using the Skalar methods: M461-318 (EPA 353.2), M155-008R (EPA 350.1) and M503-555R (Standard Method 450-P I).

4.5 STATISTICAL ANALYSES

We developed independent regression models for each of our dependent variables of particle reworking (${}^{f\text{-}SPI}L_{med}$, ${}^{f\text{-}SPI}L_{max}$, ${}^{f\text{-}SPI}L_{mean}$, SBR) and nutrient concentrations (NH_3-N, NO_3-N, PO_4-P) using the full factorial combination of independent variables (*C. fluminea* size class, temperature, salinity). As our focus is to establish the effects of different size classes of *C. fluminea*, rather than presence versus absence effects, the procedural control (*C. fluminea* absent) was removed from the statistical analysis. As our data showed evidence of a violation of homogeneity, analyses were extended to include the appropriate variance covariate structure (Minimal adequate model summaries are shown in Appendix I) using a generalised least squares (GLS) estimation procedure (Pinheiro & Bates, 2000). This procedure allows the residual spread to vary with the explanatory variables and avoids the need to transform data. For GLS, the optimal variance covariate structure was determined using restricted maximum-likelihood (REML) estimation by comparing the initial regression model without a variance covariate structure to alternative regression models that include specific variance covariate structures using AIC and visual comparisons of model residuals. The optimal fixed structure was then determined by backward selection using the likelihood ratio (L-ratio) test obtained using maximum likelihood (ML) estimation and the minimal adequate model was re-expressed using REML (Diggle et al., 2002; West et al., 2007; Zuur et al., 2009). As inferences about the relative importance of our explanatory variables, and their interactions, are based on the comparisons of the first level within each term with all other levels we used a parametric bootstrap with 999 re-samples and the percentile method to obtain the 95% CI limits around the predicted values (shown in Appendix I). All analyses were performed using the ‘R’ statistical and programming environment (R Development Core Team 2012). GLS analyses were conducted using the ‘nlme’ package (Pinheiro et al., 2014) and parametric bootstrapping were conducted assuming that the estimated parameters followed a multivariate Gaussian distribution with mean and variances provided from the output of the fitting function, using the function ‘rmvnorm’ within the package ‘mvtnorm’ (Genz et al., 2014).

5 RESULTS

We found evidence that faunal activity and behaviour, and associated nutrient concentrations, are affected by the size class of individuals of *C. fluminea*, temperature and salinity (Table 1, and Models 1-7 in Appendix I), although the observed effects did not necessarily form full factorial interactions. Size class tended to be the most important variable, followed by temperature and/or salinity (least important).

Table 1 Summary of significant terms found in the generalized least squares models, using bioturbation components and nutrient concentrations as dependent variables and size, temperature and salinity as explanatory variables

Dependent variable	**Significant terms**	**d.f.**	**L-ratio**	**p**
Bioturbation				
SBR	size x temperature	5	17.323	0.0039
$^{f\text{-}SPI}L_{mean}$	size x salinity	5	24.593	<0.001
	temperature	1	7.118	0.0076
$^{f\text{-}SPI}L_{median}$	size x temperature	5	19.761	0.0014
$^{f\text{-}SPI}L_{max}$	size	2	12.392	0.002
Nutrients				
[NH_3-N]	size x salinity	3	29.392	<0.001
	size x temperature	3	11.715	0.0084
[NO_3-N]	size	3	49.921	<0.0001
	salinity x temperature	3	18.854	<0.001
[PO_4-P]	size x temperature	3	15.499	0.0014
	size x salinity	3	14.541	0.0023

5.1 EFFECTS ON ECOSYSTEM PROCESS

Surface boundary roughness (SBR) was affected by a size class x temperature interaction, but not affected by salinity (Table 1, model structure described in Appendix I - Model 1). Temperature, and all of its interactions, was the most influential variable (L-ratio= 13.497, d.f.= 3, p= 0.0037), followed by size class and its interactions (L-ratio= 11.197, d.f.= 4, p= 0.0244). In the presence of *C. fluminea*, SBR ranged between 0.454 – 2.173 cm for the small individuals, to 0.454 - 1.853 cm for medium sized individuals and 0.214 - 2.017 cm for the large sized individuals. At lower temperature, SBR reduced considerably at intermediate body size relative to populations of small and large individuals, but this trend was less compelling at the higher temperature (Fig. 1). Small sized individuals tended to show higher SBR values, with similar values at both temperature regimes (mean ± S.E. (cm): 24ºC, 1.340 ± 0.226; 30ºC, 1.227 ± 0.194). The medium sized individuals showed a more pronounced effect of temperature, showing the smallest value of SBR (mean ± S.E., cm) at 24ºC (0.542 ± 0.050), which increased at 30ºC (1.269 ± 0.141). The lowest SBR values tended to be found in large sized individuals, with slightly higher values at 30ºC (mean ± S.E. (cm): 24ºC, 0.845 ± 0.197; 30ºC, 1.040 ± 0.245).

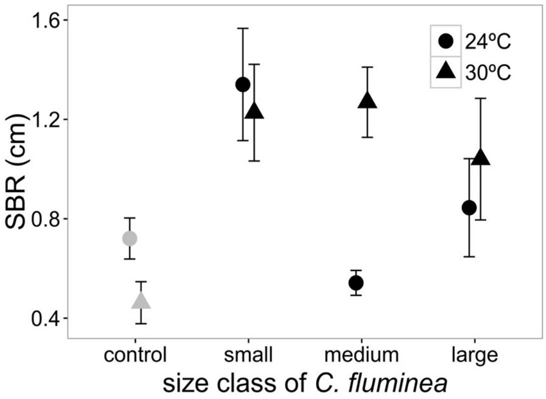

Fig. 1 The interactive effects of *Corbicula fluminea* size class x temperature on surface boundary roughness (SBR: cm, mean ± s.e.). For clarity, jitter has been applied to the x = argument of the plot function to avoid over-plotting. For comparison, SBR in the absence of C. fluminea is presented (grey, control)

$^{f\text{-}SPI}L_{mean}$ was influenced by the interaction size class x salinity and an independent effect of temperature (Table 1, model structure described in Appendix I - Model 2). Size class, and all of its interactions, was the most influential variable (L-ratio= 17.4282, d.f.= 4, p= 0.0016), followed by salinity and its interactions (L-ratio= 9.434963, d.f.= 3, p= 0.024) and temperature (L-ratio= 7.118, d.f.= 1, p= 0.0076). $^{f\text{-}SPI}L_{mean}$ values ranged between 0.483 - 2.065 cm (small size class), to 0.159 - 0.851 cm (medium size class), and 0.288 - 1.476 cm (large size class). For both salinities, small sized individuals showed the highest values for $^{f\text{-}SPI}L_{mean}$, which was even higher at salinity 5 (mean ± S.E. (cm): salinity 0, 0.754 ± 0.097; salinity 5, 1.452 ± 0.191; Fig. 2A). $^{f\text{-}SPI}L_{mean}$ values were smaller for medium sized individuals, but similar across salinity levels (mean ± S.E. (cm): salinity 0 , 0.490 ± 0.099; salinity 5, 0.458 ± 0.096, Fig. 2A). The $^{f\text{-}SPI}L_{mean}$ in the presence of larger individuals responded to increasing salinity (large sized, salinity 0 *vs* salinity 5: t-value= -2.376, d.f.= 36, p= 0.0243; mean ± S.E. (cm): salinity 0, 0.530 ± 0.083; salinity 5, 0.676 ± 0.181, Fig. 2A). $^{f\text{-}SPI}L_{mean}$ increased with increasing temperature (t-value= 4.653, d.f.= 36, p= 0.0001; mean ± S.E. (cm): 24ºC, 0.704 ± 0.132; 30ºC, 0.750 ± 0.081, Fig. 2B).

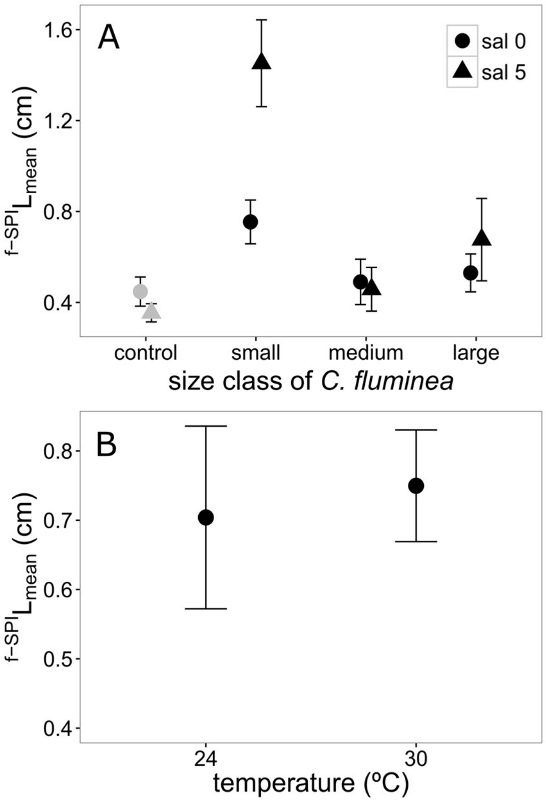

Fig. 2 The interactive effects of *Corbicula fluminea* size x salinity (A) and the independent effect of temperature (B) on mean mixed depth of luminophores redistribution ($^{f\text{-}SPI}L_{mean}$; cm, mean ± s.e.). For clarity, jitter has been applied to the x = argument of the plot function to avoid over-plotting. For comparison, SBR in the absence of C. fluminea is presented (grey, control)

$^{f\text{-}SPI}L_{median}$ was affected by the interaction size class x temperature, but unaffected by salinity (Table 1, model structure in Appendix I - Model 3). Size class, and all of its interactions, was the most influential variable (L-ratio= 18.377, d.f.= 4, p= 0.001), followed by temperature and its interactions (L-ratio= 14.846, d.f.= 3, p= 0.002). $^{f\text{-}SPI}L_{median}$ values ranged between 0.315 – 2.395 cm (small size class), to 0.164 - 0.731 cm (medium size class) and 0.214 - 0.882 cm (large size class). Despite the small sized individuals showing the highest $^{f\text{-}SPI}L_{median}$ values at both temperatures, temperature effects seemed less important (mean ± S.E. (cm): 24ºC, 1.071 ± 0.417; 30ºC, 0.975 ± 0.181, Fig. 3). Relative to small sized individuals, $^{f\text{-}SPI}L_{median}$ values decreased for medium and large size classes, at both temperatures. Interestingly, whilst $^{f\text{-}SPI}L_{median}$ increased with increasing temperature in medium sized individuals (mean ± S.E. (cm): 24ºC, 0.258 ± 0.027; 30ºC, 0.502 ± 0.062, Fig. 3), the reverse was true for large sized individuals (mean ± S.E. (cm): from 24ºC, 0.560 ± 0.095; 30ºC, 0.398 ± 0.059, Fig. 3).

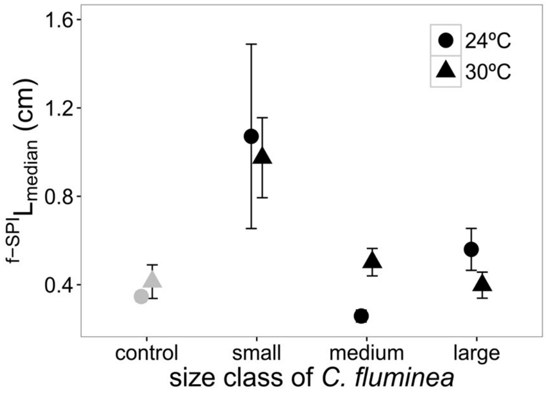

Fig. 3 The interactive effects of *Corbicula fluminea* size class x temperature on median mixed depth of luminophores redistribution ($^{f\text{-}SPI}L_{median}$; cm, mean ± s.e.). For clarity, jitter has been applied to the x = argument of the plot function to avoid over-plotting. For comparison, $^{f\text{-}SPI}L_{median}$ in the absence of C. fluminea is presented (grey, control)

For $^{f\text{-}SPI}L_{max}$, only size class was influential (Table 1, Appendix I - Model 4). Values ranged between 1.525 – 4.374 cm (small class size) to 0.756 - 2.609 cm (medium class size) and 0.681 - 5.206 cm (large class size). Mean values were highest for the small sized *C. corbicula* (mean ± S.E. (cm): 2.909 ± 0.270), followed by the large (mean ± S.E. (cm): 1.854 ± 0.425), and medium sized individuals (mean ± S.E.: 1.600 ± 0.185cm) (Fig 4). $^{f\text{-}SPI}L_{max}$ for the small sized individuals was significantly different from medium and large sizes (t-value= 4.012, d.f.= 36, p= 0.0003 and t-value= 2.093, d.f.= 36, p= 0.0441, respectively), but there was no difference between medium and large size classes (t-value= 0.555, d.f.= 36,p= 0.5828).

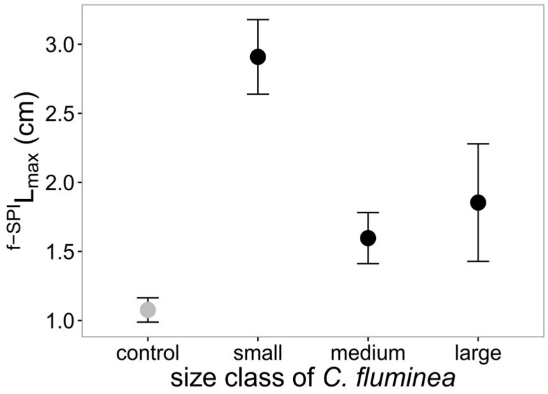

Fig. 4 The independent effect of *Corbicula fluminea* size class on maximum mixed depth of luminophores redistribution ($^{f\text{-}SPI}L_{max}$; cm, mean ± s.e.). For comparison, $^{f\text{-}SPI}L_{max}$ in the absence of C. fluminea is presented (grey, control)

5.2 EFFECTS ON NUTRIENT CONCENTRATION

[NH_3-N] was dependent on size class x salinity and size x temperature interactions (Table 1, Appendix I - Model 5). Size class, and its interactions, were more influential (L-ratio= 57.236, d.f.= 6, p< 0.0001), than salinity and its interactions (L-ratio= 29.392, d.f.= 3, p< 0.0001) and temperature and its interactions (L-ratio= 11.715, d.f.= 3, p= 0.0084). [NH_3-N] ranged from 0.200 to 1.543 mg L^{-1} in the presence of small sized individuals, from 0.118 to 1.933 mg L^{-1} in the presence of medium sized individuals and from 0.036 to 0.530 mg L^{-1} in the presence of large sized individuals of *C. fluminea*. [NH_3-N] increased with higher salinity when either medium (mean ± S.E., mg L^{-1}: salinity 0, 0.710 ± 0.289; salinity 5, 1.754 ± 0.087, Fig. 5A) or small sized individuals were present (mean ± S.E., mg L^{-1}: salinity 0, 0.243 ± 0.017; salinity 5, 1.360 ± 0.117 cm, Fig. 5A). The large sized individuals had the lowest [NH_3-N] for both salinity levels (mean ± S.E., mg L^{-1}: salinity 0, 0.148 ± 0.019;

salinity 5, 0.350 ± 0.078 cm, Fig. 5A), similar to those when small sized individuals were present at salinity 0. Temperature had positive influence on [NH_3-N] when medium sized individuals were present (mean ± S.E., mg L^{-1}: 24ºC, 0.960 ± 0.294; 30ºC, 1.504 ± 0.290 cm Fig. 5B), but was less influential when either small or large individuals were present, with the large sized clams showing the lowest [NH_3-N] (mean ± S.E., mg L^{-1}: small size, 0.687 ± 0.203 at 24ºC and 0.691 ± 0.229 at 30ºC; large size, 0.214 ± 0.074 at 24ºC and 0.283 ± 0.067 at 30ºC, Fig. 5B).

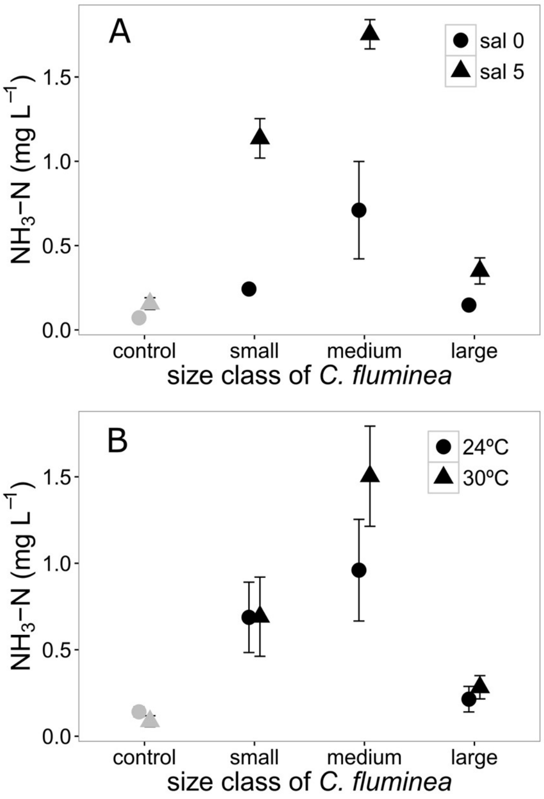

Fig. 5 The interactive effects of *Corbicula fluminea* size x salinity (A) and *Corbicula fluminea* size x temperature (B) on [NH_3-N] in the water (mg L^{-1}, mean ± s.e.). For clarity, jitter has been applied to the x = argument of the plot function to avoid over-plotting. For comparison, [NH_3-N] in the absence of C. fluminea is presented (grey, control)

[NO_3-N] was affected by the interaction salinity x temperature and an independent effect of size (Table 1, model structure in Appendix I - Model 6). Size, was the most influential variable (L-ratio= 49.921, d.f.= 3, p< 0.0001), followed by temperature and its interactions (L-ratio= 11.258, d.f.= 2, p= 0.0036) and salinity and its interactions (L-ratio= 7.999, d.f.= 2, p= 0.0183). [NO_3-N] ranged from 0.173 to

0.659 mg L^{-1} when small sized individuals were present, from 0.137 to 0.732 mg L^{-1} when medium sized individuals were present, and from 0.158 to 0.31 mg L^{-1} when large sized individuals of *C. fluminea* were present. Small and medium sized individuals showed similar values of [NO_3-N] (t-value= 1.961, d.f.= 36, p= 0.0592; mean ± S.E., mg L^{-1}: 0.411 ± 0.039 and 0.410 ± 0.046, respectively, Fig. 6A), which decreased for large sized individuals (t-value= -4.582, d.f.= 36, p= 0.0001; mean ± S.E., mg L^{-1}: 0.247 ± 0.019, Fig. 6A). A decrease in [NO_3-N] was shown with salinity 5, more accentuated at 30ºC (Fig. 6B). Also, in both salinity treatments, [NO_3-N] were lower at higher temperature (mean ± S.E., mg L^{-1}: salinity 0, 0.400 ± 0.054, at 24ºC and 0.354 ± 0.050, at 30ºC; salinity 5, 0.376 ± 0.022, at 24ºC and 0.294 ± 0.060, at 30ºC).

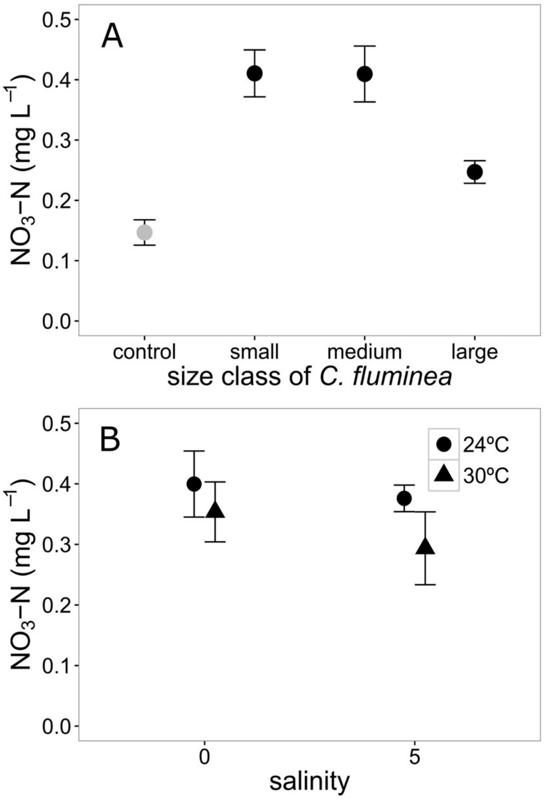

Fig. 6 The independent effect of *Corbicula fluminea* size (A) and the interactive effects of *Corbicula fluminea* size x temperature (B) on [NO_3-N] in the water (mg L^{-1}, mean ± s.e.). For clarity, jitter has been applied to the x = argument of the plot function to avoid over-plotting. For comparison, [NO_3-N] in the absence of C. fluminea is presented (grey, control)

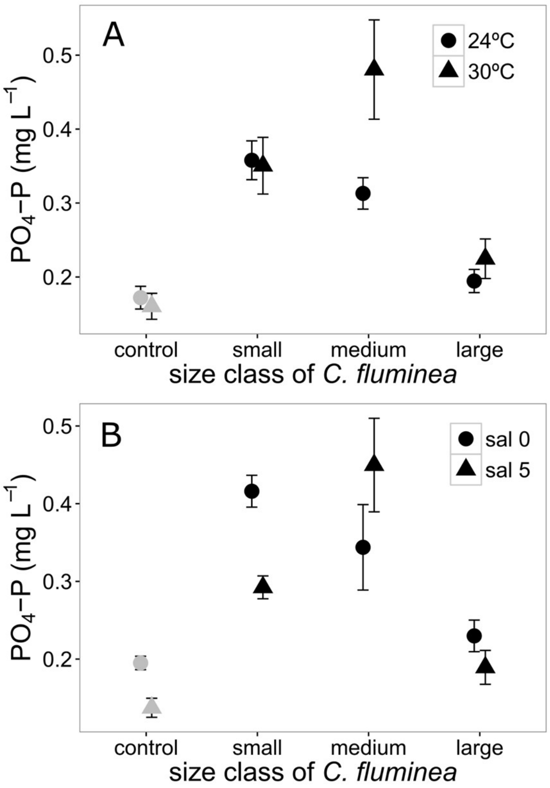

Fig. 7 The interactive effects of *Corbicula fluminea* size x temperature (A) and *Corbicula fluminea* size x salinity (B) on [PO_4-P] in the water (mg L^{-1}, mean ± s.e.). For clarity, jitter has been applied to the x = argument of the plot function to avoid over-plotting. For comparison, [PO_4-P] in the absence of C. fluminea is presented (grey, control)

[PO_4-P] was influenced by the interactions size class x temperature and size class x salinity (Table 1, model structure in Appendix I - Model 7). Size, and its interactions, was the most influential variable (L-ratio= 57.090, d.f.= 6, $p < 0.0001$), followed by temperature and its interactions (L-ratio= 15.499, d.f.= 3, p= 0.0014) and salinity and its interactions (L-ratio= 14.541, d.f.= 3, p= 0.0023). [PO_4-P] ranged from 0.240 to 0.489 mg L^{-1} for small sized individuals, from 0.253 to 0.681 mg L^{-1} for medium sized individuals and from 0.128 - 0.310 mg L^{-1} for large sized individuals of *C. fluminea*. Temperature tended to have a positive effect on [PO_4-P], particularly when medium sized individuals were present (mean ± S.E., mg L^{-1}: 0.313 ± 0.021 at 24ºC and 0.481 ± 0.067 at 30ºC, Fig. 7A). [PO_4-P] were similar at both temperatures when small sized individuals were present (t-value= -0.296, d.f.= 36, p= 0.77; mean ± S.E., mg L^{-1}: 0.358 ± 0.026 at 24ºC and 0.351 ± 0.038 at 30ºC, Fig. 7A), and also similar between temperatures when large sized individuals were present (t-value= 0.413, d.f.= 36, p= 0.683; mean ± S.E., mg L^{-1}: 0.195 ± 0.016 at 24ºC and 0.225 ± 0.027 at 30ºC, Fig. 7A). For salinity, there was a tendency for [PO_4-P] to decrease in the presence of small and large sized individuals, whilst the reverse was true when medium sized individuals were present (mean ± S.E., mg L^{-1}: 0.416 ± 0.020 for small size, 0.344 ± 0.055 for medium size and 0.230 ± 0.020 for large size, Fig. 7B).

6 DISCUSSION

When the ecological impacts of invasive species have been characterized, there is a tendency to assume that previously observed effects are uniformly expressed across novel circumstances. Our findings suggest that this assertion is not necessarily appropriate, however, as the mediation of important ecosystem functions vary with the size of the organism and with environmental context (here, temperature and salinity). Interestingly, despite strong temperature forcing across a gradient of salinity, we found a consistently prominent effect of size class on ecosystem functioning throughout all of our response variables. Whilst size class is an important trait underpinning bioturbation processes (Solan et al., 2004a; Norkko et al., 2013), it was not necessarily the most important predictor of associated nutrient dynamics (Séguin et al., 2014). Although we did not measure physiological condition directly, larger body size is known to minimize species vulnerability to the cyclic nature of physicochemical conditions (Gardner et al., 2011), which is particularly important across the freshwater-estuarine transition (Crespo et al., 2016).

The effects of body size we documented highlight the importance of species population structure for the ecosystem functioning, but our study also highlights the importance of species-environment interactions and the role on abiotic and biotic context. Small sized individuals contributed the most for bioturbation, presumably because smaller individuals may face less mechanical resistance (de la Huz et al., 2002) and are also more responsive to changes in environmental conditions (Werner & Gilliam, 1984; Gardner et al., 2011; Godbold et al., 2011). In addition, small individuals of *C. fluminea* have been reported to have higher metabolic requirements (Xiao et al., 2014) and, as they invest more in tissue growth than larger sized individuals, increased particle mixing may lead to increased feeding effort. Mattice and Dye (1976) determined the lower and upper lethal temperatures of *C. fluminea* as 2ºC and 34.8ºC, respectively, which spans our tested range. Larger individuals of *C. fluminea* seem to be less affected by temperature changes (as reported elsewhere for other bivalves, *Mytilus edulis* and *M. leucophaeata*, Rajagopal et al. 2005), which may explain why large *C. fluminea* maintain similar levels of behaviour across different temperature treatments. In contrast to the present study, however, Majdi et al (2014) tested the effects of body size on bioturbation behaviour in *C. fluminea* with a similar approach and found that medium and large sizes contributed more to bioturbation at temperatures closer to the mid-tolerance range of the species. Similarly, the effects of salinity also increased the medium mixing depth of small sized individuals, perhaps a response to increasing water salinity whilst maintaining pedal feeding on the surface. Nevertheless, salinity seems to play a less important role in moderating the mediation of ecosystem properties by *C. fluminea*. Xiao et al (2014) found that narrow salinity ranges had small effect on metabolism of *C. fluminea*. However, other mechanisms, such as reducing valve opening time and/or physiological responses to salinity (McCorkle & Dietz, 1980; Dietz et al., 1996; Ruiz & Souza, 2008) are likely to be more effective that mechanical displacement over extended periods of time. As an osmoconformer, *C. fluminea* individuals increase extracellular ionic concentrations and reduce intracellular volume to cope with hyperosmotic stress (McCorkle & Dietz, 1980; Ruiz & Souza, 2008). Evans et al (1977) found that *C. fluminea* shows different responses (and tolerance) to salinity, depending on the geographic origin of the population stock or historical acclimation status. Since older individuals are larger and will have been preexposed to salinity changes, this explanation is consistent with the effects of body size and may explain the smaller impact of salinity on bioturbation/particle reworking mediated by larger individuals.

Irrespective of the mechanisms involved, the most important finding of our study is that some species (here *C. fluminea*) are capable of invading multiple habitats and, once established, their effect on ecosystem properties reflects the environmental conditions of the locality. However, the relationship between bioturbation intensity and nutrient generation is difficult to predict based on trait values alone, especially as the organism-sediment interactions alter with context (Solan et al., 2008; Teal et al., 2010; Hale et al., 2014; Murray et al., 2014). We cannot discount the role of salinity and temperature in influencing meiofaunal and microbial communities. For instance, we observed a reduction in NO_3 and an increase in NH_3 with increasing temperature. Changes in temperature and salinity are known to influence dissimilatory nitrate reduction to ammonium (DNRA) (Giblin et al., 2010, 2013), which implies that the reduction of nitrate to ammonium either by fermentative or autotrophic DNRA is enhanced under estuarine conditions (Koop-Jakobsen & Giblin, 2010; Sousa et al., 2012a; Bonaglia et al., 2014). Simultaneously, anaerobic oxidation of ammonium will occur, but the importance of this pathway, at least in coastal and estuarine sediments, is reduced when compared to DNRA (Gilbertson et al., 2012; Giblin et al., 2013; Bonaglia et al., 2014). In addition, individuals will also contribute to nutrient release via excretion, which may be of greater relative importance than bioturbation activity at a certain threshold of body size. Certainly, larger individuals have a larger siphon and exhibit lower rates of particle mixing as they do not need to relocate to exploit food resources (Zwarts et al., 1994). For large sized *C. fluminea*, grazing on primary producers and removing particulates and sorbed phosphates might, at least in part, explain the observations for PO_4 (Phelps, 1994). Small and medium sized individuals of *C. fluminea* were responsible for greater particle reworking, which could stimulate meiofauna (via increasing aeration and solute availability) and the release of PO_4 at the sediment-water interface (Piot et al., 2014). Whilst all of these explanations are feasible, however, there is still a lot of uncertainty regarding the mechanistic basis by which species alter the functioning of an ecosystem, despite the well-known effects of NIS on the structure of communities and biodiversity (e.g. Phelps 1994; Ilarri et al. 2014). This is particularly concerning, given that biological invasions are expected to rise and ecological niches may become favorable for invasive species under climate change (Montoya & Raffaelli, 2010; Crespo et al., 2016; Gama et al., 2016).

We have looked upon the bioturbation behaviour of a single species with potential to create monocultures (Sousa et al., 2008b) and that can invade a range of environmental conditions within a habitat (Crespo et al., 2015, 2016). Our findings confirm the possibility that non-native species that can access and thrive in multiple environmental conditions along the freshwater-marine transition can have dramatic effects on ecosystem properties. Whilst these effects can vary with environmental context, they are likely to manifest at larger scales and across perceived environmental boundaries. A challenge for the management of such transitional habitats will be in determining whether residual populations, perhaps occupying suboptimal conditions, can re-establish more widely. If so, control measures will need to recognise that perceived environmental constraints may be an insufficient means of prioritising the application of control measures and that distinguishing species as freshwater versus marine may be inadequate.

Additional information to this chapter in Appendix I, starting in page 175

7 ACKNOWLEDGEMENTS

Supported by FCT (Portuguese Foundation for Science and Technology), subsidized by the European Social Fund and MCTES (Portuguese Ministry of Science, Technology and Higher Education), through the POPH (Human Potential Operational Programme), QREN (National Strategic Reference Framework) and COMPETE (Programa Operacional Factores de Competitividade) through a PhD grant awarded to D Crespo (SFRH/BD/80252/2011), post-doc grants awarded to M. Dolbeth (SFRH/BPD/110441/2015) and S. Leston (SFRH/BPD/91828/2012), and BIOCHANGED project (PTDC/MAR/111901/2009). We also acknowledge support from the MARS Network (Travel Award for Young Scientists, awarded to D. Crespo).

Chapter IV

New climatic targets for climate change: does a 2ºC temperature rise affect estuarine benthic communities?

1 ABSTRACT

Estuarine communities are able to cope with the natural oscillation in physicochemical parameters, which are highly dynamic in those systems. However, if changes in those parameters go beyond the vital range/tolerance of the estuarine species, consequences in the functioning of the communities are expected. Recent international agreements set a common goal of 2ºC as the maximum atmospheric temperature increase for global warming. Under that premise, we have tested the effects of a similar temperature increase on the benthic communities (macrofauna and microfauna) and consequent processes (bioturbation) and functions (nutrient release), in a seagrass bed and a sandflat from a temperate west-European estuary. Temperature was artificially increased *in situ* and diversity and ecosystem functioning components measured in both habitats after 10 and 30 days. Despite the detection of some effects of the warmness on the analysed components, drastic impacts were not verified. The effect of site/habitat was more important than the effects of the warmness treatment, with the seagrass habitat providing more homogenous results and being less impacted by the temperature treatments than the bare sediment. Nevertheless, the results reinforce the idea that most ecological responses to global changes are context dependent and that ecosystem stability depends not only on biological diversity but also on the availability of different habitats and niches.

2 KEYWORDS

Mondego estuary; Microfauna; Macrofauna; Bioturbation; Nutrient release; Ecosystem functioning; *In situ* experiment

3 INTRODUCTION

Benthic communities in estuaries are under regular oscillations in environmental conditions (e.g. salinity, temperature, water depth, turbidity), due to over imposed tidal cycles of different amplitude and time (e.g. daily, monthly, annual or even larger time frames) (McLusky, 1993; Pratt et al., 2014). Nevertheless, these communities are known to be extremely important for the estuarine ecosystem functioning, contributing for primary and secondary production, decomposition and nutrient dynamics (Godbold et al., 2009; Hicks et al., 2011; Dolbeth et al., 2012; Stief, 2013). Estuarine benthic communities have evolved to adapt to environments with such large range variation in physicochemical parameters (Bamber & Henderson, 1988; Bilton et al., 2002; Féral, 2002; Bozinovic & Pörtner, 2015). Despite specific adaptations to these highly dynamic habitats, estuarine organisms can only cope with environmental variation within their specific range of tolerance. As such, species richness is generally low in estuaries, as few taxa evolved towards the required broad tolerance (Attrill, 2002; Whitfield et al., 2012). Yet, estuaries are highly productive areas (Underwood & Kromkamp, 1999; Dolbeth et al., 2011), due to continuous organic matter input from river basins, nutrient dynamics through decomposition and organic matter mineralization and habitat heterogeneity (Underwood & Kromkamp, 1999; McLusky & Elliott, 2007). Simultaneously, a healthy ecosystem functioning in estuaries depends on how environmental parameters oscillate around reference conditions (Zajac, 2003; Chainho et al., 2006; Borja et al., 2013). Deviations from reference conditions of these environmental parameters can impair essential ecosystem processes and functions, with high uncertainty concerning how much ecosystem health can be affected (Bulling et al., 2010; Godbold & Solan, 2013; Bozinovic & Pörtner, 2015).

It has been accepted that biodiversity with all its components, from the microscopic to the landscape level, is fundamental for the ecosystem functioning (Solan et al., 2004a; Pinto et al., 2014; Lefcheck et al., 2015). For instance, biological and functional diversity are essential for a complementarity effect and functional redundancy in a system, i.e. different species with similar functions, which usually delivers a better functional performance towards environmental change (Dolbeth et al., 2013; Mouillot et al., 2014; Strong et al., 2015). Nevertheless, species diversity does not always cope with rapid ecosystem modification under external pressures (Mooney et al., 2009; Grilo et al., 2011), which are becoming more frequent in face of global ecological changes. On the short term, processes and functions can also depend on behavioral shifts (Godbold & Solan, 2013), and therefore, even within rich biological systems, ecosystem functions such as primary and secondary production, decomposition and nutrient cycling can be affected even without drastic changes in the community demographic figures. Multilevel faunal interactions (microfauna, meiofauna and macrofauna) offer an additional challenge for tracking flows of energy/biomass and nutrients.

Ex situ mesocosm experiences have proved to be efficient in measuring ecosystem functions and assessing the effect of changing diversity in those functions (Ieno et al., 2006; Bulling et al., 2010; Godbold & Solan, 2013) because control parameters can be easily tuned and models are straightforward to assess, with direct cause-effect responses (Bulling et al., 2006, 2010; Dossena et al., 2012; Murray et al., 2014). Nevertheless, the off-site mesocosm experiments present several limitations. The use of artificially assembled communities in laboratory represents a trade-off, because it is logistically difficult to mimic natural communities (Loreau et al., 2001; Bozinovic & Pörtner, 2015). This could be misleading, because functional replacement by close species in natural systems can only happen within a community with proper interactions. Also, the full range of cyclic environmental variation has too many variables that are difficult to emulate in laboratorial conditions. The interactive effects of multiple stressors on ecosystem functioning are difficult to predict, due to synergistic / antagonistic variation of complex natural systems (Bulling et al., 2010) or the effects of multifunctionality (Mouillot et al., 2014). As such, it becomes important to measure ecosystem processes *in situ,* under semi-controlled conditions, to understand how natural context may control the biological responses (e.g. Murray et al. 2014; Hale et al. 2014). An experimental setup in field conditions is one way to account for realistic responses, with manipulation of control/test variables, but keeping all the remaining natural variability (e.g. Crespo et al. 2016). Nevertheless, it poses additional challenge in the experimental design and for the stabilization of control parameters (Bulling et al., 2006; Godbold et al., 2011).

Global changes induced by man have risen severely during the 20th century (Goldberg, 1995; Levitus et al., 2000) and, their outcome for biological systems is still unpredictable. However, in spite of the associated uncertainty, there is a general consensus regarding environmental and socio-economic implications derived from such changes (Millennium Ecosystem Assessment, 2005; IPCC, 2014). Several international agreements on warmness gases and other climate policies were assumed by major stakeholders in recent decades (e.g. United Nations 1992; Unfccc 1998). However, a common goal regarding global warming was only assumed during the recent United Nations Conference on Climate Change, held in Paris, France, in 2015: to keep temperature rise below 2ºC, preferably 1.5ºC, above pre-industrial levels (United Nations, 2015). Limiting the emission of warmness gases and increasing the access to cleaner technology to undeveloped parties would be a way to achieve this goal. Therefore, it is highly relevant to measure responses of different functional groups within this new international framework. In fact, the Paris Conference on Climate Change appeals to the increase of scientific knowledge on the effects of climate, in order to support decision-making (article 7, paragraph 7, United Nations 2015). As such, the proposed research aims to contribute with science-based knowledge, focusing on the effect of a 2ºC temperature rise on benthic estuarine communities. For this, we performed a 30-day *in situ* experiment with benthic intertidal communities from a southern European temperate estuary, where a temperature increase was induced. Data on micro and macrofauna diversity and important ecological processes (bioturbation) and functions (nutrient balance) were measured to in order to contribute for a better knowledge of the effects of a mild temperature rise in the functioning of estuarine ecosystems. We expected changes in the benthic communities, namely decreased levels of diversity for both macrofauna and microfauna and changes in the dominant species under the effect of warmness, taking into account results from previous records on the effects of temperature increase in macrobenthic communities (e.g. Dolbeth et al., 2011; Grilo et al., 2011). As for the ecosystem processes and functions, we also expect changes owing the differential communities expected under the warmness scenario. However, we cannot anticipate the change trajectory.

4 MATERIAL AND METHODS

4.1 STUDY AREA

Field experiments were conducted during the summer of 2014 in the Mondego estuary. This is a relatively small intertidal system with 8.6 km^2, located in a warm temperate region at the western-Atlantic coast of Portugal (40°08'N, 8°50'W). The experimental set up took place in a sandflat area located in the inner part of the estuary (Fig. 1). This area is currently characterized by low water flow (0.8 – 1.2 m.s^{-1}) and fine sand sediments (median grain size according to the Folk and Ward

Method, Blott S.J. and Pye K. 2001). Currently, more than 2/3 of the intertidal area is covered by the seagrass *Zostera noltei*, with bare sediments in the remaining area. This allowed investigating the responses of benthic communities from different sites (with or without the seagrass cover) under an atmospheric warming simulation conditions.

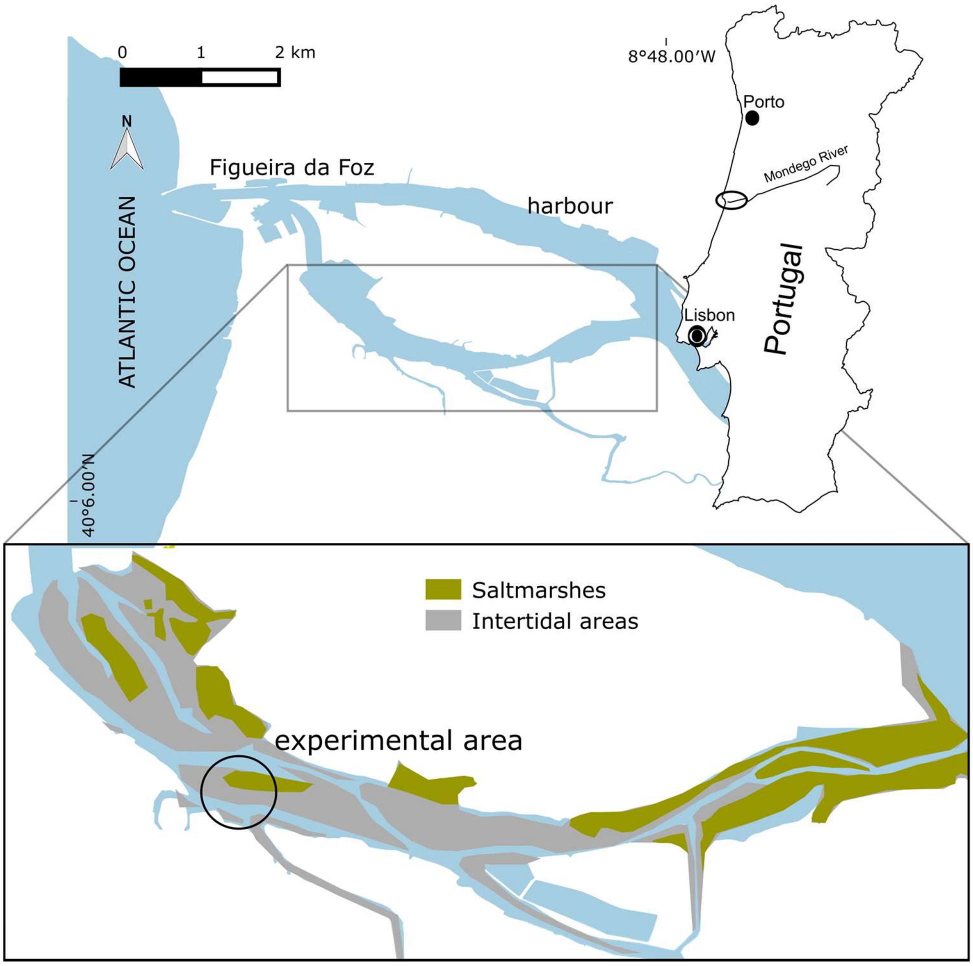

Fig. 1 The Mondego estuary, showing the experiment location in detail

4.2 EXPERIMENTAL SET-UP

As mentioned, two sites were compared: the sandflat and the *Zostera noltei* bed. Three treatments with three replicates each were run in each site: 1) control without the box; 2) control box open (with a mesh at the top to avoid disturbance/predation by crabs and shorebirds), henceforward referred as "no warmness effect"; 3) box closed, referred as “warmness effect”. Transparent plastic boxes (57x39x28 cm) were used in the experiment, thus creating a warmness effect by enabling the penetration of sunlight and preventing heat dissipation. They were placed in the sediment buried until half of its height (15 cm approximately). Tidal water had free circulation through small holes drilled in the sides of the boxes. To test for the effect of the box device, we used the control box open (“no warmness effect”). Temperature at the sediment surface was monitored in both sites at intervals of 10 minutes during 30 days by Onset® HOBO Water Temperature Pro v2 Data Loggers.

Two sampling moments were selected (T10 – after 10 days; T30 – after 30 days) to observe potential short-term and longer-term effects of warmness in the benthic communities of both sites. For each sampling time and site, three sediment cores (141 cm^2 surface area), for benthic communities, were collected after removing the boxes (total of 60 samples: 2 sites, 3 experimental treatments and 2 sampling times). These samples were washed in estuarine water through a 500 μm mesh sieve and the benthos retained was preserved in 4% buffered formalin. Afterwards, in the laboratory, animals were sorted and transferred to 70% ethanol, identified to the lowest possible taxon and counted. The ash-free dry weight (AFDW; 8 h at 450ºC) was assessed. Additional samples were taken at T10 with a 26 mm diameter core, from the superficial layers (up to 3 cm depth), for microbiological community characterization. At each sampling occasion and for each treatment, three water aliquots (20 mL) from intertidal pools (outside and inside the boxes; please notice that these intertidal pools can have a few cm^2) and three surface sediment aliquots (up to 3 cm depth) were taken for nutrient analyses (described in detail in the following sections).

4.3 MICROBIAL DIVERSITY

DNA was extracted from 250 mg of freeze-dried sediment using the PowerSoil® DNA Isolation Kit (MoBio Laboratories, Solana Beach, CA, USA) according to the manufacturer's instructions. The ITS2 region of fungal ribosomal DNA (rDNA) was amplified with the primer pair ITS3GC and ITS4, the V3 region of 16S bacterial rDNA was amplified with the primer pair 338GC and 518 (Duarte et al., 2010), and the 18S rDNA of ciliates was amplified with the primer pair 384GC and 1147 (Fernandes et al., 2013).

For PCR of fungal, bacterial and ciliate DNA, 1x Green GoTaq® Flexi Buffer, 3 mM of MgCl2, 0.2 mM of dNTPs, 0.4 μM of the appropriate primers, 1.5 U of GoTaq® G2 Flexi DNA Polymerase (Promega) and 2 μL of DNA were used in a final volume of 50 μL. PCRs were carried out in a MyCycler Thermal Cycler (BioRad Laboratories, Hercules, CA, USA). The PCR program for bacteria and fungi was initial denaturation at 95°C for 2 min, 36 cycles of denaturation at 95°C for 30 s, primer annealing at 55°C for 30 s and extension at 72°C for 1 min, and final extension at 72°C for 30 min (modified from Duarte et al., 2010). The PCR program for ciliates was initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 45 s, primer annealing at 55°C for 60 s and extension at 72°C for 90 s, and final extension at 72°C for 7 min (Dopheide et al., 2008; Fernandes et al., 2013).

DGGE analysis was performed using a DCodeTM Universal Mutation Detection System (BioRad Laboratories, Hercules, CA, USA), according to Duarte et al (2010) with few modifications. For fungi, 700 ng of the amplified DNA products with 380–400 bp was loaded on 8% (w/v) polyacrylamide gel in 1− Tris–acetate–EDTA (TAE 1×) with a denaturing gradient from 30 to 60% (100% denaturant corresponds to 40% formamide and 7 M urea). For bacteria, 700 ng of the amplified DNA products of ca. 200 bp was loaded on 8% (w/v) polyacrylamide gel in 1× TAE with a denaturing gradient from 45 to 62.5%. For ciliates, 700 ng of the amplified DNA products with 750–800 bp was loaded on 6% (w/v) polyacrylamide gel in 1× TAE with a denaturing gradient from 20 to 42.5%. Fungal and bacterial DNA was separated at 55 V and 56°C, while ciliate DNA was separated at 80 V and 60°C. All gels were run for 16 h. Gels were stained with 1x Midori Green (NIPPON Genetics EUROPE GmbH, Düren, Germany) for 10 min and gel images were captured under UV light in a gel documentation system (ChemiDoc XRS, BioRad).

4.4 MEASUREMENT OF PARTICLE REWORKING *IN SITU*

Particle reworking of the sediment (i.e. bioturbation) of each experimental unit was assessed using fluorescent sediment profile imaging (f-SPI, Solan et al. 2004b), a non-invasive method that allows tracking fluorescent-dyed sediment particles that shine under UV light (luminophores: 125–250 µm diameter, green colour; Brian Clegg, Ltd, UK) (Schiffers et al., 2011). The distribution of luminophores can be determined from high spatial resolution images from the sides of a transparent corer.

For each test box, a square section corer with open top and bottom (side 7 cm) was buried to a depth of approximately 15 cm. Luminophores (aprox. 20g corer^{-1}) were added at the beginning of the experiment. At T10 and T30 the corers were retrieved by hermetically covering the top side (creating negative pressure). After being cleaned, the squared corers were photographed on all four sides within a dark box with UV illumination. We used a Canon EOS 350D single lens reflex digital CMOS camera (8.0 megapixels; exposure 4 s, f= 4 and a film equivalent speed of ISO 100). The resulting images (red-green-blue [RGB] colour with JPEG [Joint Photographic Experts Group] compression) were cropped to the full width of the corer and all four sides were merged in one image (2188 pixels, effective resolution= 12.8 µm per pixel). These images were analyzed using a custom-made plugin that runs within ImageJ (Version 1.48c), a java-based public domain program developed at the US National Institute of Health (available at http://imagej.nih.gov/ij/). This plugin assessed the values of mean ($^{f\text{-}SPI}L_{mean}$, time dependent indication of mixing), median ($^{f\text{-}SPI}L_{med}$, typical short-term depth of mixing) and maximum ($^{f\text{-}SPI}L_{max}$, maximum extent of mixing over the long-term) mixed depths of particle redistribution, as described in Hale et al (2014). Surface Boundary Roughness (SBR), the maximum vertical deviation of the sediment-water interface, (upper – lower limit) was also measured, as an indication of surficial activity.

4.5 DISSOLVED INORGANIC NUTRIENTS

A total of 36 water aliquots (three replicates per site, treatment and sampling time) were sampled and analysed for dissolved inorganic nitrogen (DIN= NH_4-N+NO_x-N) and dissolved inorganic phosphorous (DIP= PO_4-P). Immediately after sampling, water aliquots (20 mL) were transported to the laboratory in cool boxes (dark and 4ºC), where they were filtered (0.7µm glass-fibre filter, Whatman GF/ F) and stored frozen at -18ºC until analysis. The concentrations of nitrite (NO_2-N) and nitrate (NO_3-N) were determined using a flow injection system (FIAstar 5000 Analyzer, Höganäs, Sweden), following the Strickland and Parsons (1972) method. The determination of the concentrations of ammonium (NH_4-N) and phosphate (PO_4-P) was done following the standard method described in Limnologisk Metodik (1992). To ensure the analytical quality control, calibration curves, using a standard solution, were run at the beginning of the analysis and in parallel with blanks and samples.

4.6 DATA ANALYSIS

Species richness (number of species) and Shannon-Wiener diversity were computed for the macrofauna communities. These indices and benthic community data were treated with Permutational Multivariate Analyses of Variance –

PERMANOVA, to test their differences with regard to the temperature treatment, sites and time. PERMANOVA is an analysis of variance to test one or more factors, using permutation methods and on the basis of a resemblance matrix (Anderson et al., 2008). PERMANOVA was carried out on three-factor crossed experimental design with replication for the diversity indices, upon Euclidean distance matrix for non-transformed data, and for benthic community, upon a Bray-Curtis similarity matrix, with a dummy variable to compensate for zero occurrences (Anderson et al., 2008). The explanatory factors were included as fixed factors and we considered three levels for temperature treatment (control; no warmness effect; warmness effect), two levels for time (T10 and T30) and two for site (sandflat and *Zostera*). The benthic data were further explored using Principal Coordinates Analyses (PCO), where we overlaid vectors based on Spearman correlations onto the PCO plot in order to clarify patterns of change (Anderson et al., 2008).

For microfauna, DGGE gels were aligned and normalized using BioNumerics 7.1 (Applied Maths, Sint-Martens-Latem, Belgium). Each DGGE band was considered one operational taxonomic unit (OTU), taking into account that more than one species can co-migrate to the same position in the gel. Species richness was assessed as the number of OTUs and the Shannon-Wiener diversity computed for all microfauna communities (fungi, ciliates and bacteria). The differences in diversity were tested with a two-way ANOVA for the effects of site and temperature treatment (there was only one sampling occasion for microfauna), followed by a Tukey's post-hoc test (Zar, 2010). Assumptions of the ANOVA were initially verified. Bacterial richness was Box-Cox-transformed to achieve normality. Statistical analyses were done in STATISTICA (version 8.0 for Windows; Statsoft, Tulsa, Oklahoma). We used 2-way PERMANOVA to test the effect of site and temperature on the aquatic microfauna assemblages (based on DGGE fingerprints) (Anderson et al., 2008). Prior to the analyses, data was $\sqrt{(x)}$-transformed and converted into a Bray–Curtis similarity matrix. All diversity and community data analyses for the macrofauna and microfauna were performed using PRIMER v6 software with the PERMANOVA add-on package (Anderson et al. 2008).

For particle reworking analyses, we developed independent regression models for each of our dependent variables (SBR, ${}^{f\text{-}SPI}L_{mean}$, ${}^{f\text{-}SPI}L_{med}$, ${}^{f\text{-}SPI}L_{max}$) using the full factorial combination of independent variables (site [sandflat; *Zostera*], temperature treatment [no warmness effect; warmness effect] and time [T10; T30]). The "no warmness effect" worked as control for this analysis. A generalised least squares (GLS, Pinheiro and Bates 2000) estimation procedure was used and included the appropriate variance-covariate structure (Minimal adequate models structure in Appendix II), as data violated the homoscedasticity assumption. GLS regression allows the residual spread to vary with the explanatory variables and avoids data transformation. Restricted maximum-likelihood (REML) estimation was used to determine the optimal variance-covariate structure, after finding the lowest Akaike Information Criteria (AIC) and best model residuals. The optimal fixed structure was then determined by backward selection using the likelihood ratio (L-ratio) test obtained using maximum likelihood (ML) estimation and the minimal adequate model was re-expressed using REML (Diggle et al., 2002; West et al., 2007; Zuur et al., 2009). We used a parametric bootstrap with 999 re-samples and the percentile method to obtain the 95% CI limits around the predicted values (Appendix II, Models 1- 4). GLS analyses were conducted using the 'nlme' package (Pinheiro et al., 2014) and parametric bootstrapping were conducted, assuming that the estimated parameters followed a multivariate Gaussian distribution with mean and variances provided from the output of the fitting function, using the function 'rmvnorm' within the package 'mvtnorm' (Genz et al., 2014). These analyses were performed using the 'R' statistical and programming environment (R Development Core Team 2012).

5 RESULTS

5.1 EFFICIENCY OF THE TEMPERATURE INCREASE

The warmness treatments were able to produce a differential temperature between the open boxes – “no warmness effect”, and the closed boxes – “warmness effect” (Fig. 2A). The temperature difference measured in the sediment at the daily peak was 2.35ºC for the sandflat and 1.52ºC for the *Zostera* bed (Fig. 2B), whose values were within the ones considered acceptable after the Paris United Nations Conference on Climate Change. Also, values for the continuous record (which includes high tides and night periods) denote an average temperature rise on both sites (0.36ºC in the sandflat and 0.21ºC in the *Zostera* bed, Fig. 2B).

5.2 EFFECT OF THE WARMNESS TREATMENT, HABITAT AND TIME

5.2.1 Macrobenthic communities

For the macrobenthic communities’ species richness, we found two significant interactions with site, in combination with time and in combination with the temperature treatment effect (Table 1). At T10 species richness was higher in the sandflat than in the *Zostera*, particularly for the “warmness” treatment (Fig. 3A, Table 1). However, at T30 the values were very similar between sites and treatments (Fig. 3A). The Shannon-Wiener index (H’) for macrobenthic density was higher in the sandflat for both times (Fig. 3B), with a significant interaction detected between site and time (Table 1). For the H’ estimated with biomass, only site became significant (Table 1), with the *Zostera* showing higher H’ values, except in the “no warmness” treatment, in T10 (Fig. 3C).

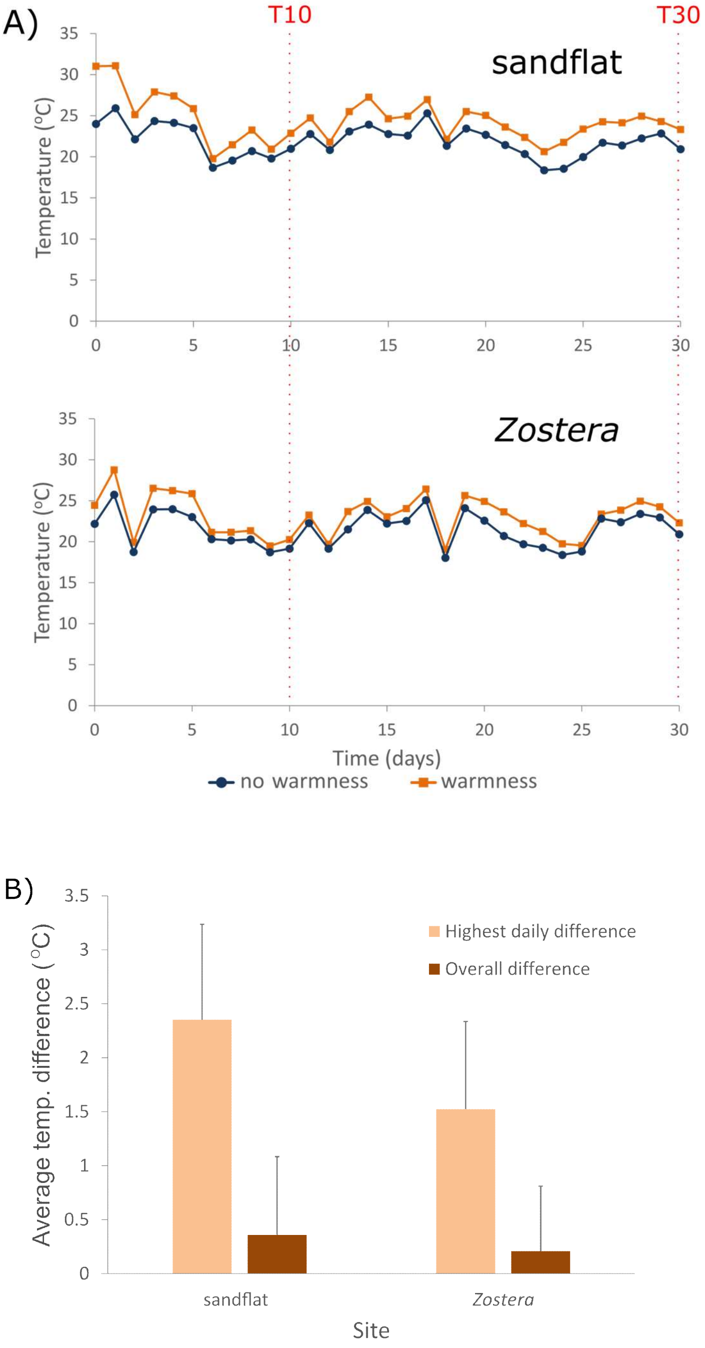

Fig. 2 Temperature registered during the experiment for "no warmness effect" (box open, acting as control for the effect of the box) and "warmness effect" (box closed) and for both sites: (A) highest daily temperature, with indication of the sampling periods, after 10 days (T10) and after 30 days (T30); (B) average difference and standard error within treatments in both sites. The highest daily difference is the average difference for every daily heat peak and the overall difference refers to all temperature records (including high tides and night periods)

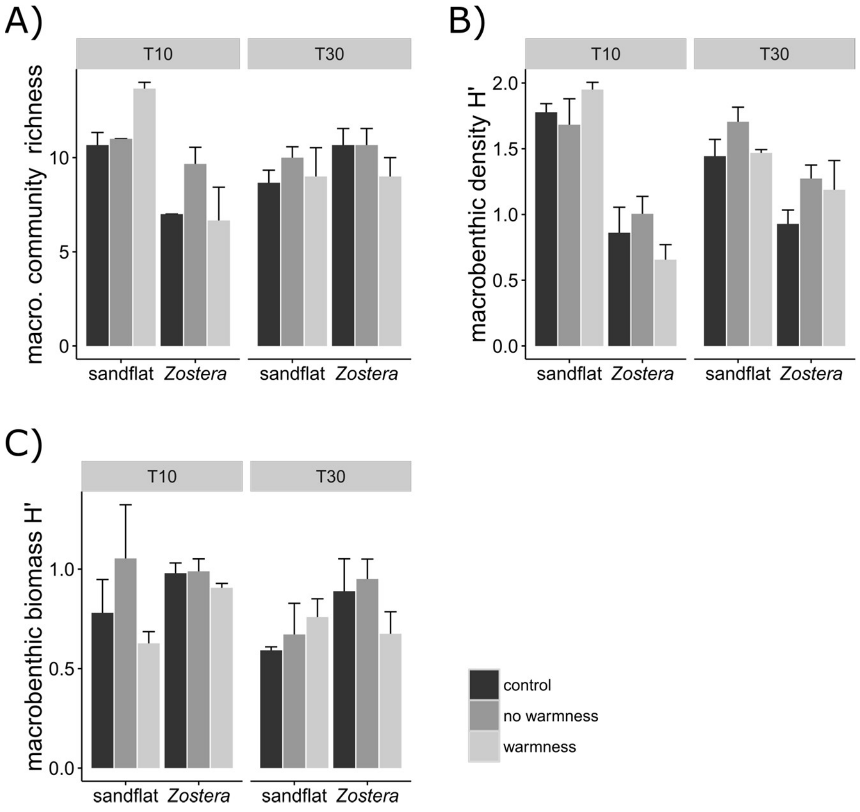

Fig. 3 Mean + s.d. (n= 3) species richness (A) and Shannon-Wiener index of the macrobenthic communities measured with density (B) and biomass (C) in the sandflat and Zostera areas, under control, no warmness and warmness treatment, for the two time periods

Regarding the macrobenthic community data, we have found significant interactions between site and treatment and site and time (Table 1). However, differences were clearer per site, with *Peringia ulvae and Hediste diversicolor* associated with the seagrass, while small opportunist polychaetes and *Ceratoderma edule* were associated with the sandflat (Fig. 4A). Despite the significant interaction found between site and time, differences were significant for both time periods and sites, while for the treatment effect statistical differences were only found between control and the warmness treatment for the sandflat alone (Table 1).

For biomass data, we had similar results as the ones for density (two significant interactions, Table 1). Again, differences were very clear per site (Fig. 4B) and there were significant differences between T10 and T30 for the sandflat samples and when comparing sites within each time period (Table 1). Regarding the effect of temperature rise on macrobenthic fauna, results were clearer than with the density (Fig. 4B). However, PERMANOVA provided the same results: no statistical differences were found between control and the “no warmness effect” treatment, used here as a control for the box device, but there were significant differences between control *vs* “warmness effect” (Table 1, Fig. 4B). These differences seemed more evident for the sandflat (Fig. 4B), but were significant for both sites (Table 1).

Table 1 Summary of significant terms from the 3-way PERMANOVA analyses, with macrobenthic community and respective diversity indices as dependent variables, and site, treatment and time as explanatory variables, with indication of the significant pairwise comparisons

Dependent variable	Significant terms	d.f.	Pseudo -F	p-perm	Terms/levels of factor	p-perm
Species Richness	site x temperature	2	4.2065	0.021	warmness [sandflat vs *Zostera*]	0.021
	site x time	1	22.011	0.002	T10 [sandflat vs *Zostera*] Sandflat, Zostera: [T10 *vs* T30]	0.001 <0.015
Shannon (density)	site x time	1	12.761	0.002	T10, T30: [sandflat vs *Zostera*] Sandflat, Zostera: [T10 *vs* T30]	<0.004 <0.05
Shannon (biomass)	site	1	4.12	0.05	sandflat vs *Zostera*	0.05
Benthic community density	site x temperature	2	1.8469	0.037	Sandflat: [Control vs warmness] All treatments: [sandflat vs Zostera]	0.056 <0.05
	site x time	1	2.8878	0.01	For T10, T30: *Zostera* vs sandflat Sandflat, *Zostera*: T10 vs T30	0.001 <0.005
Benthic community biomass	site x temperature	2	2.171	0.032	Sandflat: [Control vs warmness] All treatments: [sandflat vs Zostera]	0.017 <0.05
	site x time	1	3.2585	0.019	For T10, T30: *Zostera* vs sandflat Sandflat, Zostera: [T10 vs T30]	<0.002 <0.04

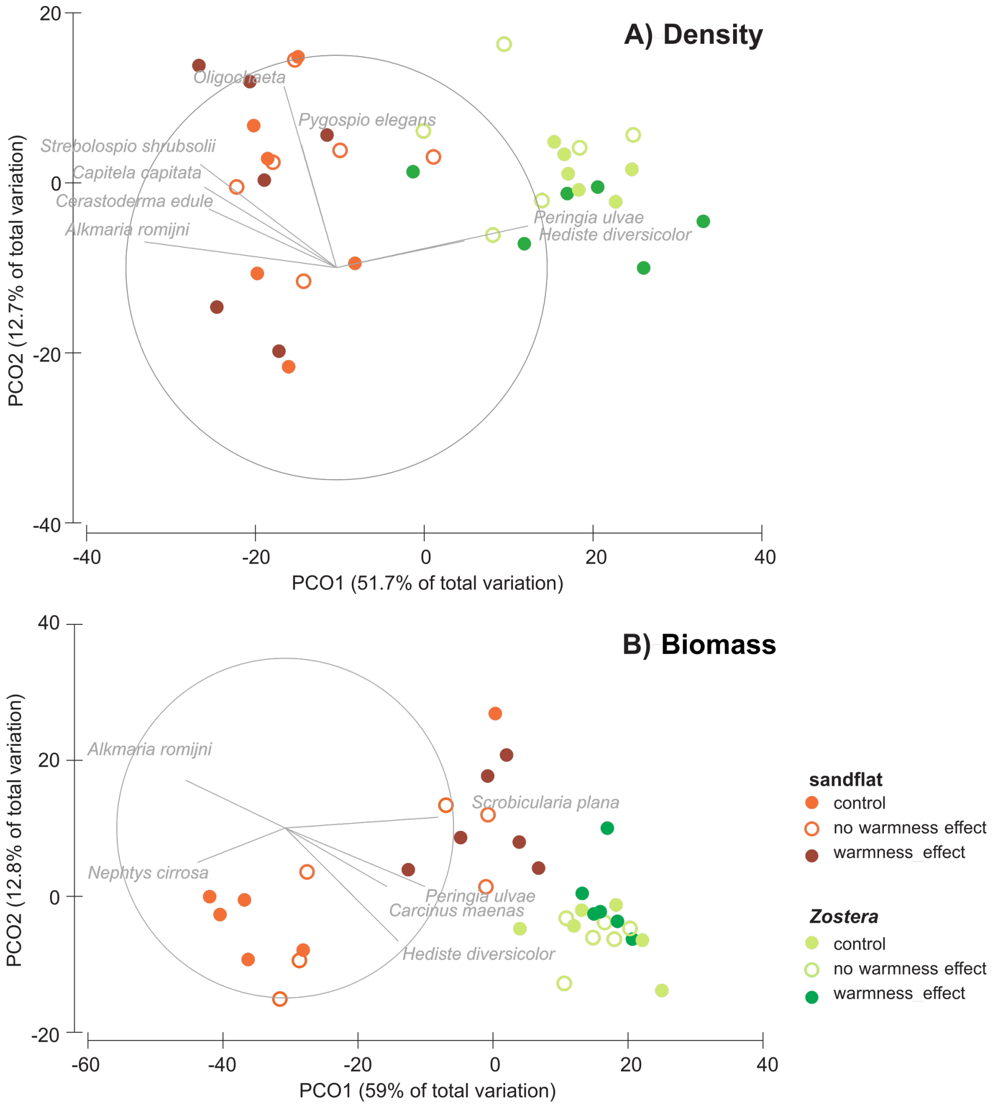

Fig. 4 PCO ordination for macrobenthic community (A) density and (B) biomass for each area and treatment. The length and direction of each vector indicates the strength and sign of the relationship between species abundance or biomass and PCO axes, based on a Spearman correlation (only vector with length >0.5 were represented)

5.2.2 Microbial communities

Microfauna diversity and composition was only assessed in T10. A similar trend variation was observed for the fungal richness and Shannon-Wiener index, but with clearer differences among areas and treatments for the richness (Fig. 5A, B). Both indices were significantly higher in the sandflat area than in *Zostera*, with the highest values found on the control treatment associated with sandflat (Table 2, Fig. 5). Differences were also evident with regard to the warmness treatment effect (Table 2), particularly for the richness (Fig. 5A, B). Richness values were significantly lower in the “warmness” compared to the “no warmness” and control treatments (Tukey’s, p< 0.05), while Shannon-Wiener values were significantly lower in the “warmness” compared to the control (Tukey’s, p= 0.0182).

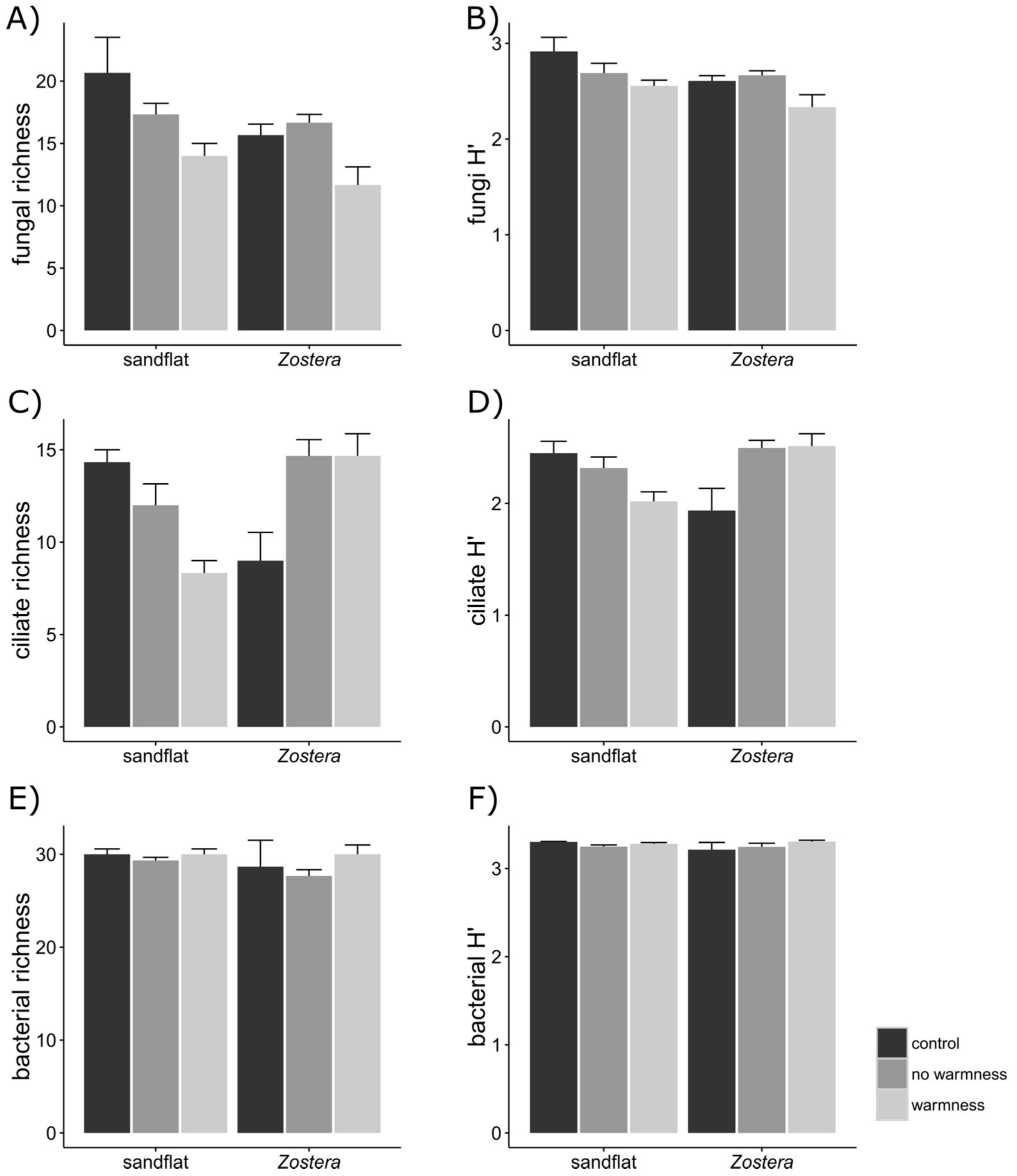

Fig. 5 Mean + s.d. (n= 3) richness of fungal (A), ciliates (C) and bacterial (E) communities and Shannon-Wiener index of fungal (B), ciliates (D) and bacterial (F) communities on sediment samples from sandflat or Zostera under control, no warmness and warmness treatment

Table 2 Results from the 2-way ANOVA for the effect of site and temperature on fungal, ciliate and bacterial communities' richness and Shannon-Wiener indices. For the bacterial Shannon-index a 2-way PERMANOVA was applied, as data did not passed the assumptions of parametric tests; ^{a} - pseudo-F, ^{b} – P(perm)

Dependent variable	Effect	S.S.	d.f.	M.S.	F-value	p-value
Fungal richness	temperature	94.33	2	47.17	7.13	0.009087
	site	32.00	1	32.00	4.84	0.048130
	temperature x site	14.33	2	7.17	1.08	0.369168
	error	79.33	12	6.61		
Fungi Shannon-Wiener index	temperature	0.32	2	0.16	5.63	0.018869
	site	0.153	1	0.15	5.30	0.040004
	temperature x site	0.06	2	0.03	1.11	0.360000
	error	0.35	12	0.03		
Ciliate richness	temperature	12.33	2	6.17	1.82	0.204069
	site	6.72	1	6.72	1.98	0.184389
	temperature x site	106.78	2	53.39	15.75	0.000440
	error	40.67	12	3.39		
Ciliate Shannon-Wiener index	temperature	0.14	2	0.07	1.70	0.225634
	site	0.01	1	0.01	0.31	0.588654
	temperature x site	0.80	2	0.40	9.50	0.003361
	error	0.50	12	0.04		
Bacterial richness	temperature	4.25×10^{12}	2	2.13×10^{12}	1.01	0.393082
	site	8.71×10^{11}	1	8.71×10^{11}	0.41	0.532230
	temperature x site	1.02×10^{12}	2	5.12×10^{11}	0.24	0.788013
	error	2.53×10^{13}	12	2.11×10^{12}		
Bacteria Shannon-Wiener index	temperature	2.11E-03	1	2.11E-03	0.46085^{a}	0.558^{b}
	site	6.69E-03	2	3.34E-03	0.73183^{a}	0.524^{b}
	temperature x site	1.04E-02	2	5.19E-03	1.1361^{a}	0.367^{b}
	Residual	5.48E-02	12	4.57E-03		

The trend variation in the diversity indices of the ciliate communities was also similar, but again, differences were clearer with richness (Fig. 5C, D). For both indices, the interaction between site and temperature was significant (Table 2). For the sandflat, both indices were highest in the control treatment and lowest in the "warmness" treatment (Fig. 5C, D). However, this trend was the opposite for the

Zostera, which had the lowest values in the control treatment compared to the others (Fig. 5E, F). The same pattern was observed for the Shannon index (2-way ANOVA, p= 0.003; Table 2; Fig. 5D), with temperature treatment decreasing ciliates communities evenness in sandflat, but increasing ciliates communities evenness in *Zostera*.

Bacterial richness and Shannon-Wiener index varied within similar values for both sites and treatments (Fig. 5E, F), with no significant differences detected among factors (Table 2). Bacterial communities contained on average 30 species (Fig. 5E).

Table 3 Summary of significant terms from the 2-way PERMANOVA analyses with fungal, ciliate or bacterial communities as dependent variables, and site and temperature as explanatory variables, with indication of the significant pairwise comparisons

Dependent variable	Significant terms	d.f.	Pseudo-F	p-perm	Terms/levels of factor	p-perm
Fungi	site x temperature	2	1.9417	0.02	*no significant terms*	*ns*
	site	1	2.1485	0.02	sandflat *vs Zostera*	0.02
	temperature	2	1.7761	0.01	control *vs* no warmness	0.04
					control *vs* warmness	0.03
Ciliate	site x temperature	2	1.7003	0.03	*no significant terms*	*ns*
	site	1	2.956	0.002	sandflat *vs* Zostera	0.004
	temperature	2	1.6075	0.03	control *vs* no warmness	0.03
					control *vs* warmness	0.03
Bacteria	site x temperature	2	5.4363	0.001	*no significant terms*	*ns*
	site	1	2.8995	0.003	sandflat *vs Zostera*	0.004
	temperature	2	9.0145	0.001	control *vs* no warmness	0.003
					control *vs* warmness	0.002
					no warmness *vs* warmness	0.002

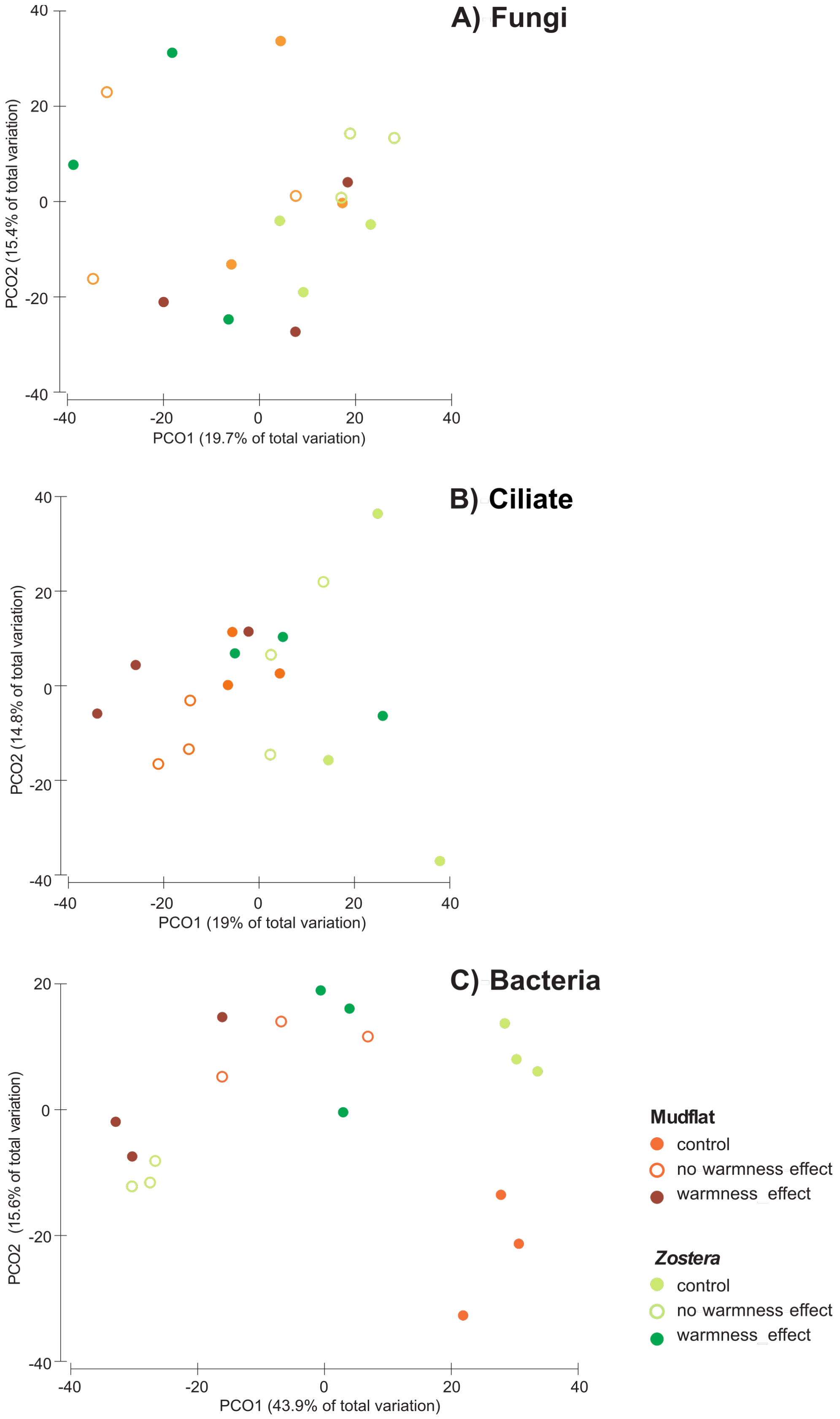

Fig. 6 PCO ordination for fungal (A), ciliates (B) and bacterial (C) communities for each site and temperature treatment

Regarding the composition and structure of the fungal, ciliate and bacterial communities, we found significant interactions between site and temperature for all communities (Table 3). However, the pairwise comparisons were not significant for any of the terms/factors pairs (Table 3). As such, we have considered the significance among main terms, which were always significant (Table 3). Despite this result, the variability explained in the PCO plots for the fungal and ciliate communities was relatively low (< 25.1%) and the discrimination among factors not that clear (Fig. 6A, B). In both these communities, significant differences were found per site and among the control treatment with the other treatments (Table 3). For the bacterial communities, statistical differences were found per site and among all temperature treatments (Table 3). The variability explained in the PCO plot was higher than for the other community (about 60%), with the control samples clearly separated from the other treatments (Fig. 6C). For the *Zostera*, differences among treatments were also clearer (Fig. 6C).

5.2.3 Biological process – particle reworking

We found evidence that bioturbation was affected by the different levels of the tested factors. The minimal adequate models were GLS regressions (Table 4), with a variance structure of different spreads for each independent fixed factor (full structure of the models, correction of the random part, pairwise comparison matrix for significant differences among levels and graphic representation of model predictions in Appendix II - Model 1 to Model 4; mean values and standard errors for each measurement in Appendix II – Table 1).

Table 4 Summary of significant terms from the GLS for particle reworking measurements, with bioturbation components as dependent variables and site, treatment and time as explanatory variables

Dependent variable	Significant terms	d.f.	L-ratio	p-value
SBR	site x temperature x time	7	16.813	0.0186
$^{f\text{-}SPI}L_{mean}$	site x temperature x time	7	20.003	0.0056
$^{f\text{-}SPI}L_{median}$	site	1	8.153	0.0043
$^{f\text{-}SPI}L_{max}$	site x temperature x time	7	20.316	0.0049

Surface Boundary Roughness (SBR), $^{f\text{-}SPI}L_{mean}$ and $^{f\text{-}SPI}L_{max}$ were significantly affected by the interaction of all factors (site x temperature x time), while $^{f\text{-}SPI}L_{median}$ only varied with site (Table 4, models structure for the four bioturbation components described in Appendix II).

SBR values ranged from 0.358 to 2.470 cm. There was always a decrease in SBR when comparing the effect of the warmness treatment, a result that was consistent for both sites and T10 (Fig. 7A, Appendix II - Table 1). For T30, SBR increased with the "warmness effect" (Fig. 7A, Appendix II - Table 1). SBR increased with time for both temperature treatments in the *Zostera* site. However, in the sandflat SBR decreased with time for the "no warmness effect" treatment and increased with time for the "warmness effect" treatment, in agreement with the significant interaction detected. Despite the observed tendencies, when comparing relevant significant differences (i.e. comparisons of treatments that share the same levels of two different factors), those were only found between the "no warmness" vs "warmness" effects in the sandflat at T10 (p= 0.0072), between T10 and T30 in the sandflat under the "no warmness effect" (p= 0.0009) and between sites at T30 under the "no warmness effect" (p= 0.039) (Appendix II - Table 2).

$^{f\text{-}SPI}L_{mean}$ values ranged from 0.689 to 6.476 cm and reflected the complex 3-way interaction. At T10, $^{f\text{-}SPI}L_{mean}$ values were lower with "no warmness effect" treatment and increased with the “warmness effect” for the sandflat (p= 0.0339, Appendix II - Table 3), while the inverse tendency was found for the *Zostera* (Fig. 7B, Appendix II - Table 1). At T30, in the *Zostera* bed, ${}^{f\text{-}SPI}L_{mean}$ increased for the “warmness effect” compared to the “no warmness” one (Fig. 7B, Appendix II - Table 1). In the sandflat, ${}^{f\text{-}SPI}L_{mean}$ values decreased with time for “warmness effect” (p= 0.0199, Appendix II - Table 3), while for the *Zostera* bed ${}^{f\text{-}SPI}L_{mean}$ decreased with time for the “no warmness effect” treatment and increased with the “warmness effect”. Generally, *Zostera* bed had higher values of ${}^{f\text{-}SPI}L_{mean}$ than the sandflat at T30, for both “no warmness effect” (p= 0.0175) and “warmness effect” (p= 0.0072, Appendix II - Table 3).

${}^{f\text{-}SPI}L_{median}$ only varied with site and values ranged from 0 to 6.949 cm (Fig. 7C). These were considerably higher for the *Zostera* bed than for the sandflat (p= 0.004, Appendix II - Table 1).

Values of ${}^{f\text{-}SPI}L_{max}$ ranged from 8.561 to 12.490 cm and again reflected the complex 3-way interaction. Overall, there was a tendency for ${}^{f\text{-}SPI}L_{max}$ to increase with time, both in the sandflat and in the *Zostera* bed (Fig. 7D). In the sandflat the increase was higher in the “no warmness effect” treatment than in the “warmness effect” treatment that showed a circumstantial decrease (Fig. 7D, Appendix II - Table 1). In the *Zostera* bed the larger increase over time was found in the “warmness effect” treatment (relevant significant differences only detected for this scenario, p= 0.0027, Appendix II - Table 4), with a small decrease in the “no warmness effect” treatment (Fig. 7D, Appendix II - Table 1). When comparing temperature treatments, the “warmness effect” showed higher or similar values to the “no warmness effect”, except at T10, for the *Zostera* bed (Fig. 7D). Nevertheless, relevant significant differences were only found between the sandflat and *Zostera* bed, at T10, under the “warmness effect” (p= 0.0001, Appendix II - Table 4).

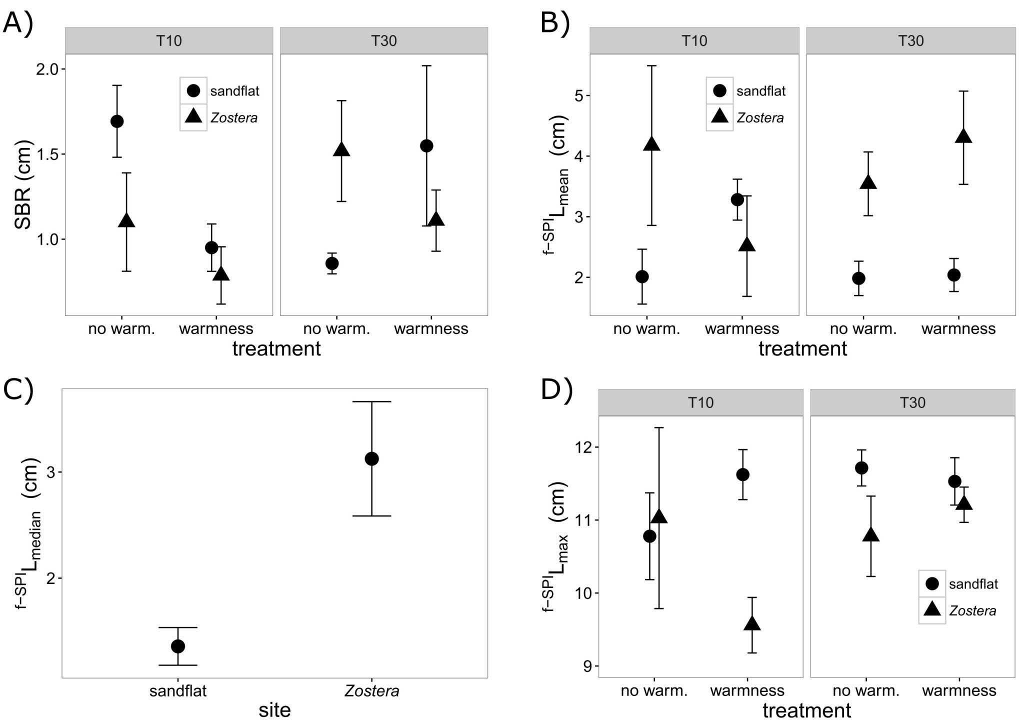

Fig. 7 The significant effects of warmness treatment, site and time on surface boundary roughness (SBR) (A), $^{f\text{-}SPI}L_{mean}$ (B), $^{f\text{-}SPI}L_{median}$ (C) and $^{f\text{-}SPI}L_{mean}$ (D) (cm, mean ± s.e.). For clarity, jitter has been applied to the x = argument of the plot function to avoid over-plotting

5.2.4 Sediment C, N and P pools and intertidal water nutrient concentrations

The average organic matter content in the sediments was 1.96 ± 0.02% in sandflat and 2.58 ± 0.02 in *Zostera*, respectively, with significant differences between sites (matched-pairs t-test= -206.621, df= 1, p-value= 0.003). Regarding C and N pools in the sediment at the beginning of the experiment, differences were observed between sites, with higher carbon and nitrogen concentrations observed in the *Zostera* meadows, as a result of the higher organic matter content ([mean ± s.d] C: 1.138 ± 0.25 mg L^{-1} in sandflat and 1.74 ± 0.36 in *Zostera;* N: 0.086 ± 0.027 mg L^{-1} in sandflat and 0.135 ± 0.027 in *Zostera*). However, these distinct sediment characteristics and distinct plant coverage were not reflected in the dissolved nutrient concentrations in the shallow low water pools. Overall, the range of nutrient concentrations at the sampling sites and time were within the described for the system, taking into account tidal and circadian cycles. However, no distinct pattern was observed in the nitrogen and phosphorus concentrations of the shallow intertidal pools of all treatments, sites and times. Concentrations of dissolved inorganic nitrogen ranged from 0.005 to 0.58 mg L^{-1}, with most values <0.25 mg L^{-1}, while PO_4-P concentrations ranged between 0.02 and 0.15 mg L^{-1}, but most values were <0.05 mg L^{-1}.

6 DISCUSSION

6.1 THE IMPORTANCE OF *IN SITU* EXPERIMENTS IN THE CONTEXT OF GLOBAL WARMING STUDIES

The use of field experiments to assess the effects of the biodiversity on ecosystem functioning could be complemented with laboratorial work (Spivak et al., 2011; Stewart et al., 2013) in order to fine tune the potential causal-effect relationship. Aquatic ecosystems are highly complex systems where organisms are challenged by other biological components, as well as by abiotic influences, with several levels of interactions. This is particularly true for estuaries, where environmental variables shift over several cycles (McLusky, 1993; Pratt et al., 2014). Therefore, the study of transitional systems in laboratory has challenges that are inherent to that complexity. Despite the high level of accuracy allowed by laboratorial work, *in situ* experiments deliver a more realistic interpretation of natural processes that actually occur in the system. Nevertheless, *in situ* experiments face limitations concerning universality (e.g. Richards et al., 2002; Crespo et al., 2016): the outcomes can be site specific as context modulates biotic responses, either due to physiological events or behavioral alteration. Simultaneously, climate changes, including global warming, may have very specific local responses. Therefore, it is of great interest to compile data from a wide geographic range in order to better understand climate change consequences. The present work brings some insight on these possible consequences on an estuarine system, in particular regarding a temperature increase, that is within the range recently preconized by the Paris United Nations Conference on Climate Change – 1.5 to 2ºC. Nevertheless, this temperature increase should be regarded as an ambitious goal, fairly more optimistic than most of global climatic models predictions.

6.2 THE TEMPERATURE INCREASE EFFECT ON DIFFERENT HABITATS AND IN BENTHIC COMMUNITIES

In our study, temperature was measured on the top layers of the sediment, suggesting that the air inside the closed boxes (warmness effect) was even warmer, as plausible during a heat wave event. This temperature increase in the sediment was within the range of the acceptable temperature increase agreed at the Paris Conference (United Nations, 2015). Although the temperature difference between the treatments was not constant, due to night temperature and high tides periods, we found a general 1.5 to 2ºC increase for the warmness effect treatment during the daily peak measurements. Even so, the *Zostera* bed seems to be acting as a temperature buffer, since temperature range was generally lower than in the bare sandflat. Three different mechanisms could explain this result: 1) a shading effect that the *Zostera'* leaves produce on the underlying sediment, which could reduce the amount of sunlight that reaches the sediment and thus warming; 2) a different colour between the *Zostera* bed and the sandflat areas, since the sandflat has darker shade and, therefore, better efficiency in light absorption, becoming warmer than the *Zostera* bed; 3) a differential thermal buffer due to distinct water retention capacities provided by the seagrass leaves and rhizomes.

Regarding the benthic communities, a differential temperature regime could limit or stimulate some natural processes and cause shifts on the community structure and functioning associated with those communities (Coma et al., 2009; Dolbeth et al., 2011; Hale et al., 2011). However, our 1.5 to 2ºC temperature increase seemed less influential than the initially expected. In fact, the differences between sites, *Zostera* bed and sandflat, were more evident than the differences between temperature treatments, for both the macrofauna and microbiological communities. Macrofauna diversity results were not consistent with the expected under the temperature increase scenario, since previous studies dealing with the effects of the temperature rise on macrofauna have mentioned increased mortalities (Coma et al., 2009; Hale et al., 2011) and decreased diversity (e.g. Grilo et al., 2011). Our temperature increase was lower than the considered in those studies, and diversity was sometimes higher in the warmness effect treatment than in the control and non-warmness ones. Similarly, the period during which the communities were exposed to the heating effect (10 days or 30 days) did not had a consistent/clear effect, except for a higher variability among treatments in the sandflat, particularly at T10. In other words, there was less variation in diversity indices among treatments for the *Zostera* bed. Still, when examining the composition and structure of the macrofauna communities, some differences were apparent due to the temperature increase (warmness effect), particularly for the biomass levels in the sandflat, the area where the temperature increase was higher. Again, these results suggest that the *Zostera* bed may mitigate the temperature increase effect on the macrobenthic community, and that time might allowed communities to adjust to the disturbance (e.g. under an acclimation process, Allen et al., 2005).

Regarding the microfauna communities, analyzed only at T10, again, there were some mild evidence that these were affected by the warmness treatments. In general, fungal and ciliate richness was higher in sandflat than in the *Zostera*, but decreased with the temperature increase in both areas for the fungal communities and for the sandflat for the ciliate ones. Significant differences were also detected for the structure of those communities regarding site and temperature. However, these differences were not clear in PCO plots, and whose variability explained was considerably low (<25.1%). On the other hand, bacterial diversity did not respond clearly to the treatments or site. However, the differences on the bacterial community structure were more expressive than with the fungal and ciliate communities: there were also significant differences with site and temperature and samples clustered taking into account these differences, particularly regarding the control and the other treatments. Overall, it seems that the warmness treatment had a larger impact in the microbial diversity than in the macrobenthic diversity. This was expected, since microfauna communities respond much faster to external factors than the macrofauna ones (Kent et al., 2007; Nydahl et al., 2013), especially due to faster generation times (Roszak & Colwell, 1987). There is also the possibility that the macrofauna under the warmness stimulus could interfere with microbial communities by competitive interaction (Kristensen et al., 1992). Despite the observed differences, in light of our initial question, of how benthic communities are affected by a 1.5 to 2ºC temperature increase, the variation trends that we obtained were not that expressive. Also, daily environmental fluctuations could conceal the influence of the warmness treatment on microbial communities, by diluting the differential temperature between treatments.

6.3 CONSEQUENCES ON ECOSYSTEM PROCESSES AND FUNCTIONS

Our next question was whether the changes in the benthic communities due to the warming would reflect in changes on the processes and functions that are usually associated to those communities. However, since differences in the benthic communities with regard to the temperature rise effect were not that consistent, we expect the same pattern to occur with these processes and functions. For almost all components of bioturbation, there were significant interactions between all factors, suggesting that temperature had an effect on bioturbation that depended on the site and sampling time. Surface boundary roughness (SBR) increased with temperature only for the sandflat and for T30, which could be related to a temperature gradient decreasing from the sediment top downwards that stimulates some activity in the top layer of the sediment, in the area that had the highest temperature increase in sediment. Nevertheless, for the other conditions, the variation was the inverse and not conclusive with regard to the temperature rise effects. For the other components of bioturbation, the variation tendency was also not clear or conclusive with regard to the temperature increase. We expected an overall bioturbation modification with temperature, as species respond differentially to thermal pressures (Ouellette et al., 2004; Godbold & Solan, 2013). However, the interaction of several factors (including those not assessed in the experiment, e.g. salinity, water flow, turbidity) resulted into indistinct tendencies, with large variation in the sediment mixing measurements within each treatment replicates. In fact, most of the studied items in this experiment showed a similar outcome: the 1.5 - 2ºC temperature increase had a smaller influence than the site (i.e., with or without vegetation).

The absence of effects as a result of the temperature increase was also visible in the nutrient dynamics from the intertidal pools, at least at the specific sampling date and time. Despite the distinct sediment characteristics between sites, due to the presence of vegetation, the sediment and shallow low water pools nutrient fluxes due to warmness treatments was not perceptible. Data on the nutrients concentrations from the low water pools presented high variability, but were within the range of previously observed values for the system (Lillebø et al., 2002, 2007; Cardoso et al., 2010; Marques et al., 2013). Due to the daily variation in physicochemical parameters in this shallow mesotidal system, which can be higher than seasonal variations (Lillebø et al., 2002, 2007), we believe that to detect the consistent differences between the temperature treatments we needed to perform 24 hours cycles, which was out of the scope of this research. Also, the great intraspecific variability observed in bioturbation was reflected in the dissolved nutrient concentrations, and may partially obscure possible effects of the temperature rise in the nutrient mineralization. Additionally, the tidal flushing and renewal of sampled intertidal pools may have hampered the evaluation of cumulative effects derived from temperature rise. Intra-treatment variability was considerable in some cases, reflecting the inherent heterogeneity of the system, and may have masked possible differences between warmness treatments. The same rationale may justify the absence of coherent differences between the sandflat and *Zostera* sites, despite the different nutrient pools observed in the sediment. Overall, our findings suggest that the 1.5 - 2ºC temperature rise will not significantly affect the nutrient biogeochemistry in this mesotidal system, as well as it does not force a clear shift in the ecosystem process of bioturbation.

6.4 IS THE SYSTEM ABLE TO COPE WITH 2°C TEMPERATURE INCREASE?

Dramatic shifts in diversity and in the rate of processes and functions with environmental disturbance are more discernible when organisms are living in the edge of their vital range (Barnosky et al., 2012; Elliott et al., 2015). Most of the organisms found in this experiment are probably well adapted to mild changes in the temperature, as part of their tolerance/ability to cope with the highly dynamic nature of the estuarine system. Also, acclimation events may occur (Allen et al., 2005; Pörtner, 2010), allowing organism to better cope with a temperature increase. The fact that at T30 the macrobenthic diversity indices became more homogenous between treatments may be another indication of this acclimation process. In this study, site was more important than all others factors in the mediation of responses of the assessed biological and ecological components. An ecosystem relies on each of its components, either biological or environmental, as well as on its spatial heterogeneity to maintain its stability. Therefore, the diversity of habitats may enhance the overall resistance and resilience of an ecosystem (Ysebaert et al., 2003; Bulling et al., 2008; Barnosky et al., 2012). One key finding of the present experiment is that different habitats respond differently to similar disturbances and that the number of possible interactions occurring in natural systems (among organisms and/or between organisms and their environment) may conceal the effects of mild disturbances. Also, our results emphases the context dependency of the ecological responses to global changes. Nevertheless, taking into account all the measured components, we may conclude that this estuarine system is able to cope with the temperature increase that is preconized as a global goal by recent international agreements.

Additional information to this chapter in Appendix II, starting in page 189

7 ACKNOWLEDGEMENTS

The authors would like to thank P. Cardoso, S. Leston e M. D’Ambrosio who helped in field and laboratorial work. This research was supported by FCT (Portuguese Foundation for Science and Technology), through the grants attributed to D Crespo (SFRH/BD/80252/2011), M Dolbeth (SFRH/BPD/110441/2015), J P Coelho (SFRH/BPD/102870/2014), I Fernandes (SFRH/BPD/97656/2013) and BIOCHANGED project (PTDC/MAR/111901/2009), subsidized by the European Social Fund and MCTES (Portuguese Ministry of Science, Technology and Higher Education), through the POPH (Human Potential Operational Programme), QREN (National Strategic Reference Framework) and COMPETE (Programa Operacional Factores de Competitividade). Thanks are also due, for the financial support to CESAM (UID/AMB/50017/2013), to FCT/MEC through national funds, and the co-funding by the FEDER, within the PT2020 Partnership Agreement and Compete 2020.

General discussion

In previous chapters, several aspects on the effects of global anthropogenic changes on the functioning of estuarine ecosystems were discussed. Estuaries are complex habitats with several paths in fluxes of energy and matter and this is clearer as the complexity in the assembled experimental communities' increases (Loreau et al., 2001; Raffaelli, 2006; Godbold et al., 2011). The effects of an introduced non-native invasive species, as well as changes in environmental parameters that are proxies for global changes, such as temperature and salinity changes, and respective interactions, affect the natural processes and the overall functioning of fundamental ecological areas such as estuaries. The maintenance of the ecosystem's stability (Emmerson et al., 2005; Donohue et al., 2016) is challenged as we face disturbances that affect the biodiversity and ecosystem processes, as well as changes in biodiversity measurements (richness and evenness). The combined use of *in situ* and *ex situ* experiments allowed us to distinguish some of the consequences of anthropogenic global changes from the background natural variation in a temperate estuary.

It is undeniable that current global changes, including climate change and biodiversity alterations, are added stressors to already naturally under-pressure areas, such as estuaries (McLusky, 1993; Power et al., 2002; McLusky & Elliott, 2004; Pratt et al., 2014). Such areas have natural variations and their biota is adapted to deal with that variation (Bamber & Henderson, 1988; Attrill, 2002; Bilton et al., 2002; Whitfield et al., 2012; Bozinovic & Pörtner, 2015). However, when the environmental variation exceeds the system natural capacity to cope with such variation, the biological components start to alter their processes levels and consequentially the functional outcome of the system is modified.

The invasion by non-native species as ecological nuisance in estuaries

We have looked upon several characteristics of the freshwater invasive species *Corbicula fluminea* (Mülller, 1774), namely global distribution, invasion history and biogeographic tendencies, as well as its survival ability and effects in ecological processes and functioning in an estuarine context. The almost universal presence of the Asian clam *C. fluminea* across the planet is, at least apparently, refrain by biogeographic features, as altitude, low order streams and winter temperature at high latitudes. Biogeographic gradients are also influential in the clam's population traits. However, one of the most obvious remarks is that the presence of the species is clearly related to human activities (Blackburn et al., 2011; Früh et al., 2012; Simberloff et al., 2013; Sousa et al., 2014), as highly invaded water bodies are in areas with high human population density. The hypothesis that the invasion is dependent on human activities is also reinforced by the fact that in Europe the first invasions were almost simultaneous across all territory: the species has notorious dispersal abilities, but simultaneous invasions on such geographically distinct sites are only possible through human mediation. This is particularly evident in a species that, like any other bivalve, has reduced mobility (Dillon, 2004; Verdelhos et al., 2005) and which natural habitats are almost isolated units, as river basins are closely independent from each other. The occurrence of recent invasions in systems neighboring old invasions sites is an indication that *C. fluminea* is still advancing towards a wider global distribution, despite the high awareness and management actions directed to control the species invasiveness and distribution (Williams et al., 2001; DeKozlowski & Page, 2006; DAISIE, 2008; Wittmann et al., 2008; Clavero et al., 2012; Holm, 2012). The current geographic range of the species extends from 39º South to 53º North, it is now present in all continents except Antarctica and its worldwide expansion took less than one century. These facts should reinforce the apprehension on the species invasion potential, as climate change and thermal pollution may contribute to extending future potential invaded areas. For instance, as average winter temperature increases, the species' latitudinal limits may expand. The reports on current distribution of *C. fluminea* may have several justifications. The species could be biologically impaired to invade new habitats, either because the new habitats features are outside the vital range of the species, or the invaded areas have structured native communities outcompeting the invaders. Another reason is the relationship with human activities, responsible for the introduction of the species in the water bodies.

C. fluminea is a particularly threatening species for estuaries. Some of the identified geographical preferences of *C. fluminea* (temperate latitude and low altitude) are also present in the Mondego River water basin. Therefore, we may find *C. fluminea* in the Mondego estuary (Franco et al., 2012; Modesto et al., 2013), at least in the uppermost areas where the salinity is low enough to allow the survival and the completion of this freshwater species life cycle. Still, the species is able to tolerate some salinity and to also occur in more saline areas. The species population in the Mondego estuary had a defined structure in the oligohaline section of the estuary, where the species found a proper environment to flourish. These oligohaline areas of the estuary held an adult stock of *C. fluminea*, and were the main source for the downstream individuals. There was a gradient in the population structure along the estuary, with the size of the dominant class decreasing as the average salinity increases. Also, there was a shift in the different sizes' abundances for different seasons, explained by changes in the hydrological regime during the summer and winter. Despite displaying an incomplete size range in the mesohaline areas, the species showed the ability to survive for extended periods in those areas, where they were previously undetected. Salinity and eventually temperature impaired the full life cycle of the species and, therefore, the establishment of a structured population downstream. Nevertheless, our findings proved that even under those physicochemical conditions it was possible to maintain basic life functions and extended survival. The salinity variation associated with the tidal cycles created an opportunity for the species to feed during low tide, when the salinity was lower. In winter, the species had a negligible mortality, while summer mortality was higher. However, some individuals were able to survive during the summer test period, even in the downstream areas. This means that the species could develop further downstream, if changes in the estuarine conditions occur, such as those during an abnormal rainfall during summer or small changes in the physical structure of the estuary. This could allow refuge to the species and the survival during critical life stages and therefore a broader invasion could take place in the Mondego estuary. The ability of *C. fluminea* to survive in the estuarine system is, therefore, proven, not only in this dissertation but also in scientific literature (Phelps, 1994; Sousa et al., 2006; Rosa et al., 2012; Ilarri et al., 2014). Nevertheless, until now the vital limits of the species were mostly tested in laboratory essays (Foe & Knight, 1986; Matthews & McMahon, 1995; Vidal et al., 2002; Rosa et al., 2012; Xiao et al., 2014). The question about the survival of the species within a natural estuarine system, with all its environmental and biological variation, was seldom studied. Our research represents a new approach to the question and the used methodology could be applied in other systems in order to assess a better understanding on how local specificities influence the survival of the species, i.e., how much local systems are able to resist the invasion.

A successful invasion of a non-native species in an estuarine system depends on the species ability to survive in that particular system and appears to have direct and indirect effects on native biodiversity and ecosystem processes and functions (Hakenkamp et al., 2001; Sousa et al., 2008b; Ilarri et al., 2012, 2014). This raises three other questions: (1) which are those processes and functions, (2) how much are they mediated by a non-native species after a successful invasion and (3) how much of the ecosystem functioning is modified by the interaction of global climatic changes with the invasive species? Here, the answer to those questions was addressed under the premise that an estuarine gradient provides multiple access options for invading species, and that invasion in estuaries, in theory, could either have a freshwater or marine origin.

The detection of ecological effects due to the interaction between warming and invasion in an estuarine context is particularly challenging because most times species behavior is context dependent (Godbold & Solan, 2009) and because natural variation in those systems can conceal the consequences of those interactions (Pimm & Redfearn, 1988; Ariño & Pimm, 1995; Solan et al., 2006; Whitfield et al., 2012). This is especially true in a species that shows high phenotypic plasticity, such as *C. fluminea* (Sousa et al., 2007; Vohmann et al., 2010; Rosa et al., 2012, 2014). In this study, we also hypothesized that individuals with different sizes (a proxy for age) perform differentially under the effect of different temperature and salinity values, and mediate different levels of processes and functions. In fact, size was the most important factor in our laboratory analysis, which brought us the perception that not only biological richness or evenness may be important to determine ecosystem functioning (Hillebrand et al., 2008; Godbold et al., 2011; Thomsen et al., 2014), but that it is also fundamental to know population structure of a species in order to predict how much ecosystem stability can be affected by anthropogenic pressures.

One of our key findings was that smaller *C. fluminea* contribute the most to the overall bioturbation. We suggested some justifications for this fact: smaller individuals may face less mechanical resistance (de la Huz et al., 2002) and therefore the displacement through the sediment may require less energy than for other sizes. Simultaneously, the smaller individuals may be more susceptible to variation in the environmental conditions (Werner & Gilliam, 1984; Gardner et al., 2011; Godbold et al., 2011). Finally, the smaller *C. fluminea* have higher metabolic requirements (Xiao et al., 2014) and their feeding effort is probably higher, contributing to an increased particle mixing. Despite the importance of the smaller size to the overall bioturbation, we found hard to predict a relationship between bioturbation intensity and nutrient release to the water column based on size. The interactions between the different levels of temperature and salinity may act over meiofaunal and microbial communities, which in turn are responsible for influencing these ecological processes and functions and this cannot be ignored (Christensen et al., 2000; Giblin et al., 2010, 2013; Gilbertson et al., 2012; Stief, 2013; Piot et al., 2014).

Nevertheless, what seems relevant is that age structure is at least as important as species identity for the overall ecosystem functioning (Dossena et al., 2012). Also relevant is the fact that invasive species are able to occupy different niches and their contribution to ecosystem functioning reflect local conditions.

Effects of climatic changes on the overall ecosystem functioning

The present dissertation has looked upon two different parameters, temperature and salinity, whose variation is predicted under current and expected climate change models and will be reflected in climatic events with different time spans such as heat waves, increasing frequency of floods and droughts or gradual temperature rise (IPCC, 2014). Temperature increase is a direct reflection of global warming, while salinity changes may be consequence of changes in the precipitation regime. Both drivers were put at test, either simultaneously or individually and in scenarios of invaded and un-invaded communities.

Global warming is due to anthropogenic activities and its consequences are already felt across several levels of biological diversity (Grilo et al., 2011; Munari et al., 2011; Verbrugge et al., 2011; Rosa et al., 2012). Salinity was here introduced under two different scopes. First, the salinity changes mirror the environmental gradient of estuaries, which implies a spatial distribution of species along these areas. Second, the manipulation of salinity within the same spatial distribution could be tested to account the effects of hydrological alterations due to the increasing frequency of extreme weather events, expected due to global climatic changes (e.g. punctual high precipitation levels, prolonged droughts). Nevertheless, the consequences of both climate change pressures are context dependent (Byers, 2002; Godbold & Solan, 2013) and its effects on BEF should be accurately measured locally in order to be integrated under a larger scale assessment.

In the present dissertation, the contribution of the invasive species for the ecosystem functioning was modified by the interactive effects of both temperature and salinity. Still, temperature effects on bioturbation and nutrients release were apparently more important. Nevertheless, the smallest effect of salinity on the *C. fluminea* could have two conclusions: (1) changes in precipitation regime and water flow could be less influential for the *C. fluminea* performance and; (2) and the species could maintain similar levels of processes along a substantial part of the freshwater/marine gradient of the estuary. This last conclusion is also reinforced by the results of the survival experiment, which means that the species effects can manifest at larger scales and outside of its natural environmental boundaries, even if performing under suboptimal conditions. However, the effects of invasions are highly context dependent (Caldwell et al., 2007; Queirós et al., 2011; Strayer & Hillebrand, 2012) and, therefore, the detected interactions between size, temperature and salinity should not be ignored.

The concept that the effects of global changes are dependent of the ecological context was a unifying idea across the present work. However, context dependency was particularly noticeable when natural benthic communities were tested under a mild warming scenario. In our *in situ* experiment with the natural benthic communities of the Mondego estuary, different habitats - seagrass and bare sandflat - displayed different BEF expression under similar testing conditions. In a scenario of atmospheric temperature increase, the seagrass bed showed the capacity for some temperature buffering, which in turn reduced the effect of the warming in the benthic component, when compared with the bare sandflat. Therefore, the physical environment was providing some protection against external fluctuations. Shade, differential light absorption or water retention among different sites may justify the distinction in heat buffering between habitats.

The warmness had some influence in macrobenthic fauna richness and biomass and density levels, particularly in the sandflat area. Seagrass beds are known for higher biological richness (Pardal et al., 2004; Dolbeth et al., 2014), and generally higher functional redundancy (Dolbeth et al., 2013), which could mitigate the effects of the temperature treatment. However, differences per habitat were far more evident than the differences due to the temperature. The microbial component had a higher response to warmness among fungi and ciliates diversity components and among the bacterial communities structure. Again, the seagrass showed a smaller effect of the warmness, stressing the importance of this habitat for the overall stability of the system. There was a high level of context dependency, visible in the amount in multiple interactions that mediated fundamental processes (bioturbation) and functions (nutrient release), with high variability inter- and intra-treatments. As the response of the benthic communities was not that clear, it also reflected their consequent processes and functions.

Conclusions

The BEF debate aims at describing the role of biodiversity for the transformation and the movement of energy and matter within an ecosystem due to physical, chemical and biological processes that occur on that ecosystem. The functioning of an ecosystem depends on the combined effects of organisms' individual performances, which in turn are influenced by the interaction with their biotic and abiotic counterparts in the system. So, the maintenance of the ecosystem functioning depends not only on biodiversity but also on the number of healthy niches that can provide quality refugia to biota.

The use of proxies for the study of Biodiversity-Ecosystem Functioning in estuaries can be useful to assess the global health status of such systems. The subset of threats under test in the current dissertation showed that ecosystem stability is under stress due to the multiple effects of global changes. Introduction of non-native species could result in disproportionate effects across several biological levels. Temperature increase and changes in salinity regime due to abnormal precipitation events are also responsible for changes in the functioning. Those pressures interact together, increasing unpredictability of future outcomes in ecosystems and their functioning. To understand the principles that govern the shifts in biological relations among the species and between species and their habitat is fundamental to reduce such unpredictability, to better design management and mitigation plans for the above mentioned threats.

Biodiversity, associated processes and functions are changing due to human actions and, ultimately, the survival of the human species depends on how well we understand those changes and how much are we able to minimize the effects of those shifts. The present work is a small contribution to that effort. Nevertheless, some new insight on the consequences of global ecological changes in the ecosystem functioning of estuaries was achieved.

Further research

Despite current advances on the theme of Biodiversity-Ecosystem Functioning, there are several issues that need to be explored with more detail, towards a better understanding on the consequences of global anthropogenic changes and more efficient mitigation plans for the ongoing shifts. Every study field in Biology should contribute to this purpose, within its own expertise. During the elaboration of the present document, some parallel questions on the effects of global anthropogenic stressors in estuaries have emerged, which are summarized below. The tools used here for the assessment of the impact of anthropogenic stressors may prove to be useful in the pursuit of the answers to those questions.

Further sources of stress in estuaries

Beside temperature, changes in the water flow/precipitation regime and the effect of biological invasions, which were under scrutiny in this dissertation, there are several other sources of stress and pressures in estuarine systems. A very recent research area is the study of the effects of noise pollution and mechanical perturbation in benthic macrofauna (Nedelec et al., 2016; Roberts et al., 2016; Solan et al., 2016). The impacts of noise in vertebrates are already well scrutinized, but the assumption that benthic invertebrates are also affected by this anthropogenic stressor is a relatively recent concept, as well as the research in this field. Therefore there is a knowledge gap in the subject. The impact of noise and vibration for the functioning of benthic communities should be explored in detail, either alone or within a more complex framework, where interactions with climate variables could be tested against different assembled communities in laboratory. The use of tools such as f-SPI in the assessment of changes in bioturbation levels, may help to detect changes in ecosystem functioning among estuarine communities under the effects of noise pollution. A regional and national characterization of underwater noise pollution could provide an insight of the type and amount of noise that affects marine and estuarine fauna. This stimulus could be emulated in the laboratory and its effects in benthic organisms assessed.

The study of interaction between age structure and global changes

Size, as a proxy for age, and its differential contribution for the overall ecosystem functioning is one of the questions raised by this dissertation. Therefore, while species identity has been consistently tested before (e.g. Ieno et al., 2006; Hillebrand et al., 2008; Solan et al., 2008; Strong et al., 2015), size/age has received little attention. This dissertation had looked upon the contribution of different size classes of *C. fluminea* and found consistent differences related to size for the ecosystem functioning. A similar protocol could be replicated for other species, in order to understand how population structure in different species mediate ecological processes and if population structure could be more relevant than identity to the overall functioning in estuaries.

Invasions and estuaries

Along this dissertation it was stated that estuaries are particularly susceptible to biological invasions by non-native species. We have looked in detail upon the effects of one of those invaders, *C. fluminea*, with implications across the marine-freshwater continuum. Nevertheless estuarine systems are naturally prone to several invasion pressures either from freshwater and marine sources. It remains to answer if some of the findings here described are universally applicable, or if the effects of biological invasions are also dependent on the invader identity, besides local context. Therefore, some of the protocols used here for *C. fluminea* can be successfully adapted for other invasive species.

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Appendixes

Appendix I - Ecological consequences of invasion across the freshwater-marine transition in a warming world

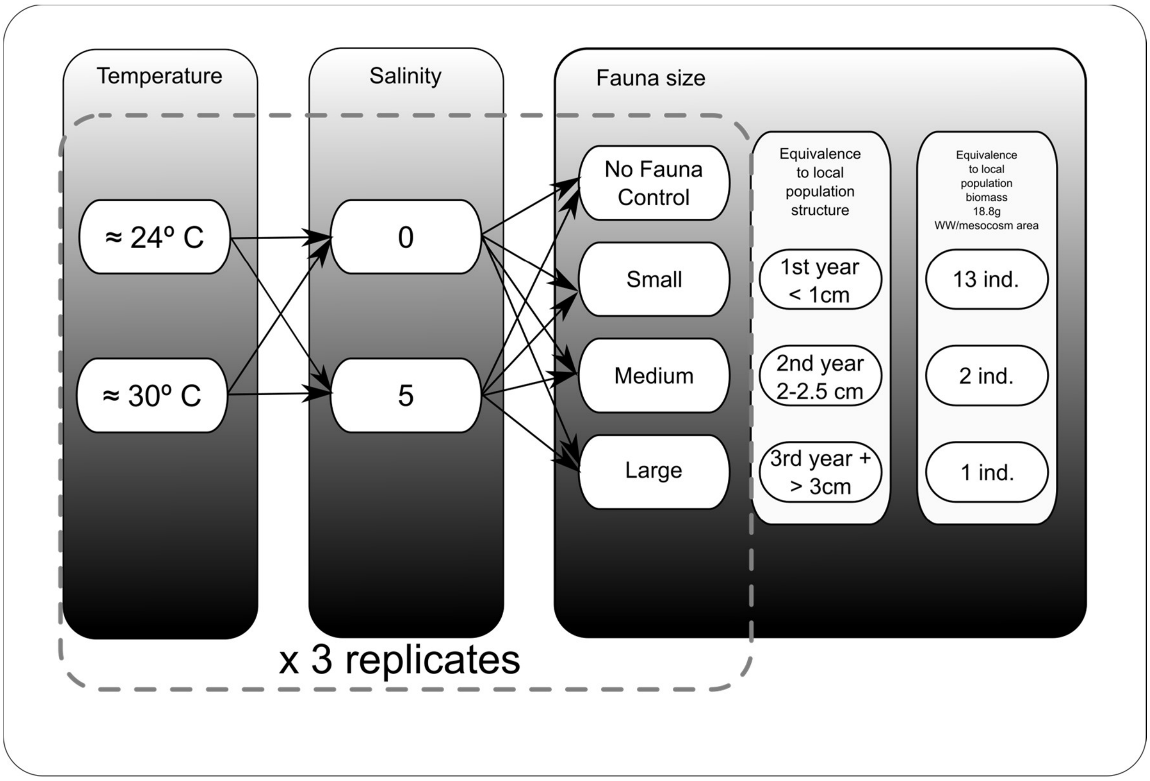

Fig. 1 Experimental design: the experiment included all possible permutations of three different size classes of *Corbicula fluminea* (small, medium and large sizes) exposed to two levels of salinity and two levels of temperature (WW: wet weight)

Table 1 Realised experimental conditions for all treatments

Factor levels	Salinity (mean ± standard deviation, PSU)	Temperature (mean ± standard deviation, ºC)	Dissolved oxygen (mean ± standard deviation, mg.L^{-1}; %)	pH
“Salinity 0”	0			8.9 ± 0.53
“Salinity 5”	4.4 ± 0.17			8.9 ± 0.53
“24ºC”		24.0 ± 1.6	8.0 ± 0.4; 95 ± 2.2	8.9 ± 0.53
“30ºC”		29.7 ± 1.2	6.4 ± 0.6; 85 ± 5.1	8.9 ± 0.53

Structure of the minimal adequate models, with body size, temperature and salinity as explanatory variables

Bioturbation

MODEL 1 – Surface Boundary Roughness (SBR)

SBR = f (size + temperature + size x temperature)

The model was a linear regression model with a GLS extension, with size as variance covariate.

Table 2 Minimal adequate model coefficient estimations for SBR

	Value	S.E.	t-value	p-value
Intercept ($size_1$;$temp_{24}$)	1.340	0.211	6.360	0.0000
$size_2$	-0.798	0.243	-3.292	0.0026
$size_3$	-0.496	0.306	-1.619	0.1163
$temp_{30}$	-0.113	0.298	-0.381	0.7062
$size_2$ x $temp_{30}$	0.840	0.339	2.475	0.0194
$size_3$ x $temp_{30}$	0.309	0.433	0.713	0.4816

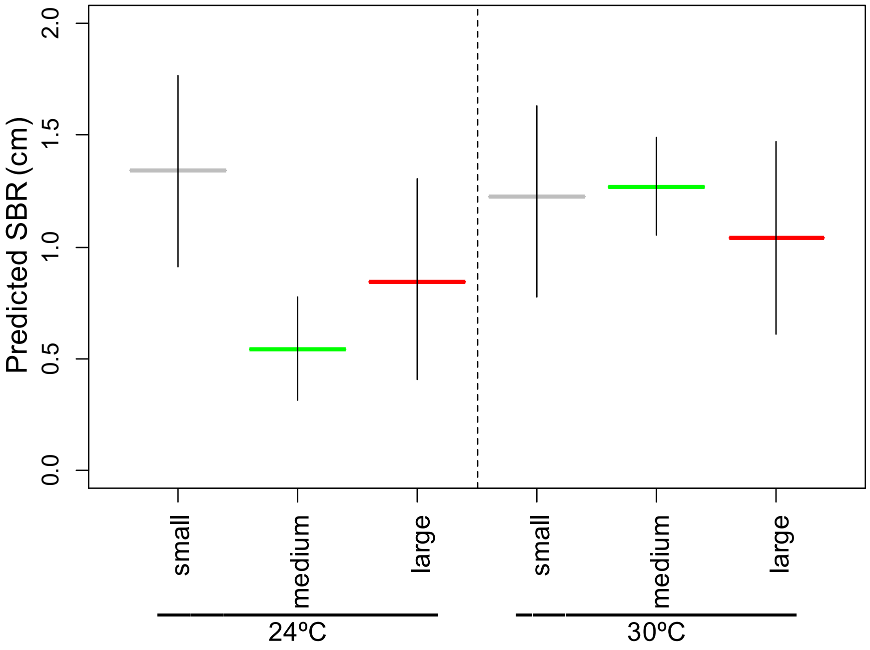

Fig. 2 Predictions of the minimal adequate regression model (GLS extension with size as variance covariate) for the Surface Boundary Roughness for varying clam size class at two levels of temperature: horizontal bars represent the predicted values from the minimal adequate regression model (n= 36) and the vertical lines represent the confidence intervals (± 95%)

MODEL 2 – Mean Luminophore Depth ($^{f\text{-}SPI}L_{mean}$)

$^{f\text{-}SPI}L_{mean}$ = f (size + salinity + temperature + size x salinity)

The model was a linear regression model with a GLS extension, with size x salinity x temperature as variance covariate.

Table 3 Minimal adequate model coefficient estimations for $^{f\text{-}SPI}L_{mean}$

	Value	S.E.	t-value	p-value
Intercept ($size_1$;$temp_{24}$;sal_0)	0.614	0.082	7.481	0.0000
$size_2$	-0.216	0.091	-2.386	0.0238
$size_3$	-0.146	0.086	-1.712	0.0975
sal_5	0.423	0.155	2.728	0.0107
$temp_{30}$	0.301	0.065	4.653	0.0001
$size_2$ x sal_5	-0.513	0.182	-2.817	0.0086
$size_3$ x sal_5	-0.720	0.188	-3.842	0.0006

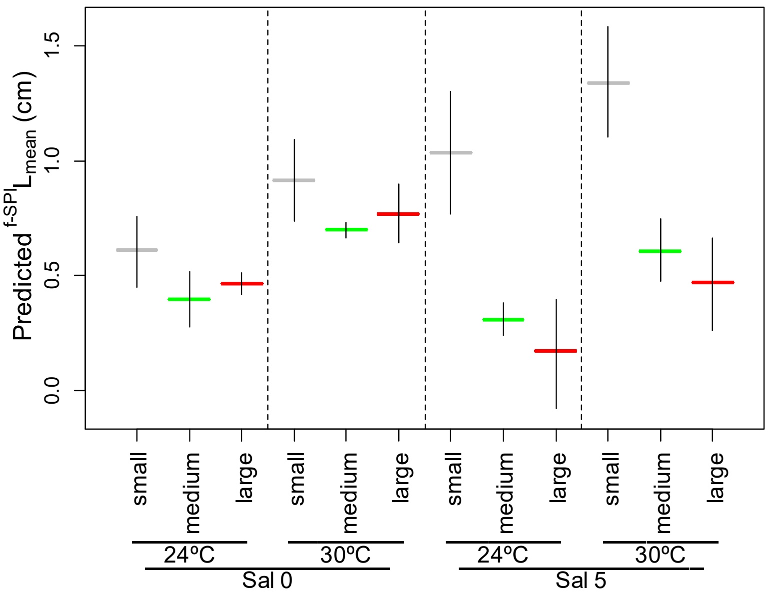

Fig. 3 Predictions of the minimal adequate regression model (GLS extension with size x salinity x temperature as variance covariate) for $^{f\text{-}SPI}L_{mean}$ for varying clam size class at two levels of salinity and temperature: horizontal bars represent the predicted values from the minimal adequate regression model (n= 36) and the vertical lines represent the confidence intervals (± 95%)

MODEL 3 – Median Luminophore Depth ($^{f\text{-}SPI}L_{median}$)

$^{f\text{-}SPI}L_{median}$ = f (size + temperature + size x temperature)

The model was a linear regression model with a GLS extension, with size x salinity as variance covariate.

Table 4 Minimal adequate model coefficient estimations for $^{f\text{-}SPI}L_{med}$

	Value	S.E.	t-value	p-value
Intercept ($size_1$;$temp_{24}$)	0.484	0.146	3.318	0.0024
$size_2$	-0.229	0.152	-1.509	0.1417
$size_3$	-0.020	0.153	-0.130	0.8974
$temp_{30}$	0.315	0.206	1.528	0.1369
$size_2$ x $temp_{30}$	-0.082	0.214	-0.382	0.7052
$size_3$ x $temp_{30}$	-0.370	0.216	-1.713	0.0970

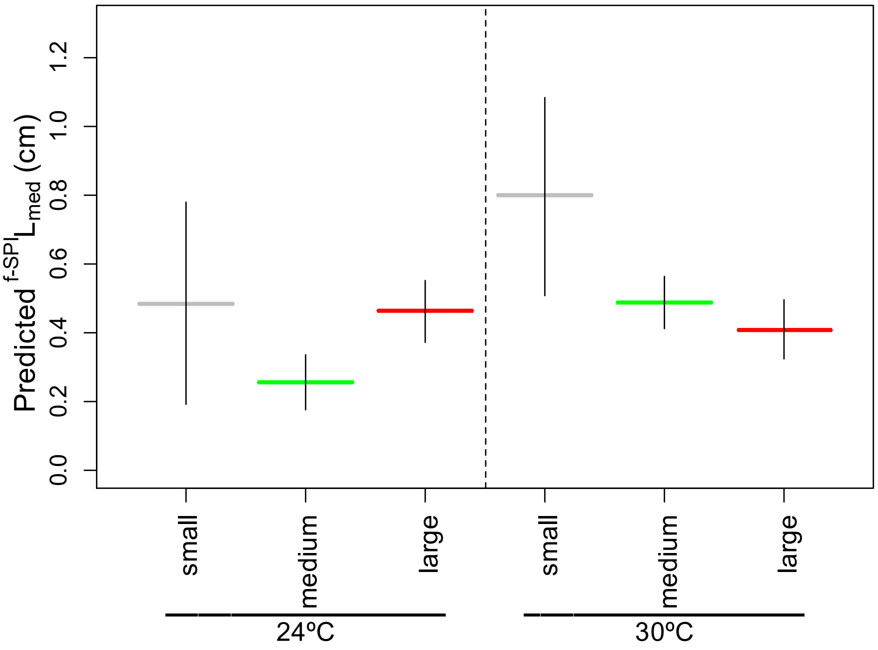

Fig. 4 Predictions of the minimal adequate regression model (GLS extension with size x salinity as variance covariate) for $^{f\text{-}SPI}L_{med}$ for varying clam size class at two levels of temperature: horizontal bars represent the predicted values from the minimal adequate regression model (n= 36) and the vertical lines represent the confidence intervals (± 95%)

MODEL 4 – Maximum Luminophore Depth ($^{f\text{-}SPI}L_{max}$)

$^{f\text{-}SPI}L_{max}$ = f (size)

The model was a linear regression model with a GLS extension, with size as variance covariate.

Table 5 Minimal adequate model coefficient estimations for $^{f\text{-}SPI}L_{max}$

	Value	S.E.	t-value	p-value
Intercept ($size_1$)	2.909	0.270	10.779	0.0000
$size_2$	-1.312	0.327	-4.012	0.0003
$size_3$	-1.055	0.504	-2.093	0.0441

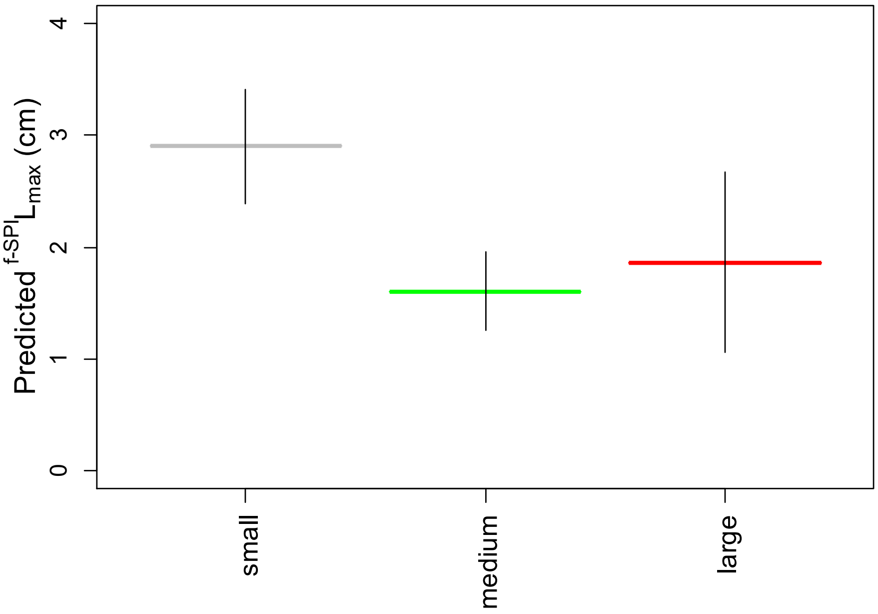

Fig. 5 Predictions of the minimal adequate regression model (GLS extension with size as variance covariate) for $^{f\text{-}SPI}L_{max}$ for varying clam size class: horizontal bars represent the predicted values from the minimal adequate regression model (n= 36) and the vertical lines represent the confidence intervals (± 95%)

Nutrients

MODEL 5 – NH_3-N concentration

NH_3-N concentration = f (size + salinity + temperature + size x salinity + size x temperature)

The model was a linear regression model with a GLS extension, with size x salinity as variance covariate.

Table 6 Minimal adequate model coefficient estimations for NH_3-N concentrations

	Value	S.E.	t-value	p-value
Intercept ($size_1$;$temp_{24}$;sal_0)	0.247	0.027	9.136	0.0000
$size_2$	0.293	0.259	1.129	0.2688
$size_3$	-0.126	0.036	-3.538	0.0015
sal_5	0.894	0.119	7.513	0.0000
$temp_{30}$	-0.009	0.038	-0.236	0.8156
$size_2$ x sal_5	-0.015	0.284	0.527	0.6023
$size_3$ x sal_5	-0.691	0.142	-4.867	0.0000
$size_2$ x $temp_{30}$	0.350	0.113	3.090	0.0046
$size_3$ x $temp_{30}$	0.062	0.050	1.236	0.2272

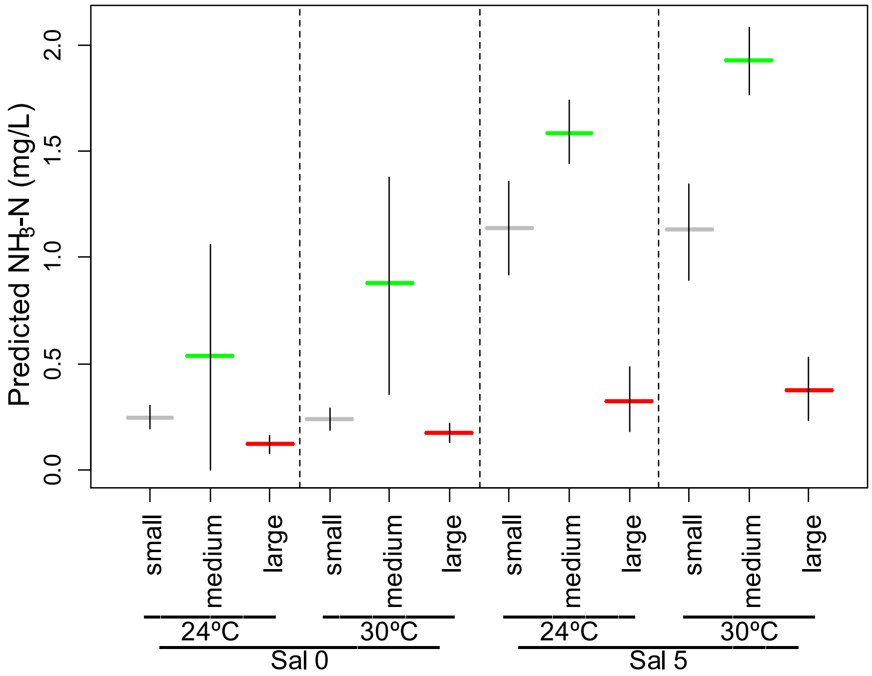

Fig. 6 Predictions of the minimal adequate regression model (GLS extension with size x salinity as variance covariate) for NH_3-N concentration for varying clam size class at two levels of salinity and temperature: horizontal bars represent the predicted values from the minimal adequate regression model (n= 36) and the vertical lines represent the confidence intervals (± 95%)

MODEL 6 – NO_3-N concentration

NO_3-N concentration = f (size + salinity + temperature + salinity x temperature)

The model was a linear regression model with a GLS extension, with size x salinity as variance covariate.

Table 7 Minimal adequate model coefficient estimations for NO_3-N concentrations

	Value	S.E.	t-value	p-value
Intercept ($size_1$;$temp_{24}$;sal_0)	0.356	0.035	10.157	0.0000
$size_2$	0.083	0.042	1.961	0.0592
$size_3$	-0.127	0.028	-4.582	0.0001
sal_5	0.080	0.034	2.342	0.0260
$temp_{30}$	0.012	0.032	0.376	0.7099
sal_5 x $temp_{30}$	-0.123	0.043	-2.858	0.0077

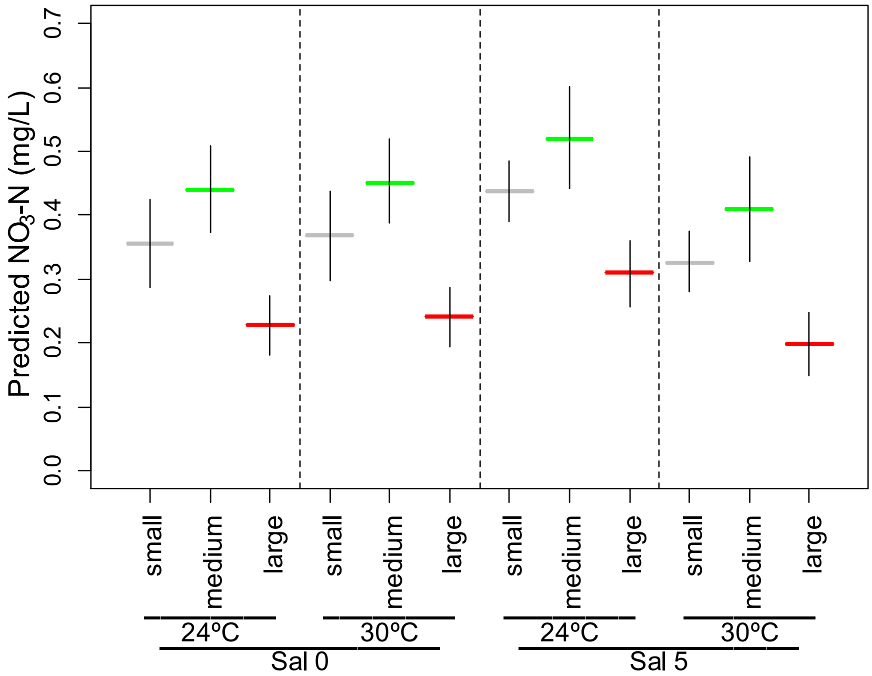

Fig. 7 Predictions of the minimal adequate regression model (GLS extension with size x salinity as variance covariate) for NO_3-N concentration for varying clam size class at two levels of salinity and temperature: horizontal bars represent the predicted values from the minimal adequate regression model (n= 36) and the vertical lines represent the confidence intervals (± 95%)

MODEL 7 - PO_4-P concentration

PO_4-P concentration = f (size + salinity + temperature + size x salinity + size x temperature)

The model was a linear regression model with a GLS extension, with salinity x temperature as variance covariate.

Table 8 Minimal adequate model coefficient estimations for PO_4-P concentrations

	Value	S.E.	t-value	p-value
Intercept ($size_1$;$temp_{24}$;sal_0)	0.412	0.013	32.902	0.0000
$size_2$	-0.123	0.018	-6.964	0.0000
$size_3$	-0.217	0.018	-12.241	0.0000
sal_5	-0.112	0.032	-3.496	0.0017
$temp_{30}$	-0.012	0.042	-0.296	0.7698
$size_2$ x sal_5	0.175	0.045	3.868	0.0006
$size_3$ x sal_5	0.101	0.045	2.230	0.0342
$size_2$ x $temp_{30}$	0.198	0.060	3.322	0.0026
$size_3$ x $temp_{30}$	0.030	0.060	0.501	0.6203

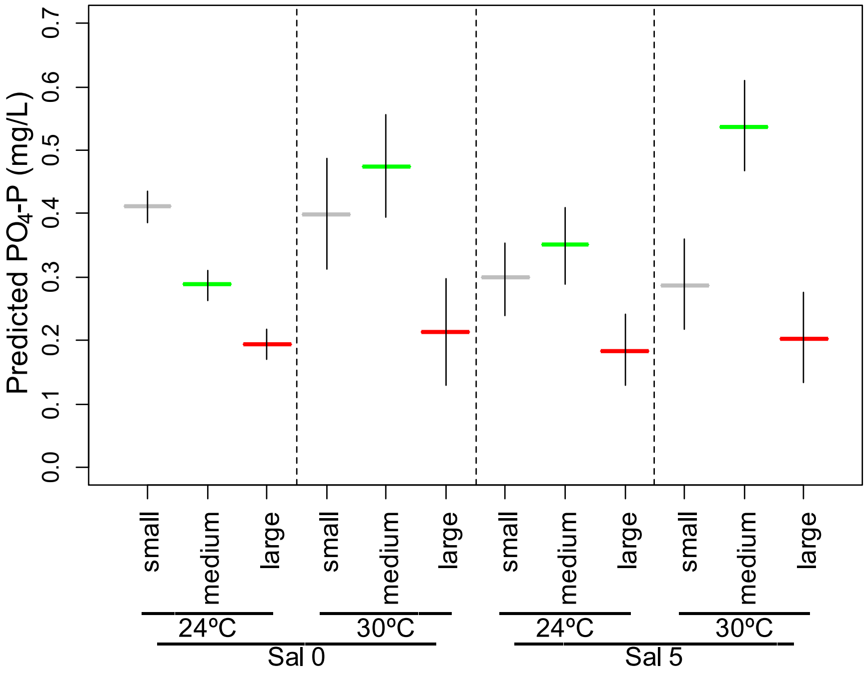

Fig. 8 Predictions of the minimal adequate regression model (GLS extension with size x temperature as variance covariate) for PO_4-P concentration for varying clam size class at two levels of salinity and temperature: horizontal bars represent the predicted values from the minimal adequate regression model (n= 36) and the vertical lines represent the confidence intervals (± 95%)

Appendix II - New climatic targets for climate change: does a 2°C temperature rise affect estuarine benthic communities?

Structure of the minimal adequate models for particle reworking, with site, treatment and time as explanatory variables

Table 1 Mean values and standard error for the particle reworking measurements, for significant levels of each factor on the minimal adequate models

variable			SBR		$^{f\text{-}SPI}L_{mean}$		$^{f\text{-}SPI}L_{median}$		$^{f\text{-}SPI}L_{max}$	
site	time	temperature treatment	Mean	± S.E.	Mean	± S.E.	Mean	± S.E.	Mean	± S.E.
sandflat	T10	no warm.	1.692	0.211	2.012	0.453	1.357	0.177	10.778	0.595
		warmn.	0.950	0.139	3.282	0.338			11.623	0.343
	T30	no warm.	0.857	0.061	1.983	0.283			11.714	0.247
		warmn.	1.548	0.471	2.039	0.272			11.530	0.325
Zostera	T10	no warm.	1.101	0.289	4.174	1.317	3.125	0.539	11.027	1.240
		warmn.	0.787	0.168	2.515	0.829			9.559	0.380
	T30	no warm.	1.518	0.296	3.543	0.526			10.778	0.551
		warmn.	1.109	0.180	4.303	0.769			11.210	0.242

MODEL 1 – Surface Boundary Roughness (SBR)

SBR = f (site x warmness treatment x time)

The model was a linear regression model with a GLS extension, with *time* as variance - covariate.

Table 2 Pairwise comparison matrix for significant differences among levels for Surface Boundary Roughness. Significant differences are highlighted

		time	T10				T30			
		site	sandflat		*Zostera*		sandflat		*Zostera*	
time	site	temperature treatment	no warm.	warm	no warm.	warm	no warm.	warm	no warm.	warm
T10	sandflat	no warm.		**.0072**	.1113	.3169	**.0009**	**.0135**	**.0133**	**.0448**
		warmn.			.3169	.4623	**.0135**	.2347	**.0448**	.6196
	Zostera	no warm.				.3580	**.0133**	**.0448**	.3235	.8451
		warmn.					**.0448**	.6196	.8451	.2035
T30	sandflat	no warm.						.1584	**.0390**	.0735
		warmn.							.1584	.3919
	Zostera	no warm.								.2497
		warmn.								

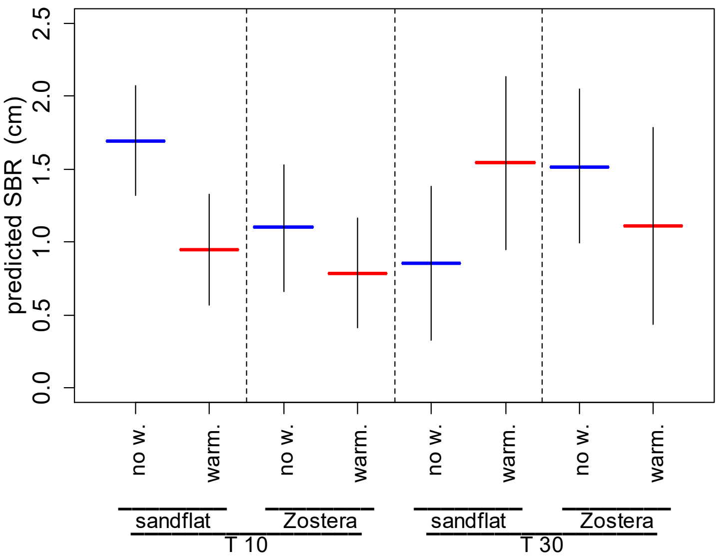

Fig. 1 Predictions of the minimal adequate regression model (GLS extension with time as variance – covariate) for the Surface Boundary Roughness for two different temperature treatments, in two different sites, at two sapling moments: horizontal bars represent the predicted values from the minimal adequate regression model (n= 32) and the vertical lines represent the confidence intervals (± 95%)

MODEL 2 – f-SPI Luminophore Mean Depth ($^{f\text{-}SPI}L_{mean}$)

$^{f\text{-}SPI}L_{mean}$ = f (site x treatment x time)

The model was a linear regression model with a GLS extension, with *site* as variance - covariate.

Table 3 Pairwise comparison matrix for significant differences among levels for f-SPI Luminophore Mean Depth. Significant differences are highlighted

		time	T10				T30			
		site	sandflat		*Zostera*		sandflat		*Zostera*	
time	site	temperature treatment	no warm.	warm	no warm.	warm	no warm.	warm	no warm.	warm
T10	sandflat	no warm.		**.0339**	.0804	.0761	.9532	.0921	.6556	.0626
		warmn.			.0761	.4702	.0921	**.0199**	.0626	**.0283**
	Zostera	no warm.				.272	.6556	.0626	.6168	.1743
		warmn.					.0626	**.0283**	.1743	.1489
T30	sandflat	no warm.						.8906	**.0175**	.4809
		warmn.							.4809	**.0072**
	Zostera	no warm.								.4060
		warmn.								

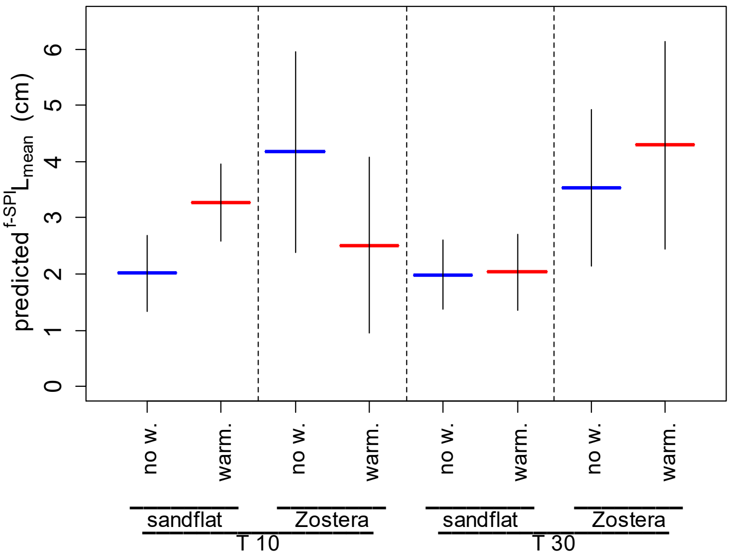

Fig. 2 Predictions of the minimal adequate regression model (GLS extension with site as variance – covariate) for the f-SPI Luminophore Mean Depth for two different temperature treatments, in two different sites, at two sapling moments: horizontal bars represent the predicted values from the minimal adequate regression model (n= 32) and the vertical lines represent the confidence intervals (± 95%)

MODEL 3 – f-SPI Luminophore Median Depth ($^{f\text{-}SPI}L_{med}$)

$^{f\text{-}SPI}L_{median}$ = f (site)

The model was a linear regression model with a GLS extension, with *site* as variance - covariate. Sandflat vs *Zostera* bed: p= 0.004.

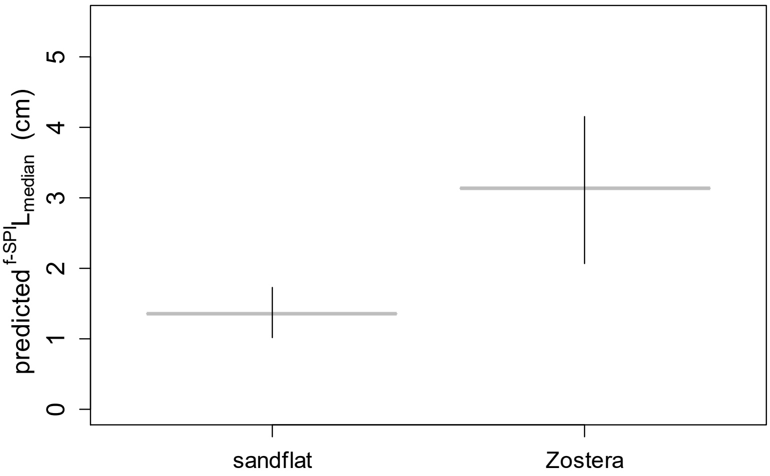

Fig. 3 Predictions of the minimal adequate regression model (GLS extension with site as variance – covariate) for the f-SPI Luminophore Median Depth for two different sites: horizontal bars represent the predicted values from the minimal adequate regression model (n= 32) and the vertical lines represent the confidence intervals (± 95%)

MODEL 4 – f-SPI Luminophore Max Depth ($^{f\text{-}SPI} L_{max}$)

$^{f\text{-}SPI} L_{max}$ = f (site x treatment x time)

The model was a linear regression model with a GLS extension, with *treatment* as variance - covariate.

Table 4 Pairwise comparison matrix for significant differences among levels for f-SPI Luminophore Max Depth. Significant differences are highlighted

time	site	temperature treatment	T10				T30			
			sandflat		*Zostera*		sandflat		*Zostera*	
			no warm.	warm	no warm.	warm	no warm.	warm	no warm.	warm
T10	sandflat	no warm.		.2455	.7991	**.0408**	.2808	.3044	.3540	.0525
		warmn.			**.0408**	**.0001**	.3044	.8517	.0525	**.0194**
	Zostera	no warm.				.0786	.3540	.0525	.7896	.0821
		warmn.					.0525	**.0194**	.0821	**.0027**
T30	sandflat	no warm.						.7876	.2531	.5251
		warmn.							.5251	.5479
	Zostera	no warm.								.5286
		warmn.								

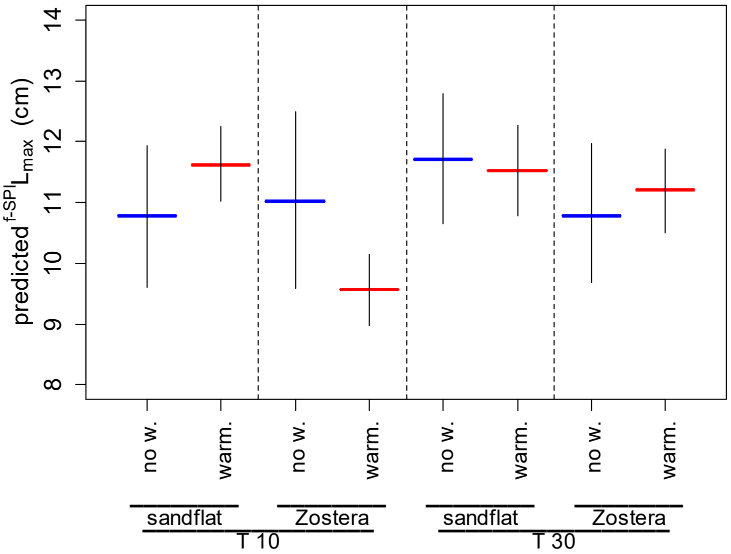

Fig. 4 Predictions of the minimal adequate regression model (GLS extension with treatment as variance – covariate) for the f-SPI Luminophore Maximum Depth for two different temperature treatments, in two different sites, at two sapling moments: horizontal bars represent the predicted values from the minimal adequate regression model (n= 32) and the vertical lines represent the confidence intervals