

Differences in the macrozoobenthic fauna colonising empty bivalve shells before and after invasion by *Corbicula fluminea*

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Abstract. Bivalve shells can potentially alter the structure of aquatic benthic communities. However, little is known about the effect that different shell morphologies have on their associated fauna. This study aimed to understand how empty shells, from four different freshwater bivalve species, affect macrozoobenthic communities, using the River Minho (Iberian Peninsula) as a study area. Three native (*Anodonta anatina*, *Potomida littoralis*, *Unio delphinus*) and one non-indigenous (*Corbicula fluminea*) species were used for this research. Comparisons among species and between scenarios (i.e. before and after invasion by *C. fluminea*) were performed. Our results suggest that macrozoobenthic community structure did not vary among treatments, with the exception of species richness, which was higher on shells of native species. Furthermore, little difference was detected when comparing scenarios with and without *C. fluminea* shells, despite dissimilarities in size and morphology between species. The empty shells of *C. fluminea* partially (in terms of density and biomass, but not in species richness) replaced the role of empty shells of native species as a physical substratum for the associated macrozoobenthic community.

Additional keywords: biodiversity, macrozoobenthos, non-indigenous species, River Minho, shell size.

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Introduction

Bivalves can significantly influence the community structure and ecosystem processes and functions (e.g. primary and secondary production and the nutrient cycle) of freshwater habitats (Vaughn and Hakenkamp 2001; Strayer 2009). This can result from several mechanisms, including filter feeding, bioturbation activities and shell production (Sousa *et al.* 2009, 2014). The changes mediated by the presence of a new substratum provided by their shells can lead to new interactions or process pathways even after the death of the bivalve (Karatayev *et al.* 2007; Sousa *et al.* 2009; Bódis *et al.* 2014). Bivalve shells influence the ecology of a variety of aquatic organisms, including sponges, cnidarians, ascidians, echinoderms, bryozoans, crustaceans, fish and many others (Nyman 1953; Corriero and Pronzato 1987; Buchman *et al.* 2007; Rabaoui *et al.* 2007). In most cases, bivalve shells are used to avoid predators and competitors and to decrease physical and physiological stress (Gutiérrez *et al.* 2003). The abundance, size and distribution range of bivalve shells confer on them a high ecological importance as hard substrata ready for colonisation (Palacios *et al.* 2000; Gutiérrez *et al.* 2003; Erwin 2008).

Biological invasions have become a common and serious concern, causing severe economic and ecological impacts, and posing serious threats to native communities (Crooks 2002; Vilà *et al.* 2010, Simberloff *et al.* 2013). Such invasions are considered one of the major threats to freshwater biodiversity (Dudgeon *et al.* 2006; Strayer and Dudgeon 2010; Strayer 2010). Particularly important may be species that become dominant or are able to introduce new ecological attributes (Gutiérrez *et al.* 2014), such as invasive bivalve species (reviewed in Sousa *et al.* 2014). The Asian clam *Corbicula fluminea* is a successful invader of fresh and brackish waters, and is consequently considered one of the most problematic invasive species in Europe (DAISIE, European Invasive Alien Species Gateway, see <http://www.europe-aliens.org/species/TheWorst.do>, accessed 4 January 2014). *C. fluminea* was first reported in Europe in the late 1970s and is now widespread throughout that continent, as well as in Africa, and North and South America (reviewed in Ilarri and Sousa 2012). This species is frequently described as a threat to several elements of ecosystems, including submerged vegetation, phytoplankton, zooplankton, macrozoobenthos and higher trophic levels,

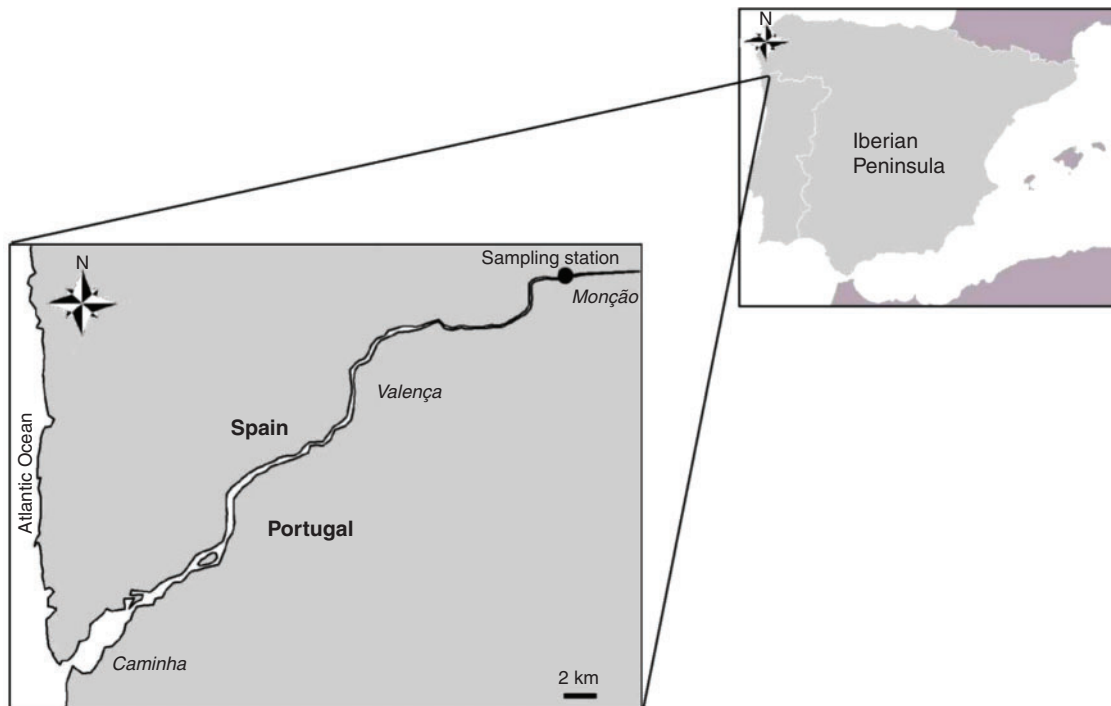


Fig. 1. Map of the study site showing where the treatments were located along the River Minho, in the north-west Iberian Peninsula.

leading to important economic losses (McMahon 2000, 2002; Sousa *et al.* 2008b, 2009).

Following the establishment of *C. fluminea* into the River Minho (in the north-west of the Iberian Peninsula) in 1989 (Araujo *et al.* 1993), the density of native bivalve species (e.g. *Anodonta anatina*, *Potomida littoralis*, *Unio delphinus* and *Pisidium amnicum*) has declined (Sousa *et al.* 2005, 2008b, 2008c, 2011). *C. fluminea* is now the dominant species in the final 70 km of the River Minho (Sousa *et al.* 2008b, 2008d, 2008e). In a recent study, Ilarri *et al.* (2012) suggested that the engineering activities by *C. fluminea* individuals significantly influenced the macrozoobenthic community with several species being more abundant in areas where the density of *C. fluminea* was also higher. In previous studies performed with other bivalve species, the same effect has been observed (Ricciardi *et al.* 1997; Spooner and Vaughn 2006; Borthagaray and Carranza 2007). Nonetheless, other studies performed with live *Corbicula* individuals in other freshwater systems have found no significant effects on the macrozoobenthic community (Hakenkamp *et al.* 2001; Karatayev *et al.* 2003; Werner and Rothhaupt 2007). However, to date very little is known about the associated fauna colonising empty bivalve shells in aquatic ecosystems. Moreover, little is known about the effects of shells with different morphologies and origins (e.g. invasive or native) on the macrozoobenthic community.

In this context, the aims of the present study are: (1) to examine whether different bivalve species, represented by their empty shells, support a distinct associated fauna; and (2) to compare the associated fauna of the most abundant native bivalve species before invasion by *C. fluminea* (through simulated scenarios) with the current associated fauna of the

bivalve species (predominantly dominated by the Asian clam *C. fluminea*).

Material and methods

Study area

The experiment was conducted in the River Minho (in the north-west of the Iberian Peninsula), ~40 km from the river mouth (Fig. 1). The headwaters of the River Minho are located at the Serra de Meira (Spain), with 95% of the Minho hydrological basin situated in Spain, with the remaining 5% in Portugal. The River Minho is 310 km long with a maximum width of 2 km near the mouth, flowing north-north-east to south-south-west into the Atlantic Ocean. The selected study area is very shallow (only 1 m deep during the summer) with permanent freshwater conditions. The substratum consists of gravel, sand, macrophytes and *C. fluminea* shells (live and empty). The water current varied from moderate to strong and these variations can be approximately correlated with the operations of a dam located 30 km upstream from the study site. However, during the study period the water current was always low. Over the 2 months of the experiment (June and July 2012), the mean water temperature ranged between 20.2 and 23.7°C, conductivity between 71 and 85 ms cm^{-1} , dissolved oxygen between 8.6 and 9.1 mg L^{-1} , pH between 7.6 and 7.8, and redox potential between 80.1 and 94.6 mV.

Experimental design and laboratory procedures

To study the effects of the bivalve shells on the associated macrozoobenthic community, empty shells of three native (*A. anatina*, *P. littoralis* and *U. delphinus*) and one non-native

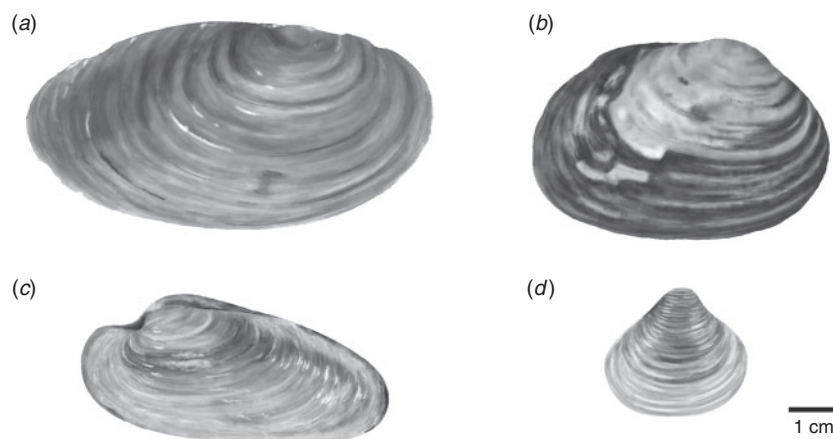


Fig. 2. Bivalve species used in the experiment: (a) *Anodonta anatina*, (b) *Potomida littoralis*, (c) *Unio delphinus*, and (d) *Corbicula fluminea*.

(*C. fluminea*) species were used (Fig. 2). The shells of the four bivalve species were manually cleaned in order to remove any traces of soft tissue, before placing the shells in individually sealed nylon net bags. As a standard we used only shells that were intact, usually belonging to an organism that recently died. Six different treatments were assigned: four treatments with shells of each individual species; one treatment with a mixture of native bivalve shells (one-third each of the three native species), simulating the River Minho scenario before the invasion by *C. fluminea* ('Mix natives'); and lastly one treatment with 90% of *C. fluminea* and 10% of native species evenly represented, simulating the scenario encountered on the River Minho now ('Mix actual'). The shell sizes used for the experiments represented sizes available in the field for each species. Furthermore, in order to avoid bias due to differences between species in the outer surface areas, an identical sum of the shells' outer surface area (1200 cm²) was used in all treatments. Bags with a mesh size of 10 mm were used in order to allow colonisation of the shells by macroinvertebrate species, while excluding larger animals. Six net bags (one per treatment) were tied with a string to a stake (a total of 10 stakes were used, $n = 10$ per treatment), and placed under water (~70 cm deep) ~10 m from the river bank (at the lowest water level). The bags remained under water for 2 months (June and July 2012), the time necessary to observe significant colonisation. At the end of the experiment, the macrozoobenthos associated with the empty shells were initially sieved using a 500- μ m mesh, and then sorted and fixed in 70% ethanol for later identification, using Tachet *et al.* (2003). Whenever species identification was not possible, specimens were identified to the lowest practical taxonomic level. After identification, all individuals were dried in an oven at 60°C for 24 h to determine their dry weight (DW).

Data analysis

Initially, a cluster analysis was performed to determine the similarities and relationships between treatments regarding the physical attributes of the shells (i.e. the size and the number of shells used in each treatment). For better data visualisation, the mean values of size and number of shells of each treatment were used in the resemblance matrix based on the Euclidean

distances. Subsequently, Permutational Multivariate Analysis of Variance (PERMANOVA) was performed to explore possible differences in the associated macrozoobenthic community. This method analyzes the variance of multivariate data explained by a set of explanatory factors, on the basis of any chosen measure of distance or dissimilarity, thereby allowing for a wide range of empirical data distributions (Anderson 2001). The overall associated macrozoobenthic density, biomass and the density of the most representative faunal groups (Platyhelminthes, Mollusca, Annelida, Crustacea and Insecta) were statistically tested using a one-way PERMANOVA (Type III), with treatment as a fixed factor (six levels: 'Anodonta', 'Potomida', 'Unio', 'Corbicula', 'Mix natives' and 'Mix actual'). Prior to the one-way PERMANOVA analyses, all variables were normalised without data transformation and a resemblance matrix based on the Euclidean distances was calculated. Species richness (S), the Shannon–Wiener index (H') and Pielou's evenness index (J') of the associated macrozoobenthic community were calculated using DIVERSE analysis. Afterward, one-way PERMANOVA (Type III) tests, using the resemblance matrix based on the normalised Euclidean distances, were used to test for differences in the ecological indexes among treatments. In all PERMANOVA tests, the statistical significance of variance ($\alpha = 0.05$) was tested using 9999 permutations of residuals within a reduced model. When the number of unique permutations was lower than 150, the Monte Carlo *P*-value was considered. One-way PERMANOVA pairwise comparisons were also performed for all PERMANOVA tests.

In order to assess the relative contributions of the size and number of shells to the associated macrozoobenthic community, a distance-based linear modelling (DistLM) was also carried out. DistLM makes it possible to test the significance ($\alpha = 0.05$) of explanatory variables for a multivariate response variable in the form of a resemblance matrix (Anderson *et al.* 2008). For the DistLM, we selected the AIC (Akaike Information Criterion) based on the analyses on the Euclidean distance resemblance after normalisation of the data.

The observed mean density and richness of the single treatments (i.e. the ones that contained only one species) were used to make the comparison of the expected mean density and

richness for the scenario treatments (i.e. before and after invasion by *C. fluminea*). The expected mean density and richness was always calculated taking into account the percentage of shells by species used in each scenario.

PRIMER analytical software (ver. 6.1.6, PRIMER-E Ltd, Plymouth, UK) with PERMANOVA+1.0.1 add-on (Anderson et al. 2008) was used for all statistical tests.

Results

Treatment characteristics: differences among species

The mean size and number of shells per bag varied among treatments (mean size: Pseudo- $F = 483.44$, $P < 0.05$; mean number: Pseudo- $F = 1946.80$, $P < 0.05$). Pairwise tests indicated that most treatments were significantly different, with respect to the mean number of shells per bag, whereas for mean size of shells per bag only the 'Potomida' v. 'Mix natives' and the 'Corbicula' v. 'Mix actual' were not significantly different.

The treatments that contained the largest shells were 'Anodonta' (80.7 ± 1.64 mm), followed by 'Potomida' (66.1 ± 0.92 mm), 'Unio' (58.7 ± 0.75 mm), 'Mix natives' (65.3 ± 1.08 mm), 'Corbicula' (29.2 ± 0.45 mm) and 'Mix actual' (28.3 ± 0.27 mm). The treatments that contained the highest number of shells per bag were those containing *C. fluminea*, namely 'Mix actual' (62.2 ± 0.57 shells per bag) and 'Corbicula' (58.6 ± 0.88 shells per bag), followed by 'Unio' (27.4 ± 0.34 shells per bag), 'Mix natives' (20.5 ± 0.31 shells per bag), 'Potomida' (17.1 ± 0.23 shells per bag) and 'Anodonta' (13.5 ± 0.27 shells per bag). Considering the mean size and number of shells per bag, the cluster analysis separated the treatments into two groups, one containing treatments with only native species and the other containing treatments with the invasive species *C. fluminea* (Fig. 3).

Macrozoobenthic community

A total of 35 macrozoobenthic taxa (14460 individuals), representing four phyla (Platyhelminthes, Mollusca, Annelida and Arthropoda) were associated with the empty bivalve shells. The two most diverse groups were the insects and molluscs, with 16 and 11 taxa respectively, accounting for 77.1% of the total number of taxa observed. The five most abundant taxa were the faucet snail *Bithynia tentaculata* (64.2%), the worms of the subclass Oligochaeta (16.2%), the Asian clam *C. fluminea* (6.7%), the waterlouse *Asellus aquaticus* (4.9%) and planarians of the family DugesIIDae (1.6%), whereas the remaining 30 taxa made up to only 6.5% (Tables 1, 2). The most abundant macrozoobenthic groups were the molluscs, annelids and crustaceans (Table 3), although no significant differences among the different treatments were observed (Table 3).

The highest values of density, biomass, species richness and diversity (Shannon–Wiener index) were observed in the treatments containing native species, with the exception of evenness (Pielou's index), where the highest value was observed for the treatment containing only *C. fluminea* shells (Fig. 4a–e). Nevertheless, significant differences in the ecological indices among treatments were recorded only for species richness (Pseudo- $F = 2.79$, $P < 0.05$) (Fig. 4b). Pairwise comparisons indicated that 'Anodonta' v. 'Corbicula' ($t = 2.26$, $P < 0.05$), 'Anodonta' v. 'Mix actual' ($t = 2.77$, $P < 0.05$) and 'Potomida' v. 'Mix actual' ($t = 2.24$, $P < 0.05$) were significantly different (Fig. 4b).

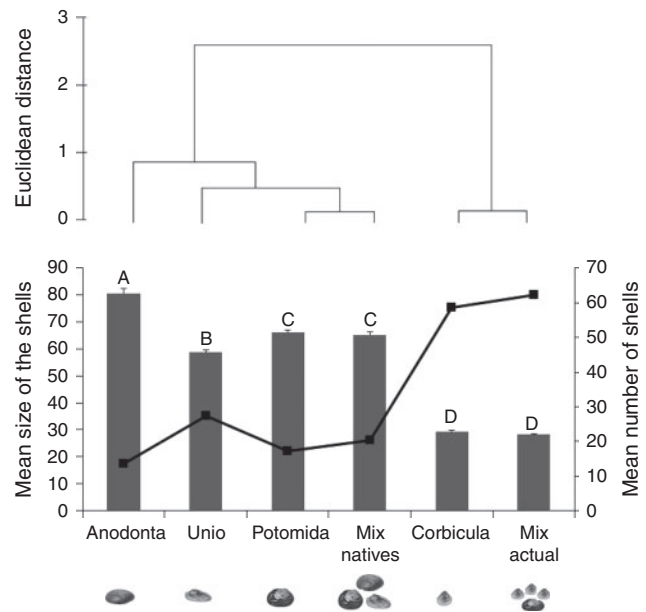


Fig. 3. Cluster with the mean (\pm s.e.) size of the shells (grey bars) and mean (\pm s.e.) number of shells (black line) used in each treatment. Different letters indicate significant differences between them (one-way PERMANOVA pairwise tests; $P < 0.05$).

The DistLM analysis indicated that the number and the size of shells significantly influenced the macrozoobenthic community (AIC = 83.29, SS-trace = 16.22, Pseudo- $F = 4.28$, $P < 0.05$); however, these two variables explained only 6.76% of the variance.

The observed mean density of macrozoobenthos between the two scenarios, before invasion by *C. fluminea* ('Mix natives') (2067.4 ± 473.0 individuals m^{-2}) and after invasion by *C. fluminea* ('Mix actual') (1533.3 ± 298.0 individuals m^{-2}), was higher than the expected mean density for each scenario ('Mix natives': 1947.8 ± 445.0 individuals m^{-2} ; 'Mix actual': 1398.5 ± 272.0 individuals m^{-2}). Furthermore, when considering the richness, the pattern was different, because the observed mean richness before invasion by *C. fluminea* (11.1 species) and after invasion by *C. fluminea* (8.8 species) was lower than the expected mean values ('Mix natives': 11.4 species; 'Mix actual': 9.7 species).

Discussion

Our results indicate that the associated macrozoobenthic community was even across treatments, and that only small differences were detected between the scenarios. The only exception was species richness, with a richer associated fauna observed for the treatments 'Anodonta', 'Potomida', 'Unio' and 'Mix natives' as compared with the treatments containing the invasive *C. fluminea* (i.e. 'Mix actual' and 'Corbicula'). It is possible that the observed differences were due to structural and morphological differences between native and non-native shells, which could have accounted for a slightly different colonisation. However, detailed studies regarding the influence of shell thickness, roughness and shape (e.g. structural morphology, edge morphology, adductor scar morphology) in attracting

Table 1. Mean (\pm s.e.) density (individuals m^{-2}) per treatment of the most representative macrozoobenthic-associated taxa

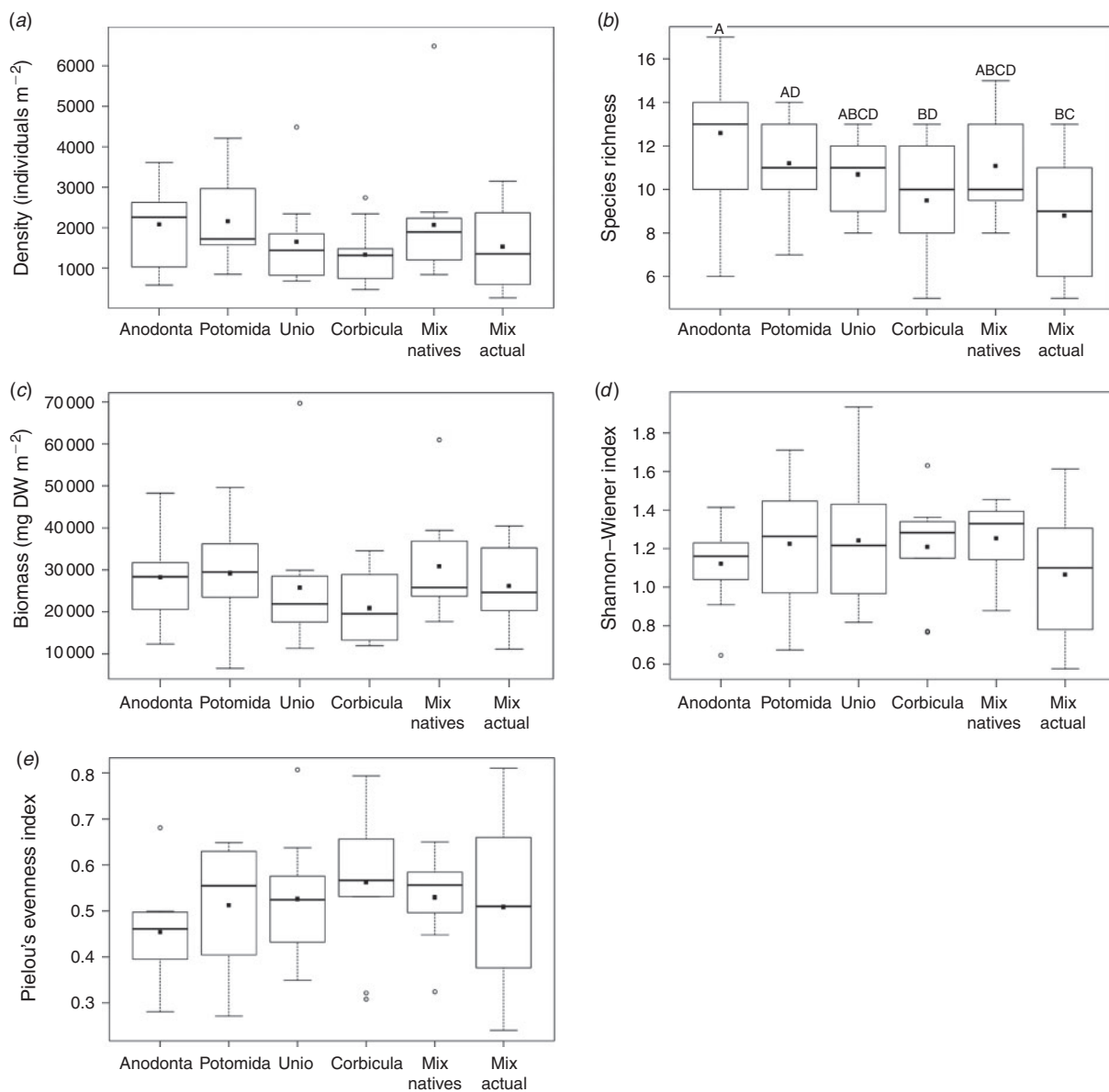
Taxonomic group	Treatment					
	'Anodonta'	'Potomida'	'Unio'	'Corbicula'	'Mix natives'	'Mix actual'
Platyhelminthes						
Planariidae	15.8 \pm 5.2	3.3 \pm 1.8	7.5 \pm 4.6	4.2 \pm 2.9	9.1 \pm 4.4	5.0 \pm 3.6
DugesIIDae	38.3 \pm 14.1	22.5 \pm 9.4	30.0 \pm 14.8	25.0 \pm 14.1	31.8 \pm 17.5	23.3 \pm 15.2
Mollusca						
<i>Bithynia tentaculata</i>	1398.3 \pm 251.7	1319.2 \pm 315.8	1100.1 \pm 313.3	777.5 \pm 135.0	1304.5 \pm 399.2	1059.2 \pm 267.5
<i>Corbicula fluminea</i>	85.8 \pm 16.8	158.3 \pm 36.8	100.8 \pm 11.9	131.7 \pm 23.0	128.0 \pm 19.5	120.0 \pm 18.7
<i>Potamopyrgus antipodarum</i>	10.8 \pm 2.8	22.5 \pm 9.2	11.7 \pm 5.4	15.0 \pm 6.9	25.0 \pm 8.2	13.3 \pm 5.9
<i>Valvata piscinalis</i>	6.7 \pm 3.3	20.0 \pm 8.3	10.8 \pm 2.2	3.3 \pm 2.6	9.1 \pm 2.7	9.2 \pm 4.9
Annelida						
Hirudinea						
Glossiphoniidae	33.3 \pm 7.3	29.2 \pm 6.8	38.3 \pm 8.2	18.3 \pm 3.2	32.6 \pm 7.6	18.3 \pm 7.1
Erpobdellidae	11.7 \pm 4.7	17.5 \pm 6.4	10.0 \pm 3.7	17.5 \pm 5.0	9.1 \pm 3.5	15.8 \pm 7.9
Oligochaeta	306.7 \pm 65.8	436.7 \pm 109.2	195.0 \pm 35.8	260.0 \pm 89.2	350.0 \pm 85.0	203.3 \pm 70.0
Arthropoda						
Crustacea						
<i>Asellus aquaticus</i>	112.5 \pm 26.7	83.3 \pm 24.2	109.2 \pm 36.7	60.0 \pm 25.8	122.0 \pm 47.5	40.0 \pm 15.0
Insecta						
Elmidae	10.0 \pm 5.7	18.3 \pm 10.0	10.0 \pm 7.3	5.8 \pm 2.8	7.6 \pm 2.8	5.0 \pm 2.2
Chironomidae	10.8 \pm 3.5	8.3 \pm 3.7	12.5 \pm 4.2	4.2 \pm 2.6	12.1 \pm 5.2	2.5 \pm 1.3
Sialidae	2.5 \pm 1.3	4.2 \pm 1.4	5.8 \pm 1.8	2.5 \pm 1.8	6.1 \pm 2.8	1.7 \pm 1.1
Limnephilidae	3.3 \pm 2.6	0.0 \pm 0.0	0.0 \pm 0.0	0.8 \pm 0.8	3.0 \pm 1.7	2.5 \pm 1.3
Hydropsychidae	1.7 \pm 1.1	0.0 \pm 0.0	1.7 \pm 1.7	3.3 \pm 1.4	1.5 \pm 1.0	0.0 \pm 0.0
Leptoceridae	5.8 \pm 4.2	5.8 \pm 1.8	3.3 \pm 1.3	3.3 \pm 1.4	5.3 \pm 2.3	5.0 \pm 5.0
Leuctridae	4.2 \pm 1.4	0.0 \pm 0.0	0.0 \pm 0.0	0.8 \pm 0.8	1.5 \pm 1.0	0.0 \pm 0.0

Table 2. Mean (\pm s.e.) biomass (mg DW m^{-2}) per treatment of the most representative macrozoobenthic-associated taxa

Taxonomic group	Treatment					
	'Anodonta'	'Potomida'	'Unio'	'Corbicula'	'Mix natives'	'Mix actual'
Platyhelminthes						
Planariidae	2.8 \pm 1.1	0.8 \pm 0.6	4.6 \pm 3.2	0.5 \pm 0.4	2.7 \pm 2.2	1.0 \pm 0.9
DugesIIDae	13.3 \pm 4.9	7.9 \pm 3.4	8.3 \pm 3.7	8.3 \pm 4.4	9.8 \pm 4.0	8.2 \pm 5.5
Mollusca						
<i>Bithynia tentaculata</i>	19166.0 \pm 2183.3	13334.3 \pm 3025.0	15838.8 \pm 4091.7	9776.2 \pm 1683.3	15671.2 \pm 3575.0	11033.3 \pm 2008.3
<i>Corbicula fluminea</i>	8699.0 \pm 1775.0	15590.9 \pm 3716.6	9578.3 \pm 1983.3	10794.2 \pm 2216.7	14477.0 \pm 2250.0	14660.9 \pm 3116.7
<i>Potamopyrgus antipodarum</i>	11.4 \pm 5.0	20.3 \pm 6.4	12.6 \pm 5.2	18.9 \pm 7.8	31.2 \pm 9.3	18.9 \pm 7.3
<i>Valvata piscinalis</i>	5.3 \pm 3.3	34.2 \pm 16.5	27.2 \pm 9.2	3.9 \pm 2.8	22.1 \pm 10.7	16.6 \pm 75.3
Annelida						
Hirudinea						
Glossiphoniidae	56.9 \pm 12.8	44.6 \pm 39.7	90.8 \pm 20.4	133.5 \pm 70.8	143.3 \pm 57.3	28.4 \pm 13.8
Erpobdellidae	26.5 \pm 11.8	44.5 \pm 18.8	50.9 \pm 16.7	56.9 \pm 17.7	22.6 \pm 9.5	48.3 \pm 27.4
Oligochaeta	60.0 \pm 13.5	67.5 \pm 16.8	25.9 \pm 3.8	50.3 \pm 14.0	51.1 \pm 13.1	247.3 \pm 195.8
Arthropoda						
Crustacea						
<i>Asellus aquaticus</i>	13.2 \pm 2.6	24.3 \pm 11.5	14.7 \pm 4.3	7.3 \pm 3.3	15.5 \pm 6.1	5.5 \pm 1.6
Insecta						
Elmidae	3.8 \pm 2.3	2.2 \pm 1.2	1.5 \pm 1.1	3.8 \pm 3.1	1.4 \pm 0.8	0.6 \pm 0.3
Chironomidae	5.8 \pm 2.9	3.1 \pm 2.0	1.3 \pm 0.4	0.5 \pm 0.3	1.8 \pm 1.2	2.9 \pm 2.5
Sialidae	8.0 \pm 4.6	12.7 \pm 4.6	20.2 \pm 9.2	9.8 \pm 6.9	22.2 \pm 9.4	7.0 \pm 4.7
Limnephilidae	41.8 \pm 40.8	0.0 \pm 0.0	0.0 \pm 0.0	11.1 \pm 11.1	57.4 \pm 32.8	22.0 \pm 11.4
Hydropsychidae	5.3 \pm 2.8	0.0 \pm 0.0	2.0 \pm 2.0	7.3 \pm 3.8	0.2 \pm 0.2	0.0 \pm 0.0
Leptoceridae	1.9 \pm 1.4	2.8 \pm 1.3	1.3 \pm 0.7	4.3 \pm 3.4	4.2 \pm 2.5	0.9 \pm 0.5
Leuctridae	4.7 \pm 3.8	0.0 \pm 0.0	0.0 \pm 0.0	0.2 \pm 0.2	2.7 \pm 2.3	0.0 \pm 0.0

Table 3. Mean (\pm s.e.) density (individuals m^{-2}) of the most representative macrozoobenthic-associated groups and PERMANOVA results

Taxonomic group	Treatment						PERMANOVA
	'Anodonta'	'Potomida'	'Unio'	'Corbicula'	'Mix natives'	'Mix actual'	
Total number of individuals	2083.3 \pm 307.0	2160.0 \pm 380.0	1659.2 \pm 359.0	1337.5 \pm 236.0	2067.4 \pm 473.0	1533.3 \pm 298.0	Pseudo- F = 0.93, P = 0.47
Platyhelminthes	54.2 \pm 18.6	25.8 \pm 10.9	37.5 \pm 19.2	29.2 \pm 15.0	40.9 \pm 20.2	28.3 \pm 17.8	Pseudo- F = 0.37, P = 0.88
Mollusca	1522.5 \pm 268.0	1526.7 \pm 307.0	1234.2 \pm 323.0	930.8 \pm 141.0	1472.7 \pm 398.0	1210.0 \pm 270.0	Pseudo- F = 0.62, P = 0.71
Annelida	351.7 \pm 72.6	483.3 \pm 112.0	243.3 \pm 40.3	295.8 \pm 87.2	391.7 \pm 86.2	237.5 \pm 80.3	Pseudo- F = 1.30, P = 0.27
Crustacea	112.5 \pm 26.7	83.3 \pm 24.0	109.2 \pm 36.9	60.0 \pm 25.8	122.0 \pm 47.2	40.0 \pm 15.4	Pseudo- F = 1.06, P = 0.40
Insecta	2930.6 \pm 605.0	4027.8 \pm 937.0	2027.8 \pm 336.0	2465.3 \pm 727.0	3263.9 \pm 719.0	1979.2 \pm 669.0	Pseudo- F = 1.62, P = 0.17

**Fig. 4.** Box plot of the (a) density (individuals m^{-2}), (b) species richness, (c) biomass (mg DW m^{-2}), (d) Shannon-Wiener index (H') and (e) Pielou's evenness index (J') of the macrozoobenthic community. The intervals represent the standard deviation, the black square symbol represents the mean and the black line represents the median. Different letters indicate significant differences among treatments (one-way PERMANOVA pairwise tests; $P < 0.05$).

fauna should be conducted in order to provide more comprehensive and definitive conclusions.

The four bivalve species used in this study presented several morphological differences, namely outer-shell surface roughness, shell hardness, thickness and three-dimensional shape. The *C. fluminea* shells are very different morphologically relative to the shells of the native species, given that the outer-shell of the Asian clams presents a rough sculpture with raised concentric ribs, in contrast to the rather smooth and somewhat flat surface of the shells of the native species. The hardnesses of the shells are also remarkably different, with *P. littoralis* presenting a robust, thick, hard shell, whereas *C. fluminea* had a slightly less hard, thinner and 3-D-complex shell. Moreover, the shells of *U. delphinus* and *A. anatina* are thin and somewhat delicate (particularly that of *A. anatina*). Furthermore, possible chemical differences between the shells are expected, and this aspect could also have influenced the associated fauna; however, future detailed studies should be performed considering the lack of knowledge on this topic. These morphological and chemical differences in the shells between species are likely to be of importance when attracting fauna, although our experimental design cannot give a definitive answer regarding this issue.

The average size of the particles in the substratum can also influence the structure of the macrozoobenthic community (Reice 1980; Erman and Erman 1984). Large shells, compared to small ones, are normally associated with a fauna of high density and richness (Beckett *et al.* 1996; Giacobbe 2002; Gutiérrez *et al.* 2003). Despite the mean shell size of native species being significantly larger than that of the non-native species, shell-size had only a marginal influence on the associated macrozoobenthos. As such, shell size should not be considered an important explanatory variable in this context, at least at our study site. Nevertheless, the diversity of structural elements in a habitat is important for the colonisation of the macrozoobenthic community (Bell *et al.* 1991). Diverse bivalve assemblages may support a denser and more diverse macrozoobenthic community than bivalve assemblages dominated by a single species or by a cluster of morphologically similar species. Indeed, heterogeneous and structurally complex habitats provide myriad microhabitats where associated fauna flourish (Allan 1975). Although distinct substratum preferences seem to be linked to different macrozoobenthic species (Allan 1975), this pattern was not recorded in the present study, with the associated fauna being evenly represented in all treatments. The only exception was species richness, which was significantly different. These results indicate that the empty shells of invasive species can partially play the same ecological function as the native species on the invaded ecosystem, as far as macrozoobenthos colonisation is concerned.

Interestingly, in this study a synergism between species could have occurred when they were available altogether, as this was observed when comparing the observed with the expected mean density values. The observed attracted much higher density values ('Mix natives': 25.8% higher; 'Mix actual': 28.2% higher) of the associated fauna than was expected. A different pattern was observed for the richness, with lower richness values ('Mix natives': 2.7% lower; 'Mix actual': 10.1% lower) observed when compared to what was expected. In this case, it is possible that the presence of *C. fluminea* could

have contributed to the lower values observed. Future detailed studies should be performed in order to better understand these results.

In this study we used empty shells. However, if we had experimented with live individuals the results could have been different. Previous studies performed with live *Corbicula* observed no effects on the benthic invertebrates (Hakenkamp and Palmer 1999; Hakenkamp *et al.* 2001; Karatayev *et al.* 2005; Werner and Rothhaupt 2007). Nonetheless, Zaiko *et al.* (2009) noted that the effect of live mussels on biodiversity was more pronounced than the effect of shell deposits. Spooner and Vaughn (2006) also observed an influence of live unionid mussels on the distribution and abundance of periphyton and invertebrates when compared with sham mussels (shells filled with sand), suggesting that the biological activities of bivalves offer ecological conditions to the benthic community beyond the physical habitat provided by the shells alone.

Given that empty shells of native and invasive species exerted a similar influence on the macrozoobenthic community, it seems reasonable to assume that *C. fluminea* shells can partially mimic the ecological role played by native bivalves, at least for the macrozoobenthic community. The high density of *C. fluminea* (represented by live individuals and empty shells) in the River Minho (see Sousa *et al.* 2008b, 2008d, 2008e) suggests that this species is probably influencing the whole system, including the macrozoobenthos that respond to the overall structural changes related mainly to the provision of a hard substrate (represented by the presence of their shells). These hard substrates can be used as a structural element for attachment, refuges to avoid predators, competitors, physical and physiological stress, and by affecting the transport of particles and solutes in the benthic environment, which may in turn influence different trophic levels (Vaughn *et al.* 2008; Ilarri *et al.* 2012, 2014). *C. fluminea* can supply prey for the higher trophic levels, and probably provide a different scenario to that observed when the native species occurred at higher densities. However, this discussion can only be speculative given the lack of detailed data regarding the macrozoobenthos before the invasion by *C. fluminea* in the River Minho, with further studies necessary in order to better understand exactly how *C. fluminea* influences different trophic levels in this system.

Recent, massive die-offs of freshwater bivalves have occurred in the River Minho owing to extreme climatic conditions (Sousa *et al.* 2008d, 2012; Ilarri *et al.* 2011), and according to the Intergovernmental Panel on Climate change (IPCC) these kinds of events will increase in frequency and intensity throughout Europe (IPCC 2007). Massive die-off events have resulted in a large accumulation of empty shells in some areas of the River Minho (Sousa *et al.* 2012). Following the die-off events, some species experienced a rapid population growth, whereas others were negatively affected (Sousa *et al.* 2008d). *C. fluminea* (e.g. dead and live) is capable of sustaining a high density of associated fauna (Ilarri *et al.* 2012, 2014 and present study). In fact, Ilarri *et al.* (2012) observed that the macrozoobenthic density, biomass and diversity positively responded to the increasing density of *C. fluminea*. Regardless of the influence of *C. fluminea* on some faunal groups (Ilarri *et al.* 2012), our results indicate that the composition of the macrozoobenthic community in the River Minho, taking into consideration only

the availability of empty shells provided, probably did not change due to the Asian clam invasion, and will likely remain partially similar if native bivalves become locally extinct. However, differences in the life cycle and density between *C. fluminea* and the native bivalves means that the absolute number of live and empty Asian clam shells on the river bed is largely higher than that of native shells, thereby covering a large area of the riverine soft bottom and providing a large amount of hard substrata ready for colonisation.

To date, few studies comparing the associated fauna of native and invasive bivalve species have been conducted (see Kochmann *et al.* 2008; Markert *et al.* 2010; Wilkie *et al.* 2012), with our study being the first to investigate the influence of empty freshwater bivalve shells. Our results were similar only to those of Wilkie *et al.* (2012), whereas other studies presented conflicting evidence. Kochmann *et al.* (2008) and Markert *et al.* (2010) observed differences in the macrofaunal community associated with the invasive species *Crassostrea gigas*, which has hard and extremely rough shells, as compared to the native species *Mytilus edulis* (which has smooth and unruffled shells) in the Wadden Sea (Germany). The results of Markert *et al.* (2010) show that the fauna associated with *C. gigas* had higher density, biomass, richness and diversity, whereas Kochmann *et al.* (2008) only observed shifts in the abundance of dominant associated species. Wilkie *et al.* (2012) suggested that the similarities between the associated fauna of two bivalve species (i.e. one invasive and the other native) are due to the fact that both species are morphologically similar ecosystem engineers, further suggesting that the degree of morphological similarity between the invasive and native species may be a good predictor of the possible impacts. However, in the present study the selected species (e.g. native and invasive species) have morphological differences in their structure, mainly due to the size and roughness, although the associated fauna was very similar.

Conclusions

The empty bivalve shells of the invasive *C. fluminea* seem to be functionally equivalent to the native species in the River Minho, with almost no distinction being recorded on the associated macrozoobenthos (with the exception that species richness was higher in the native species compared to the treatments containing the invasive *C. fluminea*). Furthermore, small differences were detected between the scenarios with respect to conditions before and after invasion. The empty shells of the invasive species *C. fluminea* have been capable of partially replacing the ecological functions of empty shells from native species, at least for associated macrozoobenthic community.

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