Integrated biomarker responses of the invasive species *Corbicula fluminea* in relation to environmental abiotic conditions: a potential indicator of the likelihood of clam's summer mortality syndrome

Cristiana Oliveira^{1,2*}, Pedro Vilares^{1,2}, Lúcia Guilhermino^{1,2}

¹ ICBAS – Institute of Biomedical Sciences of Abel Salazar, University of Porto, Department of Populations Studies, Laboratory of Ecotoxicology, Rua de Jorge Viterbo Ferreira 228, P 4050-313 Porto, Portugal

² CIIMAR/CIMAR - Interdisciplinary Centre of Marine and Environmental Research, Laboratory of Ecotoxicology and Ecology, University of Porto, Rua dos Bragas 289, P 4050-123 Porto, Portugal

Address correspondence to:

Cristiana Oliveira

CIIMAR/CIMAR - Interdisciplinary Centre of Marine and Environmental Research, University of Porto, Laboratory of Ecotoxicology and Ecology, Rua dos Bragas 289, 4050-123 Porto, Portugal

Telephone: +351223401828

*colivei@ciimar.up.pt

Abstract

The goal of the present study was to investigate the variation of a set of biomarkers in wild populations of *C. fluminea* in relation to abiotic conditions changes to identify environmental factors associated with increased stress in this species potentially leading to massive mortality events. The study was carried out from July to October in the freshwater tidal areas of the estuaries of Minho and Lima Rivers (NW Iberian Peninsula). Monthly, 7 biomarkers (biotransformation, energy production, anti-oxidant defences and lipid peroxidation damage) were determined in *C. fluminea* and 17 abiotic parameters were determined in water or sediments in 5 sampling sites: M1, M2 and M3 in the Minho river estuary (up => downstream); L in the Lima estuary. The results of biomarkers were integrated using the Integrated Biomarker Responses' Index (IBR), and also analysed in relation to environmental parameters by Redundancy Analysis (RDA). Overall, the findings of the present study indicate that July/August are particularly stressful months for the studied *C. fluminea* populations, especially at downstream sites; the increase of nutrients and ammonium water concentrations, water temperature and conductivity are major contributors for this increased stress; the biomarkers indicated that in July/August *C. fluminea* is exposed to oxidative stress inducers, environmental chemical contaminants biotransformed by esterases and glutathione S-transferase enzymes, and that organisms need additional energy to cope with the chemical and/or thermally-induced stress. The findings of the present study stress the importance of biomonitoring the health condition of *C. fluminea* because it may allow determining the likelihood of summer/post summer mortality syndrome in this species.

Key words: invasive species; biomarkers; bivalve massive mortality, summer, abiotic variation

1. Introduction

The summer/post-summer mortality syndrome in bivalves is a periodical event in temperate regions that may have significant adverse ecological and economical impacts (Ilarri et al., 2010; Mouthon and Daufresne, 2006; Tomaru et al., 2001; Vohmann et al., 2009; Weitere et al., 2009; Werner and Rothhaupt, 2008). In wild ecosystems, the sudden input of high amounts of organic matter from dead bivalves and its degradation often leads to considerable changes in nutrient cycles, energy fluxes, a deep reduction of water dissolved oxygen and massive mortalities of several other species, with important changes in ecosystem functioning and potential biodiversity losses (Baur and Schmidlin, 2007; Cooper et al., 2005; Sousa et al., 2007, 2008b,c). The dimension and severity of such events are especially high if the primary affected bivalve species is a keystone species of the ecosystem and/or if it has a considerable biomass (Power et al., 1996). Furthermore, in ecosystems invaded by non-native invasive species (NIS), massive mortality events may have a decisive influence on the competition between the bioinvasor and its native competitors, often acting in favour of the NIS. This is believed to occur because NIS generally recover faster from such adverse events than their native competitors (McMahon, 2002).

Several factors and conditions have been pointed as possible causes for the summer/post-summer mortality syndrome in bivalves, including extreme events such as draughts, temperature increase, decrease of oxygen concentration in the water, increase of pollution, post-spawning stress among several others (Cooper et al., 2005; Cotter et al., 2010; Dégremont et al., 2007; García-esquivel et al., 2001; Huvet et al., 2010; Ilarri et al., 2010; Morgan et al. 2003; Mouthon and Daufresne 2006; Tremblay et al., 1998; Urrutia et al., 1999; Vohmann et al., 2009; Weitere et al., 2009). However, the phenomenon is not yet completely understood (Rosa et al., 2011; Vohmann et al., 2009) and more research is needed.

The goal of the present study was to investigate the variation of a set of biomarkers in wild populations of *C. fluminea* in relation to abiotic conditions changes to identify environmental factors associated with increased stress in this species potentially leading to massive mortality events. This species was selected for this study mainly because it is one of the 100 worst invasive species in Europe (DAISIE, 2009), massive mortality events in their populations have been having considerable economic and ecological negative impacts (Cherry et al., 2005; Cooper et al., 2005; Ilarri et al., 2010; Sousa et al., 2008b, 2011), and these events have being pointed as decisive contributors to the decline of several native bivalve competitors .

88

89 **2. Material and Methods**

90 **2.1. Chemicals**

91 All the chemicals used were of analytical grade and purchased from Sigma-
92 Aldrich (Germany), Merck (Germany) or Bio-Rad (Germany).

93

94 **2.2. Sampling estuaries, sites and *C. fluminea* populations**

95 The populations of the tidal freshwater area (TFA) of two neighbour estuaries,
96 those of Minho and Lima Rivers (NW Iberian coast), were selected for this study
97 because summer massive mortality events have been reported for the former (Ilarri
98 et al., 2010; Sousa et al., 2007) but not for the latter. *C. fluminea* is present in the
99 Minho estuary at least since 1989 (Araújo et al., 1993). At the present, the population
100 is spread over all the TFA and has a very high density (more than 4000 individuals
101 per m² in some sites) and biomass (more than 400g of AFDW per m²) (Sousa et al.,
102 2005). In the TFA of the Minho estuary, 3 sampling sites were selected (Figure 1)
103 based on Sousa et al. (2008d) identification of three main areas with distinct
104 environmental characteristics (upstream to downstream): M1 (N42°03'09.37"
105 W8°33'42.73"), a relatively low impacted area with low organic matter (OM) and
106 environmental contamination, and residual tidal influence; M2 (N42°01'25.12"
107 W8°39'24.49"), located about 3 Km (downstream) far from the mouth of the Louro
108 River, a tributary of the Minho River that is one of the most contaminated rivers in
109 Galicia (Concha-Graña et al., 2006; Farkas et al., 2007; Filgueiras et al., 2004;
110 Lavilla et al., 2010), with a high content of OM; and M3 (N41°54'41.25"
111 W8°47'36.59"), with a greater tidal influence. *C. fluminea* was recorded for the first
112 time in the Lima estuary in 2002 and has a relatively low density in the TFA (mean of
113 60 individuals per m² and 26 g AFDW per m²) (Sousa et al., 2006 a,b). This estuary
114 has been considered more contaminated than the Minho estuary, with impacts from
115 a paper mill industry, urban settlement, agriculture crops, and harbour activities
116 (Cairrão et al., 2004; Guimarães et al., 2012; INAG, 2000; Reis et al., 2009; Sousa et
117 al., 2006a). Due to the low density of *C. fluminea* in the TFA, only one sampling site
118 was selected (Figure 1), hereafter indicated as L, in a restricted area about 15 km
119 upstream from the estuary mouth (N41°42'07.03" W8°44'37.05").

120

121 **2.3. *C. fluminea* sampling**

122 *C. fluminea* specimens were collected monthly from July to October 2011 in
123 the subtidal area (about 40 – 90 cm deep), at low tide using a hand rake with a net.
124 Clams were transported to the laboratory within 2h after their collection, being

maintained in water from the local where they were collected in thermally isolated boxes regularly open to allow water oxygenation. These conditions were found adequate for the transport of the clams and did not influence significantly the biomarkers' determinations in preliminary studies (data not shown).

2.4. Abiotic parameters determination

Monthly, at each sampling site and simultaneously to *C. fluminea* collection, water temperature, conductivity, dissolved oxygen and pH were measured in triplicate using a multiparameter probe (WTW 340i). In addition, water samples were collected, in triplicate, and maintained in appropriate bottles on ice until arrival to the laboratory where the following parameters were determined using colorimetric methods following the Photometer Systems for Water Analysis protocols (Palintest 7000 interface photometer): hardness (CaCO_3), turbidity (Turb) and the concentration of ammonium (NH_4^+), nitrates (NO_3^-), nitrites (NO_2^-), phosphates (PO_4^-), iron (Fe), phenol ($\text{C}_6\text{H}_5\text{OH}$), and silica (SiO_2).

Sediment samples were collected in triplicate with a 100 ml syringe to a 50 ml polyethylene centrifuge tube. Thirty ml of acetone (90%) and 0.2 ml of magnesium carbonate ($1 \text{ g } 100 \text{ ml}^{-1}$ ultra pure water) were added to each tube, and the mixture was shaken and maintained on ice in a thermally isolated box until arrival to the laboratory. Once in the laboratory, samples were kept at 4°C for 24 h. After 24 h, samples were centrifuged (in Kubota 5400 centrifuge) for 3 min at 2330 g, mixed and centrifuged again for 10 min in similar conditions; 3 ml of each sample supernatant were carefully collected, put in a glass spectrophotometer cuvette and its absorbance was read in a spectrophotometer (Jenway 6405 UV/VIS) at 480, 630, 645, 647 664, 665 and 750 nm; after addition of 0.2 ml of a HCl 1 N solution directly to the cuvette, agitation and 4 min waiting, the absorbance was read again at 665 and 750 nm; the concentration of chlorophyll *a*, chlorophyll *b* and chlorophyll *c* was calculated according to Jeffrey and Humphrey (1975).

The quantification of organic matter was obtained after combusting during 24h at 550°C in a muffle furnace (Fisher Scientific, Isotemp Muffle Furnace). Values are expressed in percentage relatively to the weight loss on ignition of each sample analysed.

2.5. Determination of biomarkers

In the laboratory, the anterior-posterior shell length of the clams was measured with a calliper (0-150 mm). Animals (20) with a shell length between 28 and 31 mm were selected for the study. Clams were open immediately after being

measured and gills and foot tissues were isolated on ice for biomarkers' determination.

The biomarkers included in this study were: esterase (EST) and glutathione-S transferase (GST) that are important enzymes involved in the biotransformation of several environmental contaminants; octopine dehydrogenase (ODH) and Isocitrate dehydrogenase (IDH) that are important enzymes of the anaerobic and aerobic pathways of energy production in molluscs; the anti-oxidant enzymes catalase (CAT) and glutathione reductase (GR) (GST is also involved in this system) and lipid peroxidation levels (LPO) indicative of oxidative stress and damage.

Foot tissue was used for the determination of EST, IDH and ODH activities; gill tissue was used for LPO levels, and GST, CAT and GR activity determinations. Biomarkers were determined individually per animal. A piece of muscle tissue was put in 1 ml of cold K-phosphate buffer (pH = 7.2; 0.1 M), homogenized for 1 min on ice (Ystral GmbH d-7801 Dottingen homogenizer) and centrifuged (in a Sigma, 3K30 centrifuge) at 3300 g for 3 min at 4°C; the supernatant was recovered and its protein content was determined by the Bradford method (Bradford, 1976) adapted to microplate (Frasco and Guilhermino, 2002), using bovine γ -globulin as protein standard and absorbance readings at 600 nm (in a Spectra Max M2^e spectrophotometer). After standardization of the protein content to 1 mg ml⁻¹, the supernatant was used to determine activity of EST activity following the production of thiocholine as acetylthiocholine is hydrolysed at 412 nm, by the Ellman's method (Ellman et al. 1961), adapted to microplate (Guilhermino et al., 1996). Because preliminary results on the characterization of the esterase enzymes present in *C. fluminea* foot tissue suggest that non-specific esterases may be responsible for a considerable degradation of the substrate acetylthiocholine used in the Ellman's technique (data not shown), EST activity will be used to indicate the overall esterase activity, responsible for acetylthiocholine degradation, present in this tissue.

Another piece of foot tissue was placed in 1 ml of K-phosphate buffer tris(hydroxymethyl)-aminomethan buffer (pH = 7.8; 0.5 M), homogenized for 1 min on ice (Ystral GmbH d-7801 Dottingen homogenizer) and centrifuged (in a Sigma, 3K30 centrifuge) at 3300 g for 3 min at 4 °C; the supernatant was recovered and used to determine IDH activity following the increase in NADPH at 340 nm (in a Spectra Max M2^e spectrophotometer), according to Ellis and Goldberg (1971) adapted to microplate (Lima et al., 2007), after standardization of the protein content of each sample to 1 mg ml⁻¹ as explained above.

The last piece of muscle tissue was placed in 1 ml tris buffer (pH = 7.5; 0.2 M), with 0.1 M ethylenediaminetetraacetic acid (EDTA) and 0.1 M DL-dithiothreitol

(DTT) homogenized for 5 s on ice (Ystral GmbH d-7801 Dottingen homogenizer) and centrifuged (in a Sigma, 3K30 centrifuge) at 3300 g for 3 min at 4 °C; the supernatant was recovered and used to determine ODH activity by the measure of the amount of pyruvate consumed due to NADH oxidation at 340 nm (in a Spectra Max M2^e spectrophotometer) according to Livingstone et al. (1990), after standardization of the protein content of each sample to 1 mg ml⁻¹ as explained above.

The gills were isolated on ice and homogenized (1:10 g wt v⁻¹) in K-phosphate buffer (pH = 7.4; 0.1M). Part of the homogenate was used to determine the amount of endogenous LPO (in a Spectra Max M2^e spectrophotometer) by measuring the thiobarbituric acid reactive substances at 535 nm, according to Ohkawa (1979) and Bird and Draper (1984), preventing lipid oxidation by adding 0.2 mM butylhydroxytoluene (BHT) (Torres et al., 2002). The remaining gills homogenate was centrifuged (in a Sigma, 3K30 centrifuge) for 20 min at 10000 g (4°C), to obtain post-mitochondrial supernatant used for the determination of GST, CAT and GR activities, after standardization of the protein content of each sample to 4 mg ml⁻¹ as explained above. GST activity was determined (in a Spectra Max M2^e spectrophotometer) measuring the conjugation of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm (Habig et al. 1974) adapted to microplate (Frasco and Guilhermino, 2002). CAT activity was determined by measuring the H₂O₂ consumption at 240nm (in a Spectra Max M2^e spectrophotometer) according to Clairborne (1985). GR activity was measured by following the decrease in NADPH levels, at 340 nm (in a Spectra Max M2^e spectrophotometer), according to Cribb et al. (1989).

The protein content of each sample used for enzymatic analysis was checked again at the end of enzymatic determinations in triplicate and the mean of these determinations was used to express the enzymatic activities per mg of protein.

All biochemical analyses were performed at a constant temperature of 25°C. All enzymatic activities expressed as nmol min⁻¹ mg⁻¹ protein, except catalase, which was expressed as µmol min⁻¹ mg⁻¹ protein.

2.6. Data analysis

2.6.1. Analysis of individual parameters

For each biological and abiotic parameter, measurements or determinations corresponding to different sites and months were checked for normality of distribution using the Shapiro-wilk test and for homogeneity of variance using the Levene's test. When required, appropriate data transformations were made to achieve the assumptions of The Analysis of Variance (ANOVA). Then, a two-way ANOVA (main

factors: site and month) with interaction was used to compare data of each parameter collected in different sites and months. The Statistics 18.0 package was used and the significance level was 0.05.

2.6.2. Integrated biomarker responses

The integration of the information given by the different biomarkers was performed applying the Integrated Biomarker Response index (IBR) described by Beliaeff and Burgeot (2002). Briefly, for each parameter, the mean and standard deviation (SD) per site and month were calculated; then, a standardization of mean values was made using the formula $y=(x-m)/SD$, where y is the standardized value of the biomarker for each sampling site and month; x is the mean value of the biomarker per month at each sampling site; m is the mean of the biomarker calculated for all the sampling sites and months, and SD is the standard deviation calculated per month and sampling site; using standardized data, Z is computed as equal to y or $-y$ depending if the biological effect of the correspondent biomarker is activation or inhibition, respectively. The minimum value for each biomarker (min) was determined and its absolute value was added to Z values. Finally, the index calculation is based in the triangular areas of star plots. The area (A) connecting two consecutive coordinates was calculated for each biomarker result in star plots, being B_i and B_{i+1} two consecutive biomarker scores and n the number of biomarkers considered:

Where:

$$A = \frac{B_i}{2} \sin \beta (B_i \cos \beta + B_{i+1} \sin \beta)$$

$$\beta = \arctan \left(\frac{B_{i+1} \sin \alpha}{B_i - B_{i+1} \cos \alpha} \right) \text{ and } \alpha = \frac{2\pi}{n}$$

The IBR was then calculated through the sum of the star plots triangular areas represented by two consecutive biomarker scores for a given sampling site and month. Biomarkers used for the IBR calculation were ranged clockwise as follows: LPO, GST, CAT, GR, ODH, IDH and EST. The higher the IBR value more stressed is the organism (Beliaeff and Burgeot, 2002).

2.6.3. Relating biological and environmental parameters

The integrated analysis of biological and environmental parameters data was carried out through a Redundancy Analysis (RDA), using model-based type of Monte

Carlo permutation test (ter Braak and Prentice 1986; ter Braak and Šmilauer, 2002). Quantitative environmental variables were: water temperature, dissolved oxygen, conductivity, pH, hardness, turbidity, ammonium, nitrates, nitrites, phosphates, iron, phenol and silica; and sediment chlorophyll *a*, *b* and *c* and organic matter. Multivariate analyses were performed with Canoco for Windows 4.5.

3. Results

3.1. Environmental parameters

The environmental parameters measured monthly in the water and sediments of the 4 sites are indicated in Tables 1 and 2, respectively. The comparison of the mean values (Table 3) indicated: significant differences among sites in all the parameters except in water phenol and turbidity, and in chlorophyll *b* and *c* in sediments; significant differences among months were also found in all the parameters, except turbidity; the interaction between the two factors (site and month) was also significant for all the parameters except water phenol and sediment chlorophylls *a* and *c*. In the Minho estuary (Table 3), the mean of water temperature increases from upstream (M1) to downstream (M3), and L had a mean of water temperature significantly different from all the other sites being in about the middle range of water temperatures recorded. The mean of water dissolved oxygen; pH, nitrates and silica were significantly higher in M1 than in all the other sites. The highest mean values of water conductivity and hardness, and chlorophyll *a* in sediments were recorded in M3 and L. The mean of nitrites in the water was significantly higher in all the Minho estuary sites than in Lima estuary site. The highest mean concentration of ammonium in the water was found in M2, whereas the highest mean concentrations of phosphate and iron in the water were found in M3, and the highest means of OM in sediments were recorded in M2 and M3. The highest mean of water temperature was recorded in August and the lowest in October, while the corresponding values for water dissolved oxygen were found in July and August/September, respectively. Regarding the nutrients, the highest mean of nitrates, nitrites and ammonium were found in July, whereas the highest mean of phosphates was observed in August. The mean of sediment OM increased from July to August and remained high in September and October. The lowest means of sediment chlorophylls *a*, *b* and *c* were found in July and the highest ones in September and/or October. September and October showed the highest means of water conductivity and silica. The highest mean of water pH was found in October and the lowest in July. The highest values of water iron, phenol and hardness were found in July/September, September and August/September, respectively.

3.2. *C. fluminea* biomarkers variation

Biomarker responses are given in Figure 2. Significant differences among the means calculated for different months and sites were found for all the parameters, except in GST and EST activities among sites (Table 4); a significant interaction between months and sites was also found, except in the CAT activity (Table 4). Comparing the mean of biomarkers among sampling months, the highest LPO levels were found in August/September and the lowest in July/October. The highest mean of CAT, IDH, ODH and EST activities were determined in July, and the lowest in October, August, September/October and August/September, respectively. The highest GR activity was found in August and the lowest in July. Clams from M3 had the highest means of LPO levels and ODH activity, while those from M2 had the highest GR activity. The Lima estuary site had the lowest levels of LPO, CAT and GR, and the highest IDH activity.

The results of the integrated analysis of biomarkers through the IBR index are indicated in Table 5. For all the sites, the highest values of IBR were found in July/August with, in general, higher contributions of ODH and IDH (Figure 3), whereas the lowest ones were calculated for October. In the Minho estuary, the increasing order of IBR is $M1 < M2 < M3$, except in July where the lowest value was determined for M2. Clams from L, in the Lima estuary, had the highest IBR values in all months (contributions mainly from IDH and ODH), except in August where the highest value was found in M3 (higher contributions of EST, ODH and GR) (Figure 3).

3.2. Integrated data analysis of biological and abiotic parameters

10 variables (hardness, turbidity, phosphates, iron, phenol, silica, chlorophylls *a*, *b* and *c* and organic matter) had to be removed from the final analysis because they were strongly correlated with one or more of the remaining environmental variables, producing collinearity problems. Therefore these variables were causal variables explaining the biological responses variation weakly than the other abiotic variables included in the final RDA (Nally, 2000).

The results of RDA are indicated in Figure 4. The first two axes explain 93.4% of the total variance (F-ratio = 1666.665, $p = 0.0220$, Monte Carlo permutation) with the first canonical axis (horizontal) explaining 84% of the relation between biological and environmental parameters while the second canonical axis (vertical) explains 10.5%. Regarding sites, the first axis separates M1 from M2, M3 and L; M1 and M2 are further separated by the second axis from M3 and L. M2 is associated with high

levels of GR activity and LPO levels, pH values and phenol concentration. Increased ODH activity, conductivity and temperature are associated to M3 and L. Concerning months, July and September are separated from August and October by the first axis; the second axis further separates July from September and August from October. Higher levels of nitrites, nitrates, ammonium, and CAT, EST and IDH activities are positively associated to July. High GST and GR activities, temperature and pH are associated to August. The same parameters except temperature are also associated to October.

4. Discussion

4.1. Abiotic parameters variation along sites and time

The results of abiotic parameters indicate important spatial and temporal variability in environmental conditions (Table 1). The high levels of nitrites and nitrates found in July in M1 (Table 1) reflect an environmental condition previously associated with summer massive mortality of *C. fluminea* in this area (Sousa et al., 2007). Nevertheless, M1 is located in a sort of 'bay' relatively to the main river flow and in the vicinity of the mouth of a Minho River tributary (PBH 2001) that crosses agriculture fields. Due to these conditions and the accumulation of sediments, part of the tributary discharge stays in the 'bay' instead of entering directly into the main river flow. Aquatic plants and green algae are very abundant and eutrophication conditions regularly occur which may be adverse for bivalve's physiological status (Farcy et al., 2013). The analysis of the abiotic parameters considering the overall means per site (Table 3) reveals an increasing gradient (upstream to downstream) of temperature, hardness, and conductivity in the Minho River. Furthermore, downstream sites (M2 and M3) had higher levels of OM and lower levels of DO than those recorded in M1; M3 had the highest phosphate concentrations, and M2 the highest ammonium concentrations. As a whole, these results suggest that in downstream sites, especially in M3, *C. fluminea* is exposed to more stressful conditions than in M1.

The highest means of water conductivity observed at M3, the most downstream site of the Minho River, and at L, in the Lima estuary (Table 3) associated to their location relatively to the mouth of Minho and Lima rivers suggest that these sites are under a higher tidal influence than M1 and M2, in good agreement with findings of previous studies (Ilarri et al., 2012; Sousa et al., 2008a,d).

Considering now the comparison of means per month (Table 3), the highest mean of water temperature recorded in August combined with a relatively low water DO should be highlighted since the combination of these two factors is particularly

stressful to *C. fluminea* and bivalves in general (Cherry et al., 2005; Cooper et al., 2005; Johnson and McMahon, 1998; Vohmann et al., 2009; Weitere et al., 2009; Werner and Rothhaupt, 2008). August had also the highest phosphates mean (Table 3), and these water contaminants were especially high in M3 at this time (Table 1). Concentrations of phosphates in the water above 0.25 mg l⁻¹ were associated with zebra mussel extinction (Karatayev et al., 2003).

4.2. *C. fluminea* health status variation

The highest IBR values were found in L in July (10.57) and in M3 in August (11.95), respectively. At this time, in L, the highest water concentrations of nitrates (1.84 mg l⁻¹) and ammonia (0.31 mg l⁻¹), and turbidity (6.67) were recorded (Table 1) whereas at M3, the highest water temperature (25.87 °C), phosphate concentration (0.60 mg l⁻¹) and turbidity (12.67) were recorded (Table 1). These findings suggest that increased water nutrients, ammonia, turbidity and/or temperature contributed to the high levels of stress found in *C. fluminea* collected in July (L) and August (L and M3). The highest IBR values calculated for M1 (6.21) and M2 (5.33) were observed in July and August, respectively, with a similar IBR value in M3 in July. In July, high water concentrations of nitrates and nitrites were recorded in all these sites, whereas the water temperature and phosphates were particularly high in August in M2. This finding also suggests, and supports the previous findings that increased water nutrients; ammonium, temperature and/or turbidity are important factors in the summer-induced stress in *C. fluminea*, likely acting in combination. This is in good agreement with the outcomes and hypotheses raised in previous studies with *C. fluminea* (Cherry et al., 2005; Cooper et al., 2005; Ilarri et al., 2010; Sousa et al., 2007, 2008b; Vohmann et al., 2009; Weitere et al., 2009) and other bivalves (Cotter et al., 2010; Samain et al., 2007; Samain and McCombie, 2008; Soletchnik et al., 2005). Moreover, our findings also mean that monitoring along the year the stress levels of *C. fluminea* populations will make possible to determine the likelihood of summer/post summer mortality syndrome in this species.

The biomarkers most contributing to the high IBR value found in clams from M3 in August were the enzymes EST (decreased), ODH (induced) and GR (induced) (Figures 2 and 3). EST inhibition indicates exposure to inhibitors of these enzymes that may be anticholinesterase pesticides used in neighbour crop fields that may be at increased concentrations in the water due to a reduced river flow in the summer. The induction of GR activity, an anti-oxidant enzyme, indicates exposure to oxidative stress inducers. These may be the increased water temperature observed at this time (Table 1) and/or chemical stressors present in the water as environmental

contaminants. The increase of ODH activity suggests increased energetic demands that may be need to face thermal and/or chemically induced stress. In fact, ODH is an enzyme involved in the anaerobic metabolism of several invertebrate species (Gerd et al., 1978) and its induction is often a response to cope with respiratory deficit and to supply extra ATP (Lima et al., 2007). In clams collected in July in L that had the second highest IBR value (Table 5), increased activity of the energy related enzymes ODH and IDH, and of the biotransformation GST enzymes were found. The increase of IDH and ODH suggest increased energy demands probably to cope with the stress caused by the presence of environmental contaminants (no increase of water temperature was found at this time in L), while the increase of GST activity indicates exposure to chemicals that are biotransformed by these enzymes and are known to be present Lima River estuary (Guimarães et al., 2012). Other biological factors that may have contributed to the highest levels of stress found in *C. fluminea* in July/August are those related with the reproductive cycle. In bivalves, important physiological alterations occur during the gonads development and spawning, which are very demanding processes (Cotter et al., 2010; Dégremonet et al., 2007; Huvet et al., 2010; Myrand et al., 2000; Soletchnik et al., 2005). In *C. fluminea*, an additional stress factor may be the embryos incubation process because it occurs in the adults' gills (Byrne et al., 2000). A study of the gonadal development of *C. fluminea* in the studied sites has been carried out. The preliminary evidences indicate the presence of individuals with mature gametes almost all the year (data not shown). Thus, the influence of factors related with the gonads development, spawning, and embryo incubation is unlikely to be an important factor in the variability of IBR found, despite potentially having some influence.

Overall, the IBR index provided an integrated view of *C. fluminea* stress levels allowing the discrimination of the more stressful months and sites. However, attention should be taken when using IBR index (Cravo et al., 2012) since is a dynamic index that varies with the type and number of biomarkers used, and the kind of response recorded (e.g. activation/inhibition) (Beliaeff and Burgeot, 2002; Tsangaris et al., 2011).

4.3. Relating biological and environmental parameters

The association between high ODH activity and high water conductivity and in a less extend with water temperature in M3 and L, suggests that clams from these sites are under energetic stress caused by these two abiotic variables. Because these are downstream sites (in Minho and Lima Rivers) the tidal influence is likely to significantly contribute to these high conductivity values as previously discussed

(section 3.1). Although *C. fluminea* can tolerate some salinity for relatively short periods (Sousa, 2006a), this is a freshwater species, and it seems to be sensitive to salinity fluctuations (Xiao et al., 2014). Therefore, the increased ODH activity shown by clams from M3 and L may be at least in part due to the need of getting more energy to face salinity-induced stress. *C. fluminea* is also sensitive to increased water temperature (Cooper et al., 2005; Sousa et al., 2008d; Weitere et al., 2009). In *C. fluminea*, thermal stress also requires additional energy to activate molecular defences against its effects, increase tissue repair mechanisms, and other processes (Weitere et al., 2009). M2 appears separated from the other sites in the RDA ordination diagram mainly because the high GR activity that is positively associated with water pH values. In fact, M2 clams showed increased GR activity relatively to clams from all the other sites in September and October (Figure 2), indicating increased exposure to oxidative stress inducers.

The positive association between high water concentrations of nitrites, nitrates, ammonium, and increased activities of the enzymes CAT, EST and IDH contributing to the separation of July from all the other months (Figure 2), suggests that these biological responses are, at least in part, due to these abiotic factors. A recent study also observed that the fast proliferation of algal cells could influence negatively the biomarker responses in *Mytilus edulis* L. (Farcy et al., 2013). Such enzymatic inductions indicate exposure to oxidative stress inducers (CAT), environmental contaminants hydrolysed by EST, and that additional energy is required to cope with chemically induced stress resulting in IDH induction. August and October appear separated from the other months in the RDA ordination diagram mainly due to high GST activity. In fact, the clams from all the sampling sites showed high GST activity in August, being reduced in September and increased again in October, except in L where no increase was observed in October (Figure 2). The induction of this enzyme is very important to face chemical stress induced by electrophilic agents that are biotransformed through glutathione conjugation. Common environmental contaminants of this type are organochlorine pesticides that may reach the rives through lixiviation from adjacent fields, PAHs, PCBs and several dioxin-like environmental contaminants that may reach the estuaries through contaminated tributaries and/or direct inputs. Because in August the flow of Minho and Lima rivers is reduced due to draw conditions, the water concentration of these chemicals is likely to be high. They are expected to decrease when the river flow comes back to normal values and is expected to further increase if strong rain occurs in the autumn causing the lixiviation of contaminated surrounding crop fields.

4. Conclusions

Important spatial and temporal variation in several of the 17 environmental parameters analysed were found. The most important ones were related with the increase of water temperature conductivity and nutrients in downstream sites (M3 and L) creating stressfully conditions to *C. fluminea*. The IBR analysis indicated that July/August were more stressful to clams than September/October, especially in downstream sites. The clam's stress in the summer months was mainly due to increased activity of energy-related (IDH and ODH) and biotransformation (EST and GST) enzymes. These findings indicate that in the summer *C. fluminea* populations of Minho and Lima estuaries are exposed to chemical environmental contaminants biotransformed by EST and GST enzymes and probably also to thermal stress and a higher tidal influence (especially in downstream sites), and that they need to activate energy related- enzymes to get the additional energy required to face these types of stress. Finally, the integration of environmental and biological variables through a RDA analysis provided a separation among sites ($M1 \neq M2 \neq M3 \approx L$) and among months ($J \neq A \neq S \neq O$). Increased activities of the energy related enzyme IDH, of the anti-oxidant enzyme CAT and of EST enzymes associated with increased water concentrations of ammonia, nitrate and nitrites contributed to the separation of July from the other months; induction of the GST biotransformation enzyme mainly contributed to separate August and October from the other months. Overall, the findings of the present study indicate that July/August are particularly stressful months for the studied *C. fluminea* populations, especially at downstream sites; the increase of nutrients and ammonia water concentrations, water temperature and conductivity are major contributors for this increased stress; the biomarkers indicated that in July/August *C. fluminea* is exposed to oxidative stress inducers, environmental chemical contaminants biotransformed by EST and GST enzymes, and that organisms need additional energy to cope with the chemical and/or thermally-induced stress. The findings of the present study highlight the importance of biomonitoring the health condition of *C. fluminea* because it may allow determining the likelihood of summer/post summer mortality syndrome in this species.

Acknowledgments

This research was supported by the European Regional Development Fund (ERDF) through the COMPETE - Operational Competitiveness Programme and national funds through FCT – Foundation for Science and Technology, under the projects “FCT Ref.: PTDC/AAC-AMB/102121/2008; FCOMP-01-0124-FEDER-

531 008556”, and “PEst-C/MAR/LA0015/2013. Cristiana Oliveira received a FCT grant
532 (SFRH/BD/68423/2010) supported by the Portuguese Foundation for the Science
533 and Technology (FCT), FEDER European funds and European social funds of
534 (POPH-QREN-Tipology 4.2.).

535 **References**

- 536 Araújo, R., Moreno, D., Ramos, M., 1993. The Asiatic clam *Corbicula fluminea* (Müller,
537 1774)(Bivalvia: Corbiculidae) in Europe. Am. Malacol. Bull. 10, 39–49.
- 538 Baur, B., Schmidlin, S., 2007. Effects of invasive non-native species on the native biodiversity
539 in the river Rhine. In: Nentwig, W. (Ed.) Biological Invasions 193 pp.
- 540 Bradford, M., 1976. A rapid and sensitive method for the quantification of microgram
541 quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72,
542 248–59.
- 543 Beliaeff, B., Burgeot, T., 2002. Integrated biomarker response: a useful tool for ecological risk
544 assessment. Environ. Toxicol. Chem. 21, 1316–22.
- 545 Bird, R.P., Draper, A.H., 1984. Comparative studies on different methods of
546 malondyaldehyde determination. Method. Enzymol. 90, 105–110.
- 547 Byrne, M., Phelps, H., Church, T., 2000. Reproduction and development of the freshwater
548 clam *Corbicula australis* in southeast Australia. Hydrobiologia 185–197.
- 549 Cairrão, E., Couderchet, M., Soares, a M.V.M., Guilhermino, L., 2004. Glutathione-S-
550 transferase activity of *Fucus* spp. as a biomarker of environmental contamination.
551 Aquat. Toxicol. 70, 277–86.
- 552 Cherry, D.S., Scheller, J.L., Cooper, N.L., Bidwell, J.R., 2005. Potential effects of Asian clam (
553 *Corbicula fluminea*) die-offs on native freshwater mussels (Unionidae) I: water-column
554 ammonia levels and ammonia toxicity. J. North Am. Benthol. Soc. 24, 369–380.
- 555 Clairborne, A., 1985. Catalase activity. In: Greenwald RA (ed) CRC handbook of methods in
556 oxygen radical research. CRC Press, Boca Raton 283-284.
- 557 Concha-Graña, E., Turnes-Carou, M.I., Muniategui-Lorenzo, S., López-Mahía, P., Prada-
558 Rodríguez, D., Fernández-Fernández, E., 2006. Evaluation of HCH isomers and
559 metabolites in soils, leachates, river water and sediments of a highly contaminated area.
560 Chemosphere 64, 588–95.
- 561 Cooper, N.L., Bidwell, J.R., Cherry, D.S., 2005. Potential effects of Asian clam (*Corbicula*
562 *fluminea*) die-offs on native freshwater mussels (Unionidae) II: porewater ammonia. J.
563 North Am. Benthol. Soc. 24, 381–394.
- 564 Cotter, E., Malham, S.K., O’Keeffe, S., Lynch, S. a., Latchford, J.W., King, J.W., Beaumont,
565 A.R., Culloty, S.C., 2010. Summer mortality of the Pacific oyster, *Crassostrea gigas*, in
566 the Irish Sea: The influence of growth, biochemistry and gametogenesis. Aquaculture
567 303, 8–21.
- 568 Cravo, A., Pereira, C., Gomes, T., Cardoso, C., Serafim, A., Almeida, C., Rocha, T., Lopes,
569 B., Company, R., Medeiros, A., Norberto, R., Pereira, R., Araújo, O., Bebianno, M.J.,
570 2012. A multibiomarker approach in the clam *Ruditapes decussatus* to assess the
571 impact of pollution in the Ria Formosa lagoon, South Coast of Portugal. Mar. Environ.
572 Res. 75, 23–34.
- 573 Cribb, A.E., Leeder, J.S., Spielberg, S.P., 1989. Use of a microplate reader in an assay of
574 glutathione reductase using 5,50-dithiobis (2-nitrobenzoic acid). Anal. Biochem.
575 183:195–196.

576 DAISIE (2009) Delivering alien invasive species inventories for Europe (DAISIE).
577 <http://www.europe-aliens.org> (Accessed in September 2014).

578 Dégremont, L., Ernande, B., Bédier, E., Boudry, P., 2007. Summer mortality of hatchery-
579 produced Pacific oyster spat (*Crassostrea gigas*). I. Estimation of genetic parameters for
580 survival and growth. *Aquaculture* 262, 41–53.

581 Ellis, G., Goldberg, D.M., 1971. An improved manual and semi-automatic assay for NADP-
582 dependent isocitrate dehydrogenase activity, with a description of some kinetic
583 properties of human liver and serum enzyme. *Clin. Biochem.* 2, 175–185.

584 Ellman, G.L., Courtney, K.D., Andres, V., Featherstone, R.M., 1961. A new rapid colorimetric
585 determination of acetylcholinesterase activity. *Biochem. Pharmacol.* 7, 88–95.

586 Farcy, E., Burgeot, T., Haberkorn, H., Auffret, M., Lagadic, L., et al., 2013. An integrate
587 environmental approach to investigate biomarker fluctuations in the blue mussel
588 *Mytilus edulis* in the Vilaine estuary, France. *Environ. Sci. Pollut. Res.* 20, 630–650.

589 Farkas, A., Erratico, C., Vigano, L., 2007. Assessment of the environmental significance of
590 heavy metal pollution in surficial sediments of the River Po. *Chemosphere* 68, 761–768.

591 Filgueiras, A. V., Lavilla, I., Bendicho, C., 2004. Evaluation of distribution , mobility and binding
592 behaviour of heavy metals in surficial sediments of Louro River (Galicia , Spain) using
593 chemometric analysis : a case study. *Sci. Total Environ.* 330, 115–129.

594 Frasco, M.F., Guilhermino, L., 2002. Effects of dimethoate and betanaphthoflavone on
595 selected biomarkers of *Poecilia reticulata*. *Fish Physiol. Biochem.* 26, 149–156.

596 García-esquivel, Z., Bricelj, V.M., Gonzalez-gomez, M.A., 2001. Physiological basis for
597 energy demands and early postlarval mortality in the Pacific oyster , *Crassostrea gigas*.
598 *J. Exp. Mar. Biol. Ecol.* 263, 77–103.

599 Gerd, G., Weeda, E., Gabbott, P.A., Zoophysiology, I., Bonn, U., Avz, I., Allee, E., 1978.
600 Changes in the Level of Octopine during the Escape Responses of the Scallop *Pecten*
601 *maximus* (L.). *J. Comp. Physiol.* 124, 121–127.

602 Guilhermino, L., Lopes, M.C., Carvalho, A.P., Soares, A.M., 1996. Acetylcholinesterase
603 activity in juveniles of *Daphnia magna* Straus. *Bull. Environ. Contam. Toxicol.* 57, 979–
604 985.

605 Guimarães, L., Medina, M.H., Guilhermino, L., 2009. Health status of *Pomatoschistus microps*
606 populations in relation to pollution and natural stressors: implications for ecological risk
607 assessment. *Biomarkers* 17(1), 62–77.

608 Habig, W.H., Pabst, M.J., Jacoby, W.B., 1974. Glutathione S-transferases. The first enzymatic
609 step in mercapturic acid formation. *J. Biol. Chem.* 249, 7130–7139.

610 Huvet, A., Normand, J., Fleury, E., Quillien, V., Fabioux, C., Boudry, P., 2010. Reproductive
611 effort of Pacific oysters: A trait associated with susceptibility to summer mortality.
612 *Aquaculture* 304, 95–99.

613 Ilarri, M.I., Antunes, C., Guilhermino, L., Sousa, R., 2010. Massive mortality of the Asian clam
614 *Corbicula fluminea* in a highly invaded area. *Biol. Invasions* 13, 277–280.

615 Ilarri, M.I., Freitas, F., Costa-Dias, S., Antunes, C., Guilhermino, L., Sousa, R., 2012.
616 Associated macrozoobenthos with the invasive Asian clam *Corbicula fluminea*. J. Sea
617 Res. 72, 113–120.

618 INAG, 2000. Planos das bacias Hidrograficas dos rios Luso-Espanhois-Sintese.
619 Caracterização recursos Hídricos, Divisão e Diagnóstico. Instituto da Água, Direcção de
620 serviço de Recursos Subterrâneos, 398 pp.

621 Johnson, P.D., McMahon, R.F., 1998. Effects of temperature and chronic hypoxia on
622 survivorship of the zebra mussel (*Dreissena polymorpha*) and Asian clam (*Corbicula*
623 *fluminea*). Can. J. Fish. Aquat. Sci. 55, 1564–1572.

624 Jeffrey, S.W., Humphrey, G.F., 1975. New spectrophotometric equations for determining
625 chlorophylls a, b, c1 and c2 in higher plants, algae and natural phytoplankton.
626 Biochemie und Physiologie der Pflanzen, 167:191–194.

627 Karatayev, A.Y., Burlakova, L.E., Padilla, D.K., Johnson, L.E., 2003. Patterns of spread of the
628 zebra mussel (*Dreissena polymorpha* (Pallas)): the continuing invasion of Belarussian
629 Lakes. Biol. Invasions 5, 213–221.

630 Lavilla, I., Garrido, J., Bendicho, C., 2010. A biogeochemical approach to understanding the
631 accumulation patterns of trace elements in three species of dragonfly larvae : evaluation
632 as biomonitors. J. Environ. Monit. 12, 724–730.

633 Lima, I., Moreira, S.M., Osten, J.R.-V., Soares, A.M.V.M., Guilhermino, L., 2007. Biochemical
634 responses of the marine mussel *Mytilus galloprovincialis* to petrochemical environmental
635 contamination along the North-western coast of Portugal. Chemosphere 66, 1230–1242.

636 Livingstone, D.R., Stickle, W.B., Kapper, M.A., Wang, S., Zurburg, W., 1990. Further studies
637 on the phylogenetic distribution of pyruvate oxidoreductase activities. Comp. Biochem.
638 Phys. B 97, 661–666.

639 McDowell, B., Byers, J., Rosemond, A., McDowell, W., 2013. Effects of Mass Mortality of an
640 Abundant Invasive Species on Ecosystem Function 30602.

641 McMahon, R.F., 2002. Evolutionary and physiological adaptations of aquatic invasive
642 animals : r selection versus resistance. Can. J. Fish. Aquat. Sci. 59, 1235–1244.

643 Morgan, D.E., Keser, M., Swenarton, J.T., Foertch, J.F., 2003. Population dynamics of the
644 Asiatic clam, *Corbicula fluminea* (Muller) in the Lower Connecticut River: Establishing a
645 foothold in New England. J. Shellfish Res. 22, 193–203.

646 Mouthon, J., Daufresne, M., 2006. Effects of the 2003 heatwave and climatic warming on
647 mollusc communities of the Saone: a large lowland river and of its two main tributaries
648 (France). Glob. Chang. Biol. 12, 441–449.

649 Myrand, B., Guderley, H., Himmelman, J., 2000. Reproduction and summer mortality of blue
650 mussels *Mytilus edulis* in the Magdalen Islands, southern Gulf of St. Lawrence. Mar.
651 Ecol. Prog. Ser. 197, 193–207.

652 Ohkawa, H., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction.
653 Anal. Biochem. 95, 351–358.

654 PBH (2001) Plano de Bacia Hidrográfica do Rio Minho, Relatório Final, Ministério do
655 Ambiente e do Ordenamento do Território, Instituto da Água, IP.

- 656 Power, M., Tilman, D., Estes, J., Menge, B., 1996. Challenges in the quest for keystones.
657 Bioscience 46, 609–620.
- 658 Reis, P.A., Antunes, J.C., Almeida, C.M.R., 2009. Metal levels in sediments from the Minho
659 estuary salt marsh: a metal clean area? Environ. Monit. Assess. 159, 191–205.
- 660 Rosa, I.C., Pereira, J.L., Gomes, J., Saraiva, P.M., Gonçalves, F., Costa, R., 2011. The Asian
661 clam *Corbicula fluminea* in the European freshwater-dependent industry: A latent threat
662 or a friendly enemy? Ecol. Econ. 70, 1805–1813.
- 663 Samain, J.F., McCombie, H., 2008. Summer Mortality of Pacific Oyster *Crassostrea gigas*.
664 Ed. Ifremer/Quae, 379 pp.
- 665 Samain, J.F., Dégremont, L., Soletchnik, P., Haure, J., Bédier, E., Ropert, M., Moal, J., Huvet,
666 A., Bacca, H., VanWormhoudt, A., Delaporte, M., et al., 2007. Genetically based
667 resistance to summer mortality in the Pacific oyster (*Crassostrea gigas*) and its
668 relationship with physiological, immunological characteristics and infection process.
669 Aquaculture 268, 227–243.
- 670 Soletchnik, P., Lambert, C., Costil, K., 2005. Summer mortality of *Crassostrea gigas*
671 (Thunberg) in relation to environmental rearing conditions. J. Shellfish Res. 24, 197–
672 207.
- 673 Sousa, R., Antunes, C., Guilhermino, L., 2006a. Factors influencing the occurrence and
674 distribution of *Corbicula fluminea* (Müller, 1774) in the River Lima estuary. Ann. Limnol.
675 42, 165–171.
- 676 Sousa, R., Antunes, C., Guilhermino, L., 2007. Species composition and monthly variation of
677 the Molluscan fauna in the freshwater subtidal area of the River Minho estuary. Estuar.
678 Coast. Shelf Sci. 75, 90–100.
- 679 Sousa, R., Dias, S., Antunes, C., 2006b. Spatial Subtidal Macrobenthic Distribution in
680 Relation to Abiotic Conditions in the Lima Estuary, NW of Portugal. Hydrobiologia 559,
681 135–148.
- 682 Sousa, R., Dias, S., Freitas, V., Antunes, C., 2008a. Subtidal macrozoobenthic assemblages
683 along the River Minho estuarine gradient (northwest Iberian Peninsula). Aquat. Conserv.
684 Mar. Freshw. Ecosyst. 1077, 1063–1077.
- 685 Sousa, R., Guilhermino, L., Antunes, C., 2005. Molluscan fauna in the freshwater tidal area of
686 the River Minho estuary, NW of Iberian Peninsula. Ann. Limnol. - Int. J. Limnol. 41, 141–
687 147.
- 688 Sousa, R., Ilarri, M., Souza, A.T., Antunes, C., Guilhermino, L., 2011. Rapid decline of the
689 greater European peacclam at the periphery of its distribution. Ann. Limnol. - Int. J.
690 Limnol. 47, 211–219.
- 691 Sousa, R., Nogueira, A., Antunes, C., Guilhermino, L., 2008b. Growth and production of
692 *Pisidium amnicum* in the freshwater tidal area of the River Minho estuary. Estuar. Coast.
693 Shelf Sci. 79, 467–474.
- 694 Sousa, R., Nogueira, A., Gaspar, M., Antunes, C., Guilhermino, L., 2008c. Growth and
695 extremely high production of the non-indigenous invasive species *Corbicula fluminea*
696 (Müller, 1774): Possible implications for ecosystem functioning. Estuar. Coast. Shelf.
697 Sci. 80, 289–295.

- 698 Sousa, R., Rufino, M., Gaspar, M., Antunes, C., Guilhermino, L., 2008d. Abiotic impacts on
699 spatial and temporal distribution of *Corbicula fluminea* (Müller, 1774) in the River Minho
700 Estuary, Portugal. Aquat. Conserv. Mar. Freshw. Ecosyst. 110, 98–110.
- 701 ter Braak, C.J.F., 1986. Canonical correspondence analysis: a new eigenvector technique for
702 multivariate direct gradient analysis. Ecology, 67, 1167–1179.
- 703 ter Braak, C.J.F. & Smilauer, P. 2002. CANOCO Reference manual and CanoDraw for
704 Windows User's guide: software for Canonical Community Ordination (version 4.5).
705 Microcomputer Power, Ithaca.
- 706 Tomaru, Y., Kawabata, Z., Nakano, S., 2001. Mass mortality of the Japanese pearl oyster
707 *Pinctada fucata martensii* in relation to water temperature, chlorophyll a and
708 phytoplankton composition. Dis. Aquat. Organ. 44, 61–8.
- 709 Torres, M.A., Testa, C.P., Gaspari, C., Masutti, M.B., Panitz, C.M.N., Curi-Pedrosa, R.,
710 Almeida, E.A., Di Mascio, P., Filho, D.W., 2002. Oxidative stress in the mussel *Mytella*
711 *guyanensis* from polluted mangroves on Santa Catarina Island, Brazil. Mar. Pollut. Bull.
712 44, 923-932.
- 713 Tremblay, R., Myrand, B., Guderley, H., 1998. Temporal variation of lysosomal capacities in
714 relation to susceptibility of mussels, *Mytilus edulis*, to summer mortality. Mar. Biol. 132,
715 641–649.
- 716 Tsangaris, C., Hatzianestis, I., Catsiki, V.-A., Kormas, K.A., Stroglyoudi, E., Neofitou, C.,
717 Andral, B., Galgani, F., 2011. Active biomonitoring in Greek coastal waters: application
718 of the integrated biomarker response index in relation to contaminant levels in caged
719 mussels. Sci. Total Environ. 412-413, 359–65.
- 720 Urrutia, M.B., Ibarrola, I., Iglesias, J.I.P., Navarro, E., 1999. Energetics of growth and
721 reproduction in a high-tidal population of the clam *Ruditapes decussatus* from Urdaibai
722 Estuary (Basque Country, N. Spain). J. Sea Res. 42, 35–48.
- 723 Vohmann, A., Borchering, J., Kureck, A., bij de Vaate, A., Arndt, H., Weitere, M., 2009.
724 Strong body mass decrease of the invasive clam *Corbicula fluminea* during summer.
725 Biol. Invasions 12, 53–64.
- 726 Weitere, M., Vohmann, A., Schulz, N., Linn, C., Dietrich, D., Arndt, H., 2009. Linking
727 environmental warming to the fitness of the invasive clam *Corbicula fluminea*. Glob.
728 Chang. Biol. 15, 2838–2851.
- 729 Werner, S., Rothhaupt, K.-O., 2008. Mass mortality of the invasive bivalve *Corbicula fluminea*
730 induced by a severe low-water event and associated low water temperatures.
731 Hydrobiologia 613, 143–150.
- 732 Xiao, B.-C., Li, E.-C., Du, Z.-Y., Jiang, R.-L., Chen, L.-Q., Yu, N., 2014. Effects of temperature
733 and salinity on metabolic rate of the Asiatic clam *Corbicula fluminea* (Müller, 1774).
734 Springerplus 3, 455.

735

736

Captions

Figure 1. Map of Minho and Lima Rivers showing the four sampling sites location.

Figure 2. Gills lipid peroxidation (LPO) levels, and glutathione S-transferase (GST), catalase (CAT) and glutathione reductase (GR) activities and foot muscle esterase (EST), isocitrate dehydrogenase (IDH) and octopine dehydrogenase (ODH) activities determined for each sampling site and month in the tidal freshwater area of Minho River estuary and Lima River estuary, from July to October 2011. Values are the mean of 20 clams per sampling site per month with the corresponding standard error bars.

Figure 3. Star plots representing the contribution of each biomarker (Gills lipid peroxidation (LPO) levels, glutathione S-transferase (GST), catalase (CAT) and glutathione reductase (GR) activities and foot muscle esterase (EST), isocitrate dehydrogenase (IDH) and octopine dehydrogenase (ODH) activities), used to compute the IBR index calculated for each sampling site and month in the tidal freshwater area of Minho River estuary and Lima River estuary, from July to October 2011.

Figure 4. Redundancy analysis ordination diagram displaying the scores for biological variables (represented by straight arrows) showing correlations with quantitative environmental variables (represented by dotted arrows): the first axis (horizontal) significantly explained 84.0% and second (vertical) 10.5% of the variability. The biological variables are: Gills lipid peroxidation (LPO) levels, and glutathione S-transferase (GST), catalase (CAT) and glutathione reductase (GR) activities and foot muscle esterase (EST), isocitrate dehydrogenase (IDH) and octopine dehydrogenase (ODH) activities. The quantitative environmental variables are: temperature (T), dissolved oxygen (DO), pH, NH_4^+ (Ammonium), NO_2^- (nitrites) and NO_3^- (nitrates). Centroids of the sampling sites are represented by black triangles and centroids of months by black circles.

769

770 **Figure 1**

771

772

777

778

779 **Figure 4**

780

781

783 **Table 1.** Abiotic water variables values determined for each sampling site and month in the tidal freshwater area of Minho River estuary and
784 Lima River estuary, from July to October 2011. Values are mean±standard error of temperature (T, °C), dissolved oxygen (DO, mg l⁻¹),
785 conductivity (Cond, µS cm⁻¹), pH (pH units), nitrites (NO₂⁻, mg l⁻¹), nitrates (NO₃⁻, mg l⁻¹), ammonium (NH₄⁺, mg l⁻¹), phosphates (PO₄³⁻, mg l⁻¹),
786 silica (SiO₂, mg l⁻¹), phenol (C₆H₅OH, mg l⁻¹), iron (Fe, mg l⁻¹), hardness (CaCO₃, mg l⁻¹) and turbidity (Turb, FTU).

787

Sampling site	Month	Abiotic water variable												
		T	DO	Cond	pH	NO ₂ ⁻	NO ₃ ⁻	NH ₄ ⁺	PO ₄ ³⁻	SiO ₂	C ₆ H ₅ OH	Fe	CaCO ₃	Turb
M1	July	18.27±0.09	9.17±0.22	14.43±0.03	7.28±0.02	0.13±0.00	4.48±0.07	0.04±0.01	0.01±0.00	6.32±0.21	0.07±0.01	0.03±0.00	27.22±1.11	0.00±0.00
	August	25.27±0.12	10.11±0.12	88.23±2.40	7.77±0.10	0.05±0.02	0.41±0.03	0.29±0.10	0.09±0.01	6.02±0.74	0.07±0.01	0.04±0.01	36.11±0.56	3.33±2.69
	September	19.57±0.15	8.47±0.03	79.67±0.33	7.28±0.07	0.01±0.00	0.65±0.06	0.14±0.02	0.11±0.01	6.96±0.38	0.07±0.01	0.02±0.00	33.33±2.55	7.33±0.38
	October	19.03±0.27	10.21±1.79	85.67±0.12	9.90±0.12	0.00±0.00	0.40±0.05	0.13±0.06	0.04±0.01	7.24±0.10	0.06±0.00	0.02±0.01	31.67±0.96	6.67±3.42
M2	July	22.33±0.03	10.10±0.22	17.40±0.02	7.33±0.02	0.13±0.00	1.96±0.09	0.94±0.06	0.01±0.00	1.38±0.06	0.08±0.00	0.08±0.01	40.00±2.54	8.00±2.00
	August	25.73±0.24	8.01±0.04	34.00±1.36	7.69±0.12	0.01±0.00	0.56±0.04	0.13±0.01	0.15±0.02	0.98±0.03	0.07±0.00	0.05±0.01	42.22±0.56	5.78±0.22
	September	22.50±0.17	8.55±0.05	117.33±0.33	7.46±0.02	0.01±0.00	0.60±0.02	0.09±0.01	0.09±0.01	1.98±0.05	0.11±0.01	0.05±0.02	43.89±2.22	3.56±1.74
	October	20.50±0.23	8.35±0.05	122.67±0.33	7.47±0.02	0.01±0.00	0.26±0.02	0.08±0.05	0.02±0.01	1.65±0.06	0.09±0.02	0.03±0.01	54.44±2.42	6.67±2.69
M3	July	23.50±0.00	9.10±0.04	37.67±1.45	6.96±0.03	0.12±0.03	2.16±0.10	0.33±0.09	0.00±0.00	1.04±0.01	0.04±0.02	0.04±0.00	263.33±14.24	3.11±1.94
	August	25.87±0.12	8.25±0.03	29.4±2.56	7.49±0.04	0.00±0.00	0.61±0.04	0.08±0.02	0.60±0.05	1.15±0.04	0.08±0.02	0.05±0.02	257.22±3.09	12.67±1.39
	September	23.20±0.31	8.22±0.18	1477.00±3.51	7.82±0.04	0.01±0.00	0.41±0.00	0.17±0.03	0.16±0.03	2.16±0.01	0.10±0.01	0.16±0.05	228.89±10.82	5.33±1.54
	October	20.87±0.09	9.67±0.07	987.00±7.21	7.93±0.05	0.01±0.00	0.19±0.02	0.14±0.08	0.11±0.03	2.09±0.03	0.06±0.01	0.02±0.00	155.00±8.66	1.33±0.67
L	July	21.33±0.19	10.52±0.04	32.00±0.58	7.49±0.04	0.00±0.00	1.84±0.07	0.31±0.05	0.05±0.01	2.17±0.16	0.07±0.01	0.10±0.02	21.11±1.11	6.67±2.52
	August	26.13±0.15	7.84±0.04	29.30±3.37	7.46±0.06	0.00±0.00	0.72±0.06	0.08±0.01	0.05±0.01	4.28±0.12	0.08±0.02	0.03±0.00	132.78±4.75	2.89±1.35
	September	22.20±0.15	8.53±0.00	1566.67±0.88	7.26±0.12	0.00±0.00	0.49±0.07	0.12±0.04	0.06±0.01	2.51±0.10	0.09±0.01	0.04±0.01	149.44±10.56	1.33±1.33
	October	19.13±0.12	8.25±0.23	824.33±28.59	8.08±0.15	0.00±0.00	0.21±0.01	0.22±0.06	0.07±0.01	2.13±0.08	0.09±0.01	0.03±0.00	90.56±5.47	5.33±2.40

Table 2. Sediments chlorophyll *a*, *b* and *c* values and organic matter percentage for each sampling site and month in the tidal freshwater area of Minho River estuary and Lima River estuary, from July to October 2011. Values are mean±standard error of chlorophyll *a*, *b* and *c* (Chl *a*, Chl *b* and Chl *c*, respectively, units are $\mu\text{g l}^{-1}$), very coarse sand (VCS, %), coarse sand (CS, %), medium sand (MS, %), fine sand (FS, %), very fine sand (VFS %) and silt + clay (S+C, %) for granulometry and organic matter (%).

Sampling site	Month	Sediment chlorophyll ($\mu\text{g l}^{-1}$)			Organic matter (%)
		Chl <i>a</i>	Chl <i>b</i>	Chl <i>c</i>	
M1	July	1.20±0.15	0.84±0.03	2.04±0.07	0.99±0.23
	August	1.88±0.58	1.06±0.18	2.13±0.14	1.40±0.40
	September	1.85±0.30	0.95±0.04	2.09±0.09	0.86±0.07
	October	2.16±0.36	1.39±0.15	2.41±0.19	1.60±0.09
M2	July	0.75±0.01	0.69±0.01	1.85±0.02	1.35±0.17
	August	0.91±0.05	0.77±0.00	1.98±0.05	2.40±0.16
	September	2.11±0.26	1.18±0.11	2.25±0.11	2.75±0.74
	October	1.31±0.18	0.98±0.12	2.15±0.09	4.58±0.82
M3	July	1.32±0.10	0.79±0.02	1.95±0.03	0.72±0.05
	August	1.78±0.25	0.97±0.06	2.25±0.06	3.74±0.32
	September	3.14±0.54	1.20±0.10	2.64±0.09	4.65±0.45
	October	1.77±0.28	0.87±0.08	2.24±0.14	0.73±0.05
L	July	2.46±0.58	0.87±0.06	2.10±0.11	1.76±0.12
	August	2.03±0.40	0.99±0.16	2.55±0.45	1.62±0.06
	September	1.93±0.64	0.82±0.08	2.12±0.09	1.44±0.08
	October	3.08±0.58	0.96±0.04	2.58±0.27	1.39±0.03

Table 3. Results of the one-way analysis of variance (one-way ANOVA) and post-hoc test (Tukey) of abiotic water and sediment variables performed to investigate significant differences among sampling sites and among months. Small letters represents significant differences between sampling sites and capital letters represents significant differences between sampling months. Temperature (T), dissolved oxygen (DO), conductivity (Cond), pH (pH), nitrites (NO₂⁻), nitrates (NO₃⁻), ammonium (NH₄⁺), phosphates (PO₄³⁻), silica (SiO₂), phenol (C₆H₅OH), iron (Fe), hardness (CaCO₃), turbidity (Turb) chlorophylls a, b and c (Chl a, Chl b and Chl c, respectively) and organic matter (OM).

Parameters	Factor	df	F	p	Tukey groups			
					M1 July	M2 August	M3 September	L October
T	site	3	210.999	<0.001	20.53 ^a	22.77 ^c	23.36 ^d	22.20 ^b
	month	3	892.900	<0.001	21.36 ^B	25.75 ^D	21.87 ^C	19.88 ^A
	site x month	9	31.461	<0.001				
DO	Site	3	12.543	<0.001	9.49 ^b	8.75 ^a	8.81 ^a	8.78 ^a
	Month	3	34.832	<0.001	9.72 ^C	8.55 ^A	8.44 ^A	9.12 ^B
	site x month	9	17.202	<0.001				
Cond	Site	3	7150.701	<0.001	67.00 ^a	72.85 ^a	632.77 ^c	613.08 ^b
	Month	3	10140.528	<0.001	25.38 ^A	45.23 ^B	810.17 ^U	504.92 ^C
	site x month	9	2854.141	<0.001				
pH	Site	3	46.174	<0.001	8.06 ^b	7.49 ^a	7.55 ^a	7.57 ^a
	Month	3	148.979	<0.001	7.27 ^A	7.60 ^C	7.45 ^B	8.35 ^U
	site x month	9	55.685	<0.001				
NO ₂ ⁻	Site	3	21.653	<0.001	0.05 ^b	0.04 ^b	0.03 ^b	0.00 ^a
	Month	3	93.474	<0.001	0.09 ^B	0.01 ^A	0.01 ^A	0.01 ^A
	site x month	9	12.196	<0.001				
NO ₃ ⁻	Site	3	148.372	<0.001	1.49 ^b	0.84 ^a	0.84 ^a	0.81 ^a
	Month	3	1625.051	<0.001	2.61 ^C	0.58 ^B	0.54 ^B	0.26 ^B
	site x month	9	136.232	<0.001				
NH ₄ ⁺	Site	3	7.923	<0.001	0.15 ^a	0.31 ^b	0.18 ^a	0.18 ^a
	Month	3	27.217	<0.001	0.41 ^B	0.15 ^A	0.13 ^A	0.14 ^A
	site x month	9	17.529	<0.001				
PO ₄	Site	3	71.300	<0.001	0.06 ^a	0.07 ^a	0.22 ^b	0.06 ^a
	Month	3	89.124	<0.001	0.02 ^A	0.22 ^D	0.11 ^C	0.06 ^B
	site x month	9	43.242	<0.001				
SiO ₂	Site	3	458.692	<0.001	6.64 ^c	1.50 ^a	1.61 ^a	2.77 ^b
	Month	3	6.811	0.001	2.73 ^A	3.11 ^{AB}	3.40 ^B	3.28 ^B
	site x month	9	10.219	<0.001				
C ₆ H ₅ OH	Site	3	2.710	0.061	0.07 ^a	0.09 ^a	0.07 ^a	0.08 ^a
	Month	3	3.960	0.017	0.07 ^A	0.08 ^{AB}	0.09 ^B	0.07 ^{AB}
	site x month	9	1.695	0.131				
Fe	Site	3	3.933	0.017	0.03 ^a	0.05 ^{ab}	0.07 ^b	0.05 ^{ab}
	Month	3	5.508	0.004	0.06 ^B	0.04 ^{AB}	0.07 ^B	0.03 ^A
	site x month	9	4.975	<0.001				
CaCO ₃	Site	3	842.181	<0.001	32.08 ^a	45.14 ^b	226.11 ^d	98.47 ^c
	Month	3	32.995	<0.001	87.92 ^A	117.08 ^B	113.89 ^B	82.92 ^A
	site x month	9	40.919	<0.001				
Turb	Site	3	1.021	0.396	4.33 ^a	6.00 ^a	5.61 ^a	4.06 ^a
	Month	3	0.770	0.520	4.45 ^A	6.17 ^A	4.39 ^A	5.00 ^A
	site x month	9	4.022	0.002				
Chl a	site	3	5.758	0.003	1.78 ^{ab}	1.27 ^a	2.00 ^b	2.38 ^b
	month	3	3.853	0.019	1.44 ^A	1.65 ^{AB}	2.26 ^B	2.08 ^{AB}
	site x month	9	1.732	0.125				
Chl b	Site	3	2.427	0.085	1.07 ^a	0.90 ^a	0.96 ^a	0.91 ^a
	Month	3	5.802	0.003	0.80 ^A	0.95 ^{AB}	1.04 ^B	1.05 ^B
	site x month	9	2.984	0.012				
Chl c	Site	3	2.094	0.122	2.17 ^a	2.06 ^a	2.27 ^a	2.33 ^a
	Month	3	3.487	0.028	1.99 ^A	2.23 ^{AB}	2.27 ^{AB}	2.34 ^B
	site x month	9	1.199	0.332				
Organic matter	Site	3	18.991	<0.001	1.21 ^a	2.77 ^b	2.46 ^b	1.55 ^a
	Month	3	10.575	<0.001	1.20 ^A	2.29 ^B	2.43 ^B	2.07 ^B
	site x month	9	14.322	<0.001				

Table 4. Results of the two-way analysis of variance (one-way ANOVA) and post-hoc test (Tukey) of biomarkers values performed to investigate significant differences among sampling sites, among months, and the respective interaction. Small letters represents significant differences between sampling sites and capital letters represents significant differences between sampling months. Lipid peroxidation (LPO), glutathione S-transferase (GST), catalase (CAT), glutathione reductase (GR), isocitrate dehydrogenase (IDH), octopine dehydrogenase (ODH) and esterase (EST).

Parameters	Factor	df	F	p	Tukey groups			
					M1	M2	M3	L
					July	August	September	October
LPO	site	3	12.716	<0.001	0.43 ^a	0.39 ^a	0.48 ^b	0.40 ^a
	month	3	18.195	<0.001	0.38 ^A	0.47 ^B	0.47 ^B	0.39 ^A
	site x month	9	6.063	<0.001				
GST	Site	3	0.953	0.416	16.38 ^a	18.68 ^a	18.22 ^a	18.17 ^a
	Month	3	12.837	<0.001	14.24 ^A	20.53 ^B	15.76 ^A	20.92 ^B
	site x month	9	4.151	<0.001				
CAT	Site	3	2.323	0.075	4.94 ^b	5.06 ^b	5.02 ^b	4.42 ^a
	Month	3	30.640	<0.001	6.16 ^C	4.63 ^B	4.96 ^B	3.69 ^A
	site x month	9	1.026	0.419				
GR	Site	3	5.147	0.002	2.76 ^{ab}	3.10 ^b	2.62 ^a	2.47 ^a
	Month	3	17.746	<0.001	2.19 ^A	3.42 ^C	2.61 ^{AB}	2.74 ^B
	site x month	9	8.673	<0.001				
IDH	Site	3	23.666	<0.001	4.18 ^a	4.15 ^a	4.62 ^a	6.15 ^b
	Month	3	22.796	<0.001	5.96 ^C	3.93 ^A	4.92 ^B	4.28 ^{AB}
	site x month	9	3.105	0.001				
ODH	Site	3	49.984	<0.001	2.61 ^a	3.03 ^a	4.40 ^C	4.01 ^b
	Month	3	20.585	<0.001	3.99 ^B	3.97 ^B	2.91 ^A	3.18 ^A
	site x month	9	7.641	<0.001				
EST	Site	3	0.482	0.695	0.62 ^a	0.64 ^a	0.63 ^a	0.59 ^a
	Month	3	5.803	0.001	0.71 ^B	0.56 ^A	0.57 ^A	0.65 ^{AB}
	site x month	9	2.351	0.014				

Table 5. IBR index values calculated for clams from each sampling site and month in the tidal freshwater area of Minho River estuary and Lima River estuary, from July to October 2011. Values are computed using the following biomarkers responses: lipid peroxidation (LPO), glutathione S-transferase (GST), catalase (CAT), glutathione reductase (GR), isocitrate dehydrogenase (IDH), octopine dehydrogenase (ODH) and esterase (EST). Higher values indicate higher stressed condition.

Sampling site	Sampling month			
	July	August	September	October
M1	6.21	3.28	1.65	0.47
M2	4.08	5.33	3.95	1.15
M3	6.25	11.95	4.25	3.95
L	10.57	8.92	5.74	4.23