

# Relative sensitivity of soft-bottom intertidal macrofauna to increased CO<sub>2</sub> and experimental stress

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**ABSTRACT:** We used a controlled CO<sub>2</sub> perturbation experiment to test hypotheses about changes in diversity, composition and structure of soft-bottom intertidal macrobenthic assemblages, under realistic and locally relevant scenarios of seawater acidification. Patches of undisturbed sediment were collected from 2 types of intertidal sedimentary habitat in the Ria Formosa coastal lagoon (South Portugal) and exposed to 2 levels of seawater acidification (pH reduced by 0.3 and 0.6 units) and 1 unmanipulated (control) level. After 75 d the assemblages differed significantly between the 2 types of sediment and between field controls and the *ex situ* treatments, but not among the 3 pH levels tested. The naturally high values of total alkalinity buffered seawater from the changes imposed on carbonate chemistry and may have contributed to offsetting acidification at the local scale. Observed differences on biota were strongly related to the organic matter content and grain-size of the sediments, particularly to the fractions of medium and coarse sand. Soft-bottom intertidal macrofauna was significantly affected by the stress of being held in an artificial environment, but not by CO<sub>2</sub>-induced seawater acidification. Given the previously observed variations in the sensitivities of marine organisms to seawater acidification, direct extrapolations of the present findings to different regions or other types of assemblages do not seem advisable. However, the contribution of *ex situ* studies to the assessment of ecosystem-level responses to environmental disturbances could generally be improved by incorporating adequate field controls in the experimental design.

**KEY WORDS:** Carbon dioxide · Global change · Ocean acidification · Mesocosms · Arthropoda · Annelida · Mollusca · Coastal ecosystems

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## INTRODUCTION

The combustion of fossil fuels has increased exponentially during the past 200 yr, raising atmospheric concentrations of carbon dioxide (CO<sub>2</sub>) by approximately 40%, from 280 ppm (estimated pre-industrial level) to an average of 395.37 ppm in 2013 (NOAA 2013). This level of atmospheric CO<sub>2</sub> has not been

exceeded during at least the last 800 000 yr, and there is widespread acknowledgement that it is causing global environmental changes, with noticeable increases in global temperature and changes to marine carbon chemistry (Meehl et al. 2007). In fact, the uptake of anthropogenic CO<sub>2</sub> by the oceans is increasing the concentration of hydrogen (H<sup>+</sup>) and bicarbonate (HCO<sub>3</sub><sup>-</sup>) ions, while decreasing the con-

centration of carbonate ions ( $\text{CO}_3^{2-}$ ). This process of ocean acidification (OA) is well documented and follows simple thermodynamic relationships (Waldbusser & Salisbury 2014). It has been occurring at a rate that is unprecedented in the past 55 million years ( $-0.0017$  to  $-0.0019$  pH units  $\text{yr}^{-1}$ ), having already decreased the average pH of ocean surface waters by 0.1 units since pre-industrial times (Haugan & Drange 1996, Gattuso et al. 2011). Modelling projections based on the emissions scenarios estimated by the Intergovernmental Panel on Climate Change (IPCC) for the 21st century indicates further declines of ocean pH ranging between 0.21 and 0.36 units (Brewer 1997, Joos et al. 2011). Under unrestricted emissions scenarios, larger reductions, varying between 0.7 and 1.3 pH units, have been hypothesised for the year 2300 (Caldeira & Wickett 2003, 2005). These past and future changes in seawater carbonate chemistry caused by the uptake of anthropogenic  $\text{CO}_2$  of the oceans are known with a high degree of certainty, but the effects on marine organisms, ecosystems, and the goods and services they provide are much less certain (Gattuso et al. 2011).

In coastal waters, carbonate chemistry is known to depend on the balance between anthropogenic  $\text{CO}_2$  emissions and watershed processes affecting the export of nutrients, organic and inorganic carbon, acids and carbonate alkalinity to the ocean (Borges & Gypens 2010, Duarte et al. 2013). These multiple coastal drivers contribute to increased variability (spatial and temporal), relative to the open ocean, which may exacerbate or counteract OA in ways that are still not fully understood. Investigating the sensitivity of marine organisms to OA and other anthropogenic perturbations of marine pH in coastal areas is, therefore, particularly relevant, because: (1) such investigations provide ample opportunities to assess the generality of response patterns and underlying mechanisms, (2) human interactions with marine organisms mainly occur within these areas and (3) local patterns of ecological response to climate change are considered to be more relevant than global averages (Philippart et al. 2007, Duarte et al. 2013).

Global change clearly has the potential to modify the composition of benthic marine assemblages, by promoting tolerant species and reducing sensitive ones, but direct evidence for changes in response to OA is still scarce (Gattuso et al. 2011). Shifts in species composition—with increased abundances of non-calcifying organisms, to the detriment of calcifying taxa; reductions of local biodiversity (i.e. number and evenness of taxa and functional groups); modifi-

cations of competitive interactions; decreased resistance to predation; and greater vulnerability to invasion by non-native species—are the most commonly perceived effects (see reviews by Hendriks et al. 2010, Hofmann et al. 2010, Kroeker et al. 2010, 2013, Gattuso & Hansson 2011).

Until recently the effects of OA were only being tested over short timescales and on single species, although impacts on whole assemblages were frequently hypothesised. Laboratory experiments with single species do not allow, however, the assessment of assemblage-level responses, which can modify interactions among organisms and induce cascading effects along the trophic web. A limited number of  $\text{CO}_2$ -perturbation experiments have been done at the community scale, the majority of which were focused on subtropical or tropical calcifying communities (reviewed by Andersson et al. 2011). These studies clearly showed that benthic organisms will not only be affected by OA directly, but also indirectly by the consequences of this process for other organisms, including competitors, prey, predators, and the quantity and quality of food supply. *In situ* studies in submarine volcanic  $\text{CO}_2$  vents are also producing significant advances in this respect. Hall-Spencer et al. (2008) observed significant alterations of benthic community structure, with the loss of calcifying species (i.e. echinoderms and molluscs) from natural assemblages around  $\text{CO}_2$  vents in the Mediterranean Sea. Reductions in coral diversity, recruitment and abundance were also observed in shallow  $\text{CO}_2$  vents in Papua New Guinea (Fabricius et al. 2011). Furthermore, enhanced variability has been found in the responses to OA in multi-species mesocosms (Kroeker et al. 2013, Stewart et al. 2013), so direct extrapolations of results from experiments with single species are not possible. Accordingly, predicting the assemblage level effects of OA remains an important gap in our knowledge.

Other earlier studies have directly measured the impacts of  $\text{CO}_2$  on sediment-dwelling fauna, but they generally intended to simulate the leakage from deep-sea sequestration sites used in future Carbon Capture and Storage (CCS) programmes (Tamburri et al. 2000, Barry et al. 2004). Most of these studies reported deleterious effects of increased  $\text{CO}_2$ , although associated with pH reductions far beyond the predicted changes for the global ocean during the following centuries. Furthermore, given that pH varies most in the upper ocean, deep-sea organisms are generally perceived to be more susceptible to pH variations than those of shallow waters. Accordingly, the relevance of these  $\text{CO}_2$  sequestration studies to

the more subtle, yet longer term, impacts of OA is limited (Widdicombe & Spicer 2008).

Macrofauna inhabiting nearshore and intertidal habitats has generally been regarded as being particularly tolerant to changes in pH, because it is routinely subjected to tidal, diurnal and seasonal fluctuations, which can exceed 1 pH unit (Duarte et al. 2013). Nevertheless, there is increasing awareness that many intertidal species may already be living close to their tolerance limits, such that further increases in environmental stress are likely to have significant physiological and ecological impacts (Godbold & Solan 2013). For instance, Widdicombe et al. (2009) reported significant changes to community structure, with decreased abundance and diversity loss in macrofaunal and nematode assemblages exposed to acidified seawater. This study also highlighted the variability of responses between sandy and muddy sediment types, with greater and faster impacts on the sandy sediment fauna than on the muddy sediment fauna. Two recent studies focused on temperate rocky shore assemblages using artificial substrate units. Hale et al. (2011) observed significant changes in assemblage structure, with reductions of species diversity and evenness of assemblages with reduced pH. Christen et al. (2013) reported a similar pattern, with the dominance of assemblages shifting from calcareous to non-calcareous organisms in response to reduced pH. These studies generally related the sensitivity of organisms to seawater acidification to its phylogeny, with vulnerability ranking from echinoderms (most sensitive), to molluscs, crustaceans and polychaetes (least sensitive), which matches the sequence proposed in meta-analyses (Kroeker et al. 2010, 2013).

Previous CO<sub>2</sub>-forcing experiments have shown that pH profiles of sediments are generally affected by changes in the carbonate chemistry of the overlying seawater and that these effects are generally stronger at the sediment surface (Dashfield et al. 2008, Wood et al. 2008, Widdicombe et al. 2013). Net increases in the dissolution of carbonate sediments have been observed in previous field and laboratory experiments, where the overlaying seawater was naturally or artificially acidified (Yates & Halley 2006, Andersson et al. 2007). Recent studies in coral reefs also indicate that OA can significantly accelerate bioerosion rates and reduce the sediment grain size (Dove et al. 2013, Wisshak et al. 2013). Despite granulometry no longer being considered the primary determinant of macrobenthic assemblages, its interactions with other factors like organic matter or microbial content (food availability), pore-water

chemistry and hydrodynamics can surely have direct or indirect effects on the distribution of benthic macrofauna (Snelgrove & Butman 1994).

Generally, the effects of OA have been tested over short timescales and on single species, but impacts on whole assemblages have been predicted. In the present experiment we compared *ex situ* CO<sub>2</sub> perturbation treatments with adequate controls (*ex situ* and *in situ*) to test hypotheses about changes in diversity, composition and structure of intertidal macrobenthic assemblages and the associated sediment characteristics, under realistic and locally relevant scenarios of seawater acidification. Results of previous studies, mentioned above, allowed us to hypothesise the following outcomes for this experiment:

Sediment characteristics:

- Grain size (Hypothesis s1), pH (s2) and carbonate content (s3) of sediments will be reduced in treatments with acidified seawater, relative to controls;

Macrobenthic assemblages:

- The composition or structure of assemblages will differ between treatments with acidified seawater and controls (b1);
- The number of taxa (b2), total abundance (b3), diversity (b4) and evenness (b5) of the assemblages will be reduced in treatments with acidified seawater, relative to controls;
- Densities of bivalves (b6) and gastropods (b7) will be reduced in treatments with acidified seawater, relative to controls;
- Densities of crustaceans (b8) and polychaetes (b9) will not differ among treatments or will increase in treatments with acidified seawater, relative to controls;

Relationship between sediments and biota:

- Effects of seawater acidification on macrobenthic assemblages will differ, in frequency or magnitude, between sedimentary habitats of distinct granulometry (r1).

## MATERIALS AND METHODS

### Experimental design and sampling

On 28 and 29 September 2011, during an equinoctial spring low tide, 2 types of sedimentary habitat were sampled from an extreme low intertidal area in the Ria Formosa coastal lagoon, SW Iberian Peninsula (37° 7' 17.7" N, 7° 37' 12.2" W). According to the classification of Flemming (2000), both sediment types were predominantly comprised of muddy sand,

but differed in the relative contributions of secondary components: slightly muddy sand (Sand) and sandy mud (Mud). These sedimentary habitats are well represented in the 100 km<sup>2</sup> of total wet area of the lagoon (Gamito 2008). A small box-corer (0.4 × 0.25 × 0.15 m) and a spade were used to cut patches of sediment, which were immediately placed in 25 l tanks filled with natural seawater. A layer of 60 µm nylon mesh was used to support the sediment 2 cm above the bottom of the tanks, to allow water flow and prevent anoxia. Replicate patches of each type of sediment were collected within an area of approximately 10 × 10 m, to assure that they were exposed to similar environmental conditions. On the same day, the tanks were transported to a nearby bivalve hatchery operated by IPMA (Instituto Português do Mar e da Atmosfera). This hatchery is equipped with a flow-through system to manipulate the carbonate chemistry of natural seawater, by bubbling pure CO<sub>2</sub> gas, which has previously been described in detail (Range et al. 2011, 2012).

During the following week, the tanks with sediment were maintained at ambient temperature and photoperiod and were gradually exposed to 2 levels of seawater acidification ( $\Delta\text{pH}$ -0.3 and  $\Delta\text{pH}$ -0.6) and 1 unmanipulated control level ( $\Delta\text{pH}$ -CT), while maintained at ambient temperature and photoperiod. The reduction of 0.3 pH units corresponds to the average drop in oceanic pH predicted for the year 2100, under the IPCC-SRES A2 emission scenario; the reduction of 0.6 pH represents a more distant future (year 2300), under a scenario of unconstrained exploitation of fossil fuels (Nakicenovic et al. 2000, Caldeira & Wickett 2003, 2005, Joos et al. 2011). Each combination of pH level and habitat type was replicated in 3 tanks (18 in total). In addition, there were 2 control tanks, for each level of pH, with no sediment, for water analysis. Three other sediment patches (field controls) were collected from each type of habitat in the first (F-Sep) and last (F-Dec) days of the experiment. One sediment core (5 cm diameter × 15 cm length) was collected at the same time as each sample of macrobenthos for the determination of sediment characteristics (grain size, carbonate content, organic content and sediment pH).

#### Maintenance of the experiment

The experiment lasted 75 d, and during this period organisms in each tank were fed daily with a 1 l mixture of 2 microalgae (*Isochrysis galbana* and *Chaetoceros calcitrans*), and once a week, with a

supplement of fish food (TetraMin®). Two hours after this supplement was distributed, the water in the experimental tanks was always completely renewed and the flow rate was re-adjusted to 6 l h<sup>-1</sup>. To avoid confounding the effects of treatments with artefacts eventually caused by the spatial arrangement of the experimental units, the tanks assigned to different treatments were interspersed and their positions rotated once per week.

#### Determination of seawater variables

Temperature, salinity and pH were continuously monitored during the experiment using dedicated electrodes and the data logger function of the controller (Aqua Medic—AT control). These automatic records were validated against weekly determinations with a handheld probe (YSI ProPlus), which was also used to measure dissolved oxygen (DO). Salinity was calibrated with an AutoSal salinometer using IAPSO standard seawater, with a variation coefficient of 0.003%. The pH electrodes were standardised against Tris seawater buffers (ionic strength: 0.7 M), at a precision of 0.14%.

On Day 67 of the experiment (7 December 2011), seawater samples for chemical analyses were collected in all the experimental tanks and at 4 field locations within the Ria Formosa lagoon (Barra nova/Farol, 36° 58' 32.5" N, 7° 52' 32.2" W; Canal de Faro, 36° 59' 44.2" N, 7° 54' 41.8" W; Canal do Ramalhete, 37° 0' 20.2" N, 7° 58' 13.4" W; Marina de Faro, 37° 0' 54.7" N, 7° 56' 10.7" W). Samples for total alkalinity (TA) were immediately poisoned with 50% saturated HgCl<sub>2</sub> to stop biological activity and refrigerated until further processing. Within the next 48 h TA determinations were done by automatic titration with HCl (0.25 M HCl in a solution of 0.45 M NaCl) past the endpoint of 4.5 (Dickson et al. 2007) with an accuracy of ±2 µmol kg<sup>-1</sup>. The accuracy of TA measurements was controlled against certified reference material supplied by A. G. Dickson (Scripps Institution of Oceanography, San Diego, USA). Values of pH at *in situ* temperature were calculated from TA, pH and *in situ* temperature. For these calculations the carbon dioxide constants of Millero et al. (2006) were applied. Dissolved inorganic carbon (DIC) and partial pressure of CO<sub>2</sub> in seawater ( $p\text{CO}_2$ ) were calculated from the *in situ* temperature and corrected pH and TA using the carbonic acid dissociation constants given by Millero et al. (2006) and the CO<sub>2</sub> solubility coefficient of Weiss (1974). The CaCO<sub>3</sub> saturation state for calcite ( $\Omega_{\text{cal}}$ ) and aragonite ( $\Omega_{\text{ara}}$ ) were

calculated using the thermodynamic solubility product of calcite and aragonite taken from Morse et al. (1980). Determinations for nitrites (NO<sub>2</sub><sup>-</sup>), nitrates (NO<sub>3</sub><sup>-</sup>), ammonium (NH<sub>4</sub><sup>+</sup>) and phosphates (PO<sub>4</sub><sup>3-</sup>) were done on a MERCK NOVA 60 spectrophotometer, using Spectroquant® Test kits.

### Sediment characteristics

Sediment cores were carefully opened, measured and longitudinally sectioned in half. Each half was further sectioned into 3 layers, according to distance from the surface: 0–5 cm, 5–10 cm and 10–15 cm. Determination of pH in each layer was done directly in the wet sediment, after stabilisation of values, by using multiparameter equipment (Consort C861 with a SP10T DD6 electrode). Results were recorded once values had stabilised. Sediment sections were subsequently dried at 40°C and processed separately for determinations of other variables.

The method of loss on ignition was used to quantify the organic matter content of sediments. About 500 mg of dry sediment was burned at 450°C for 2 h (ash weight). The organic matter content (ash-free dry weight) was estimated by the ratio: [(dry weight) – (ash weight)] / (dry weight).

Grain size was first determined by wet separation of the silt and clay fraction from the sandy fraction with a 63 µm sieve. The silt and clay fraction (<63 µm) was recovered by sequential decantation of percolated material, dried and weighed. Sediments retained in the 63 µm sieve were subsequently dried and passed through a stack of 8 other sieves in a vibratory sieve shaker (5 min, 50 kpa), to quantify the coarser fractions: very fine sand (63 to <125 µm), fine sand (125 to <250 µm), medium sand (250 to <500 µm), coarse sand (500 µm to <1 mm), very coarse sand (1 to <2 mm), fine gravel (2 to <4 mm), medium gravel (4 to <8 mm) and coarse gravel (>8 mm). Finally, the percentages of the distinct fractions were calculated.

Sediment sub-samples were ground by mortar and pestle for determinations of carbonate content. The sample quantity to be used was estimated according to the reaction of a sample to HCl: a strong reaction required <1–2.5 g sediment, a weak reaction 5–10 g sediment. Carbonate content was determined with a Eijkelkamp calcimeter (Ref. No.: 08.53), which measures the volume of carbon dioxide produced during the reaction of the sample with hydrochloric acid. The carbonate content is expressed as equivalent calcium carbonate content.

### Taxonomic identification

The macrofaunal samples were sieved through a 500 µm mesh under a constant seawater flow. All organisms retained were preserved in 4% buffered formalin and stained with Rose Bengal. Macrofauna was firstly hand sorted into major taxonomic groups and subsequently counted and identified under a binocular stereomicroscope. Polychaeta, Echinodermata and Echiura were identified to the family level, while Crustacea, Gastropoda and Bivalvia were generally identified to genus or species. Given the large densities of the gastropod *Bittium* spp., individuals were only identified to the genus level. The other phyla present (Nemertea and Platyhelminthes) were represented by a residual number of individuals, so no further identification was attempted.

### Data analyses

Two orthogonal factors were considered in all the analyses: Habitat was considered a random factor with 2 levels (Sand and Mud), while Treatment was a fixed factor with 5 levels (F-Sep, ΔpH-CT, ΔpH-0.3, ΔpH-0.6, F-Dec). Permutational multivariate analysis of variance (PERMANOVA) was used to test for statistical differences in normalised sediment characteristics (Euclidean distance) and untransformed densities of macrofaunal assemblages (Bray-Curtis similarity). Preliminary analyses were also done on data transformed for presence/absence, revealing that differences in resemblance largely reflected differences in the abundances of individual taxa rather than changes in composition or frequency of occurrence. Accordingly, only the analyses for untransformed densities are presented here. All tests were done with unrestricted permutations (999) of raw data with Type III (partial) sums of squares and fixed effects summed to zero for mixed terms. Ordination by non-metric multidimensional scaling (MDS) and principal components analysis (PCA) were used to visualise patterns in the biological dataset and sediment characteristics, respectively. All multivariate analyses were done using the PRIMER 6 statistical package with the PERMANOVA+ add-on (PRIMER-E, Plymouth Marine Laboratory).

The similarity percentages routine (SIMPER) was used to examine the contribution of each variable to average resemblances between sample groups. The DIVERSE routine was used to calculate diversity indices for the entire assemblages and the dominant groups of organisms: number of organisms (*N*), num-



ber of taxa ( $S$ ), diversity ( $d$ ) and evenness ( $J'$ ). Two-way analyses of variance (ANOVA) were subsequently used on the densities of individual taxa highlighted by the SIMPER routine, diversity indices and sediment variables. Prior to these analyses, the assumption of homogeneity of variances was tested using Cochran's test. Differences between means have been considered statistically significant for  $p < 0.05$ . Whenever the interaction term was not a significant source of variation ( $p > 0.25$ ; Underwood, 1997), it was pooled with the residual and the mean squares [Res + (Ha  $\times$  Tr)] was used as denominator for the  $F$ -ratios of main effects. Multiple comparisons for means were done on significant Treatment effects, after pooling, using Student-Newman-Keuls (SNK) tests with significance levels adjusted using a sequential Bonferroni procedure. Univariate analyses and graphs were done with SigmaPlot software (Version 11.0, Systat Software, Inc.).

Distance-based linear models (DistLM) were used to examine the relationship between the biological resemblance matrix (Bray-Curtis similarity) of densities of macrofaunal and sediment characteristics. Firstly, the significance of the relationship was assessed for individual sediment variables with marginal tests (999 permutations). Significant variables ( $p < 0.05$ ) were subsequently included in model selection using the BEST procedure (i.e. all possible variable combinations). Distance-based redundancy analysis (dbRDA) was used for the ordination and visualisation of the best overall DistLM solution, according to the Akaike information criterion (AIC).

## RESULTS

### Physical-chemical characteristics of seawater

Measurements of pH during the 75 d of exposure in the experimental tanks ranged between 0.43 and 0.53 units within each pH level:  $\Delta\text{pH-CT}$ , 7.76–8.18;  $\Delta\text{pH-0.3}$ , 7.23–7.76; and  $\Delta\text{pH-0.6}$ , 7.12–7.55. The average daily temperature consistently decreased during exposure, from 22.6°C in September to 17.7°C in December. Salinity varied in a narrow range (28.62–31.93), with no discernible temporal pattern or trend associated with the experimental treatments. TA ranged from 3396 to 3861  $\mu\text{mol kg}^{-1}$ , with consistently smaller values in the Mud habitat, although this pattern was not significant. DO and nutrient concentrations of seawater in the exposure tanks also varied independently of the experimental factors. The measured ranges for these variables were: DO, 60–107%; nitrates, 0.45–0.64  $\text{mg l}^{-1}$ ; and phosphates, 0.15–0.21  $\text{mg l}^{-1}$ . Nitrites and ammonium were generally under the detection limit for the test kits (0.007 and 0.013  $\text{mg l}^{-1}$ , respectively).

All the variables described above, except for TA and nutrients, were continuously monitored in the experimental tanks (or controlled in the case of pH) during the exposure. Nevertheless, given that field measurements in the Ria Formosa were only done on Day 67, all values presented in Table 1 are relative to that day. Field measurements of temperature, TA, DIC and  $p\text{CO}_2$  were generally under the range observed in our experimental system, while field val-

Table 1. Seawater carbonate chemistry variables (means  $\pm$  SE) in different habitats, including Blank tanks without sediment. Measurements of pH (total scale), temperature (T), salinity (S) and total alkalinity (TA) were done on Day 67 (7 December 2011); dissolved inorganic carbon (DIC), partial pressure of  $\text{CO}_2$  in seawater ( $p\text{CO}_2$ ) and saturation state for calcite ( $\Omega_{\text{cal}}$ ) and aragonite ( $\Omega_{\text{ara}}$ ) were calculated from *in situ* T and S, and corrected pH and TA

$\Delta\text{pH}$	T (°C)	S	$\text{pH}_T$	TA ( $\mu\text{mol kg}^{-1}$ )	DIC ( $\mu\text{mol kg}^{-1}$ )	$p\text{CO}_2$ ( $\mu\text{atm}$ )	$\Omega_{\text{cal}}$	$\Omega_{\text{ara}}$
<b>Mud (n = 3)</b>								
CT	17.53 $\pm$ 0.03	31.59 $\pm$ 0.04	7.93 $\pm$ 0.01	3563 $\pm$ 2	3345 $\pm$ 15	868 $\pm$ 46	4.72 $\pm$ 0.24	3.02 $\pm$ 0.15
-0.3	17.50 $\pm$ 0.06	31.62 $\pm$ 0.04	7.60 $\pm$ 0.05	3499 $\pm$ 89	3448 $\pm$ 60	1979 $\pm$ 350	2.33 $\pm$ 0.58	1.49 $\pm$ 0.38
-0.6	17.60 $\pm$ 0.06	31.64 $\pm$ 0.03	7.41 $\pm$ 0.04	3554 $\pm$ 42	3596 $\pm$ 82	3180 $\pm$ 632	1.49 $\pm$ 0.28	0.95 $\pm$ 0.19
<b>Sand (n = 3)</b>								
CT	17.47 $\pm$ 0.03	31.65 $\pm$ 0.01	7.97 $\pm$ 0.01	3625 $\pm$ 83	3390 $\pm$ 77	788 $\pm$ 22	5.03 $\pm$ 0.18	3.21 $\pm$ 0.12
-0.3	17.53 $\pm$ 0.03	31.65 $\pm$ 0.03	7.64 $\pm$ 0.02	3690 $\pm$ 85	3628 $\pm$ 91	1857 $\pm$ 175	2.52 $\pm$ 0.17	1.61 $\pm$ 0.11
-0.6	17.53 $\pm$ 0.03	30.80 $\pm$ 0.86	7.39 $\pm$ 0.02	3558 $\pm$ 13	3611 $\pm$ 12	3300 $\pm$ 182	1.39 $\pm$ 0.04	0.89 $\pm$ 0.03
<b>Blank (n = 2)</b>								
CT	17.60 $\pm$ 0.00	31.70 $\pm$ 0.00	7.97 $\pm$ 0.01	3592 $\pm$ 13	3356 $\pm$ 3	798 $\pm$ 11	5.04 $\pm$ 0.29	3.22 $\pm$ 0.20
-0.3	17.55 $\pm$ 0.05	31.70 $\pm$ 0.00	7.61 $\pm$ 0.02	3637 $\pm$ 92	3583 $\pm$ 97	2012 $\pm$ 160	2.43 $\pm$ 0.00	1.56 $\pm$ 0.00
-0.6	17.65 $\pm$ 0.05	31.70 $\pm$ 0.02	7.31 $\pm$ 0.03	3663 $\pm$ 199	3747 $\pm$ 213	4131 $\pm$ 508	1.27 $\pm$ 0.04	0.81 $\pm$ 0.02
<b>Field (n = 4)</b>								
	14.23 $\pm$ 0.65	35.86 $\pm$ 0.13	8.04 $\pm$ 0.03	2684 $\pm$ 117	2415 $\pm$ 133	474 $\pm$ 86	4.79 $\pm$ 0.29	3.09 $\pm$ 0.19

ues of pH and salinity clearly exceeded that range. Seawater was generally supersaturated ( $\Omega > 1$ ) with respect to CaCO<sub>3</sub>, except for aragonite ( $\Omega_{\text{ara}}$ ) in the most extreme pH reductions ( $\Delta\text{pH-0.6}$ ).

### Sediment characteristics

Measurements of sediment cores revealed that the deepest layer (10–15 cm) was generally absent in cores collected from the exposure tanks, so it was excluded from the analyses. Preliminary tests also revealed that sediment characteristics generally did not differ between the 2 remaining layers (0–5 cm and 5–10 cm), which were pooled in subsequent analyses. PERMANOVA showed that sediment composition differed significantly between the 2 habitats considered, but was generally not affected by the experimental treatments (Table 2). The Sand habitat was dominated by a mixture of 34% fine sand (125  $\mu\text{m}$ ) and 32% silt and clay (<63  $\mu\text{m}$ ), while the Mud habitat was clearly dominated by silt and clay (46%). Despite this clear dominance, the SIMPER routine showed that the relative contributions of sediment variables to the average squared distance between habitats varied in a narrow range, between 10.61% for coarse sand (500  $\mu\text{m}$ ) and 4.58% for pH. This general pattern is clearly visible in the PCA ordination of normalised sediment variables (Fig. 1). The coarser fractions (4 and 8 mm) and pH were the only sediment variables that were unaffected by the 2 experimental factors (2-way ANOVAs, see Table S1 in the Supplement at [www.int-res.com/articles/suppl/m509p153\\_supp.pdf](http://www.int-res.com/articles/suppl/m509p153_supp.pdf)). Two sediment fractions

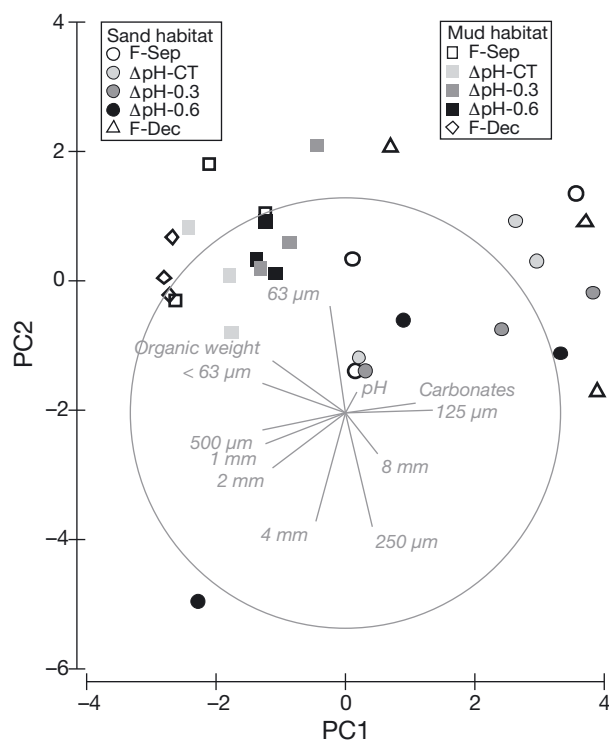


Fig. 1. Principal component analysis (PCA) of normalised values of sediment variables. For Mud and Sand habitats: lengths of overlaid vectors indicate the relative contribution of each variable to these 2 PC axes, which explain 59% of total variation. F: field control; CT: control

(63  $\mu\text{m}$  and 1 mm) and organic weight showed a significant Habitat  $\times$  Treatment interaction. In these cases there were significant differences among treatments only in the Mud habitat, but the pattern was inconsistent (see Table S2 in the Supplement). In

Table 2. PERMANOVA on normalised sediment characteristics (Euclidean distance) and untransformed densities of macrofauna (Bray-Curtis similarity). Habitat (Ha) is a random factor and Treatment (Tr) is fixed; unrestricted permutations (999) of raw data were used with Type III (partial) sums of squares and fixed effects summed to zero for mixed terms. df: degrees of freedom; MS: mean square ( $n = 3$ )

Source of variation	df	Sediment			All taxa			Bivalvia		
		MS	Pseudo- <i>F</i>	<i>p</i>	MS $\times 10^{-2}$	Pseudo- <i>F</i>	<i>p</i>	MS $\times 10^{-2}$	Pseudo- <i>F</i>	<i>p</i>
Habitat	1	102.25	13.18	0.001	278.29	20.29	0.001	253.38	11.65	0.001
Treatment	4	11.12	0.97	0.503	50.06	1.12	0.399	41.53	1.37	0.242
Ha $\times$ Tr	4	11.52	1.48	0.095	44.62	3.25	0.033	30.36	1.40	0.122
Residual	20	7.76			13.72			21.76		
Source of variation	df	Crustacea			Gastropoda			Polychaeta		
		MS $\times 10^{-2}$	Pseudo- <i>F</i>	<i>p</i>	MS $\times 10^{-2}$	Pseudo- <i>F</i>	<i>p</i>	MS $\times 10^{-2}$	Pseudo- <i>F</i>	<i>p</i>
Habitat	1	127.71	8.02	0.001	128.32	4.87	0.003	271.22	14.94	0.001
Treatment	4	43.47	1.19	0.330	65.25	1.37	0.158	32.99	0.88	0.610
Ha $\times$ Tr	4	36.63	2.30	0.004	47.51	1.80	0.030	37.41	2.06	0.006
Residual	20	15.92			26.36			18.16		

both types of habitat considered, the smallest values of sediment pH were recorded in field samples.

### Macroinvertebrate assemblages

A total of 3154 individuals, distributed across 74 taxa, were identified during this study. Polychaeta clearly dominated the assemblages in terms of the number of taxa (22 families) and abundance (1418 individuals). Other relevant groups were: Gastropoda (16 taxa, 942 individuals), Bivalvia (16 taxa, 314 individuals) and Crustacea (14 taxa, 471 individuals). Four other groups of organisms were scarcely represented in terms of abundance: Nemertea (5 individuals), Echinodermata (2 individuals), Echiura (1 individual) and Platyhelminthes (1 individual; for further details see Table S3 in the Supplement).

PERMANOVA showed that the compositions of the entire macrofaunal assemblages and of the dominant groups of organisms differed significantly between the 2 types of habitat considered (Table 2). In all the major groups, except Bivalvia, there was also a significant Habitat  $\times$  Treatment interaction. Pairwise tests showed that, in the Sand habitat, December field controls differed significantly from all the other treatments, while in the Mud habitat, both field controls (September and December) differed from the *ex situ* treatments, which did not differ among themselves.

Considering the large dissimilarities between habitats and the PERMANOVA results, we decided to proceed with separate non-metric multi-dimensional scaling (nMDS–Bray–Curtis similarities) analyses for each habitat. The nMDS of untransformed densities

of organisms clearly shows smaller similarities among samples in the *ex situ* treatments, relative to field controls (Fig. 2). The SIMPER routine ranked taxa according to their contributions to overall dissimilarities among treatments. The main contributors (>5%) in each habitat type were: Anthuridae (Crustacea, 14.69%), *Diogenes pugilator* (Crustacea, 11.83%), Maldanidae (Polychaeta, 7.6%) and *Upogebia pusilla* (Crustacea, 6.23%) in Sand and *Bittium* spp. (Gastropoda, 32.27%), Ampharetidae (Polychaeta, 9.62%), Nephtyidae (Polychaeta, 9.50%), *Melita palmata* (Crustacea, 9.07%) and Oweniidae (Polychaeta, 6.27%) in Mud.

Two-way ANOVA showed that, among the strongest contributors to dissimilarities, only the polychaete family Maldanidae was unaffected by the habitat type (Table S4 in the Supplement). Three other taxa also showed significant Habitat  $\times$  Treatment interactions: Anthuridae (Sand) and Ampharetidae and *Bittium* spp. (Mud). Pairwise SNK tests showed that these interactions were always caused by significant differences between field controls and the pH manipulations, which did not differ among themselves (Fig. 3). Vectors representing the correlations between densities of these 3 taxa and the MDS ordination show particularly strong associations with field controls (Fig. 2). Furthermore, in 8 of the 9 taxa analysed, the maximum densities were recorded in the field samples. The only exception to this pattern was the amphipod *Melita palmata*, which was consistently more abundant in the *ex situ* treatments, relative to field controls. Additionally, densities of *M. palmata* among the 3 pH levels considered varied inversely to the pH gradient, although this pattern was not significant (Fig. 3).

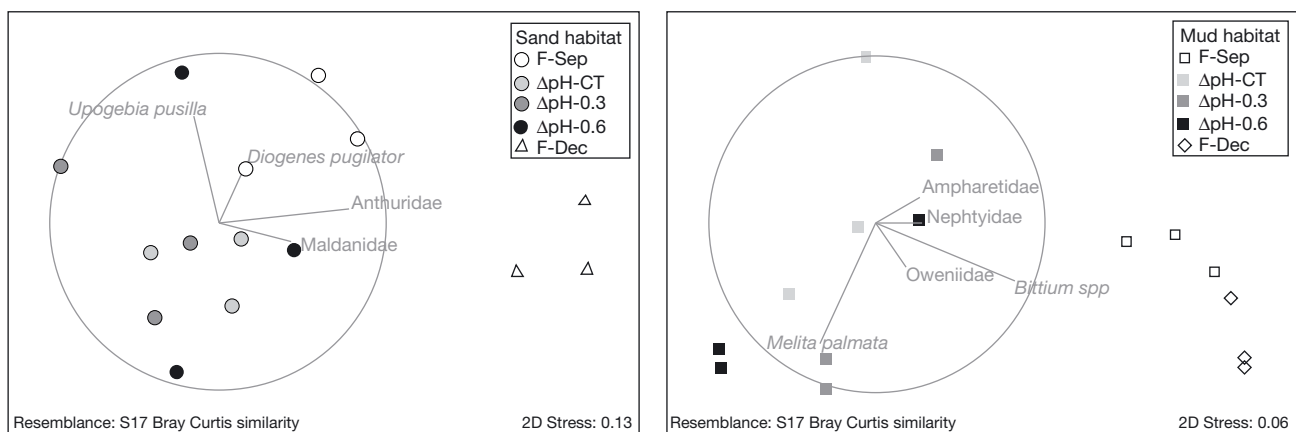


Fig. 2. Non-metric multi-dimensional scaling (MDS) on untransformed densities of the entire faunal assemblage. For Sand habitat (left panel) and Mud habitat (right panel) vectors represent the correlations between the MDS ordination and densities of taxa contributing >5% to overall dissimilarities. F: field control; CT: control



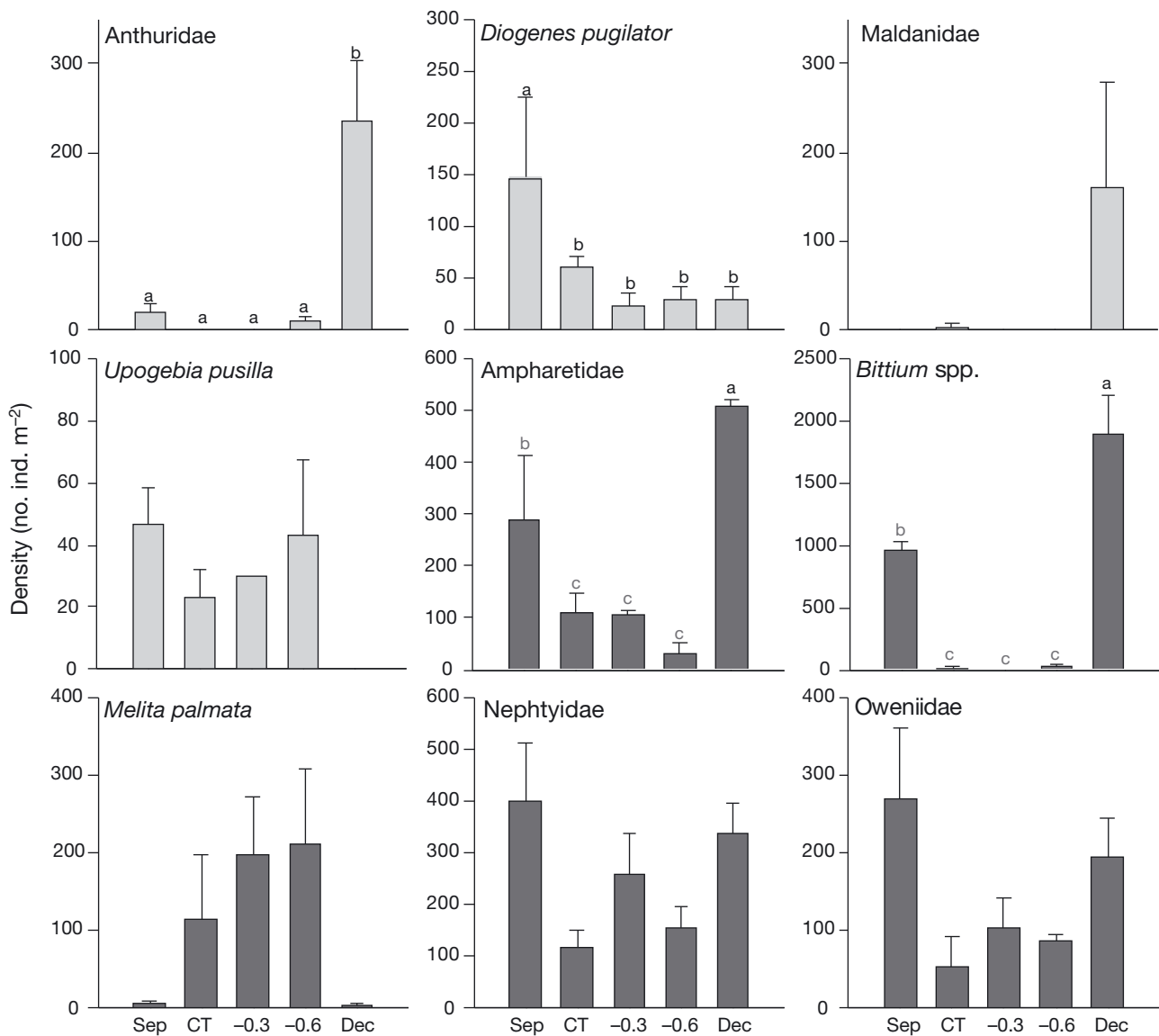


Fig. 3. Densities (means  $\pm$  SE) of taxa contributing  $>5\%$  to overall dissimilarities among treatments (2 field controls: September and December; 3 pH levels: control (CT),  $-0.3$  and  $-0.6$ ) within each habitat (Sand: light grey and Mud: dark grey). Letters (a, b) identify distinct groups of means within each habitat (Student-Newman-Keuls tests)

The 2-way ANOVA on the diversity indices for the entire assemblage consistently revealed significant Habitat  $\times$  Treatment interactions (Table S5 in the Supplement). Pairwise (SNK) tests showed that significant differences among treatments were always between field controls and *ex situ* treatments, which did not differ among themselves (Table 3). Densities of organisms ( $N$ ) were consistently larger in field samples, relative to *ex situ* treatments. The number of taxa ( $S$ ) generally followed the same pattern, except for the December samples in the Sand habitat ( $F$ -Dec  $<$   $\Delta$ pH-0.6). In contrast, the average values for

diversity ( $d$ ) and evenness ( $J'$ ) generally peaked in one of the *ex situ* treatments.

With the exception of Crustacea, densities ( $N$ ) and numbers of taxa ( $S$ ) were consistently larger in Mud than in Sand habitat. The 4 dominant groups of organisms also showed a significant Habitat  $\times$  Treatment interaction for densities. Polychaeta, Bivalvia, Gastropoda and Crustacea in Sand followed the pattern described above for the entire assemblage, while Crustacea in Mud showed the opposite pattern:  $N$  was larger in *ex situ* treatments, relative to field samples (Table 3). Gastropoda was the only group to show

Table 3. Diversity indices (means  $\pm$  SE,  $n = 3$ ) for the major taxonomic groups in each Habitat (Ha) and Treatment (Tr) combination ( $S$ : number of taxa;  $N$ : density;  $d$ : diversity;  $J'$ : evenness). 'All taxa' also includes Echinodermata, Echiura, Nemertea and Platyhelminthes. Significant terms in the ANOVA are indicated for each variable; superscript letters identify consistent groups of means within each Habitat for significant Ha  $\times$  Tr interactions (Student-Newman-Keuls tests). Empty cells for Gastropoda denote zero individuals in at least one replicate. F: field control; CT: control

Index	ANOVA term	Sand habitat			Mud habitat					
		F-Sep	$\Delta$ pH-CT	$\Delta$ pH-0.6	F-Dec	$\Delta$ pH-CT	$\Delta$ pH-0.6	F-Dec		
<b>Polychaeta</b>										
$S$	Ha	7.0 $\pm$ 1.0	3.0 $\pm$ 0.6	7.0 $\pm$ 1.5	7.3 $\pm$ 0.3	10.7 $\pm$ 0.9	6.7 $\pm$ 0.9	8.7 $\pm$ 0.3	6.7 $\pm$ 1.8	11.7 $\pm$ 1.9
$N$	Ha $\times$ Tr	200 $\pm$ 66	43 $\pm$ 19	107 $\pm$ 24	310 $\pm$ 143	1197 $\pm$ 268 <sup>b</sup>	400 $\pm$ 21 <sup>a</sup>	660 $\pm$ 64 <sup>a</sup>	393 $\pm$ 113 <sup>a</sup>	1383 $\pm$ 70 <sup>b</sup>
$d$	Ha $\times$ Tr	1.15 $\pm$ 0.16	0.54 $\pm$ 0.10	1.27 $\pm$ 0.27	1.16 $\pm$ 0.11	1.37 $\pm$ 0.13	0.94 $\pm$ 0.14	1.18 $\pm$ 0.04	0.94 $\pm$ 0.25	1.47 $\pm$ 0.25
$J'$		0.90 $\pm$ 0.04	0.96 $\pm$ 0.04	0.95 $\pm$ 0.01	0.81 $\pm$ 0.12	0.71 $\pm$ 0.04	0.82 $\pm$ 0.01	0.73 $\pm$ 0.03	0.82 $\pm$ 0.04	0.70 $\pm$ 0.03
<b>Crustacea</b>										
$S$	Ha	4.3 $\pm$ 0.9	3.7 $\pm$ 0.3	4.0 $\pm$ 1.2	2.7 $\pm$ 0.3	2.7 $\pm$ 0.3	4.0 $\pm$ 1.2	4.3 $\pm$ 0.3	2.7 $\pm$ 0.3	3.0 $\pm$ 0.6
$N$	Ha $\times$ Tr	240 $\pm$ 106	113 $\pm$ 19	70 $\pm$ 17	287 $\pm$ 67	47 $\pm$ 17	160 $\pm$ 70	243 $\pm$ 73	233 $\pm$ 95	63 $\pm$ 23
$d$		0.62 $\pm$ 0.12	0.56 $\pm$ 0.06	0.46 $\pm$ 0.11	0.30 $\pm$ 0.06	0.45 $\pm$ 0.09	0.65 $\pm$ 0.26	0.64 $\pm$ 0.12	0.34 $\pm$ 0.10	0.49 $\pm$ 0.09
$J'$		0.80 $\pm$ 0.10	0.82 $\pm$ 0.03	0.88 $\pm$ 0.04	0.59 $\pm$ 0.10	0.86 $\pm$ 0.10	0.72 $\pm$ 0.19	0.54 $\pm$ 0.16	0.50 $\pm$ 0.23	0.93 $\pm$ 0.04
<b>Bivalvia</b>										
$S$	Ha $\times$ Tr	3.0 $\pm$ 0.6	2.0 $\pm$ 0.0	3.3 $\pm$ 0.7	0.7 $\pm$ 0.7	4.0 $\pm$ 1.2	6.0 $\pm$ 0.6	3.7 $\pm$ 0.3	4.3 $\pm$ 1.2	6.3 $\pm$ 0.3
$N$	Ha $\times$ Tr	47 $\pm$ 13	20 $\pm$ 0	33 $\pm$ 7	10 $\pm$ 10	133 $\pm$ 38 <sup>a</sup>	153 $\pm$ 20 <sup>a</sup>	110 $\pm$ 65 <sup>a</sup>	110 $\pm$ 42 <sup>a</sup>	417 $\pm$ 41 <sup>b</sup>
$d$	Ha $\times$ Tr	0.52 $\pm$ 0.12	0.33 $\pm$ 0.00	0.65 $\pm$ 0.16	0.29 $\pm$ 0.00	0.60 $\pm$ 0.22	1.00 $\pm$ 0.13	0.62 $\pm$ 0.10	0.70 $\pm$ 0.22	0.88 $\pm$ 0.04
$J'$	Ha $\times$ Tr	0.92 $\pm$ 0.06 <sup>b</sup>	1.00 $\pm$ 0.00 <sup>b</sup>	1.00 $\pm$ 0.00 <sup>b</sup>	0.92 $\pm$ 0.00 <sup>a</sup>	0.84 $\pm$ 0.12	0.86 $\pm$ 0.03	0.93 $\pm$ 0.05	0.90 $\pm$ 0.04	0.72 $\pm$ 0.01
<b>Gastropoda</b>										
$S$	Ha	1.00 $\pm$ 0.58	2.33 $\pm$ 0.33	0.33 $\pm$ 0.33	0.67 $\pm$ 0.67	2.67 $\pm$ 0.33	3.33 $\pm$ 0.33	3.00 $\pm$ 1.15	0.67 $\pm$ 0.33	3.00 $\pm$ 1.15
$N$	Ha $\times$ Tr	27 $\pm$ 15	23 $\pm$ 3	7 $\pm$ 7	7 $\pm$ 7	983 $\pm$ 61 <sup>b</sup>	53 $\pm$ 7 <sup>a</sup>	50 $\pm$ 23 <sup>a</sup>	23 $\pm$ 19 <sup>a</sup>	1953 $\pm$ 299 <sup>c</sup>
$d$	Tr	0.13 $\pm$ 0.13	0.42 $\pm$ 0.08	0.33 $\pm$ 0.00	0.33 $\pm$ 0.00	0.24 $\pm$ 0.05	0.59 $\pm$ 0.07	0.47 $\pm$ 0.26	0.00 $\pm$ 0.00	0.27 $\pm$ 0.16
$J'$	Tr	0.7 $\pm$ 0.0	1.0 $\pm$ 0.0	1.0 $\pm$ 0.0		0.1 $\pm$ 0.0	0.9 $\pm$ 0.0	0.9 $\pm$ 0.0		0.2 $\pm$ 0.1
<b>All taxa</b>										
$S$	Ha $\times$ Tr	15.3 $\pm$ 2.3	11.0 $\pm$ 1.2	15.0 $\pm$ 2.6	11.7 $\pm$ 1.2	20.3 $\pm$ 0.7	20.0 $\pm$ 0.0	20.0 $\pm$ 1.7	15.0 $\pm$ 3.1	24.3 $\pm$ 1.9
$N$	Ha $\times$ Tr	513 $\pm$ 113	200 $\pm$ 40	263 $\pm$ 65	617 $\pm$ 159	2363 $\pm$ 338 <sup>b</sup>	770 $\pm$ 79 <sup>a</sup>	1067 $\pm$ 75 <sup>a</sup>	767 $\pm$ 80 <sup>a</sup>	3827 $\pm$ 401 <sup>c</sup>
$d$	Ha $\times$ Tr	2.3 $\pm$ 0.3	1.9 $\pm$ 0.2	2.5 $\pm$ 0.4	1.7 $\pm$ 0.1	2.5 $\pm$ 0.1	2.9 $\pm$ 0.0	2.7 $\pm$ 0.2	2.1 $\pm$ 0.4	2.8 $\pm$ 0.2
$J'$	Ha $\times$ Tr	0.86 $\pm$ 0.05 <sup>b</sup>	0.88 $\pm$ 0.01 <sup>b</sup>	0.92 $\pm$ 0.03 <sup>b</sup>	0.70 $\pm$ 0.03 <sup>a</sup>	0.63 $\pm$ 0.00 <sup>a</sup>	0.83 $\pm$ 0.02 <sup>b</sup>	0.75 $\pm$ 0.01 <sup>b</sup>	0.77 $\pm$ 0.02 <sup>b</sup>	0.59 $\pm$ 0.02 <sup>b</sup>

significant differences among treatments independently of the habitat, for diversity ( $d$ ) and evenness ( $J'$ ). It was also the only group where  $S$  and  $N$  consistently varied according to the gradient in the pH manipulations, although this pattern was not significant (Table 3). Bivalves were the only major group that did not differ, in terms of composition or structure, between *ex situ* treatments and field controls (Table 2), although significant differences were detected in the diversity indexes ( $J'$  for Sand and  $N$  for Mud; Table 3).

### Relationship between sediment characteristics and macrofaunal assemblages

The DistLM marginal tests indicated that 9 of the 12 sediment variables considered were significantly related to the densities of macrobenthos: silt and clay (<63  $\mu\text{m}$ ), very fine sand (63 to <125  $\mu\text{m}$ ), fine sand (125 to <250  $\mu\text{m}$ ), medium sand (250 to <500  $\mu\text{m}$ ), coarse sand (500  $\mu\text{m}$  to <1 mm), very coarse sand (1 to <2 mm), fine gravel (2 to <4 mm), carbonates and organic weight. Marginal tests were not significant for medium gravel (4 to <8 mm), coarse gravel (>8 mm) or sediment pH, which were excluded from subsequent analyses. The overall best DistLM solution included: organic weight, medium sand and coarse sand. Vector overlays were found for these 3 variables on the dbRDA plot; this can be interpreted as the effect of each variable on the ordination, where the length of the vector is proportional to the contribution of that variable for the dbRDA axes (Fig. 4). Organic weight of the sediment was the strongest contributor to Axis 1, which explained most of the variation in the ordination. Polychaetes were strongly associated with this variable, particularly the families Nephthyidae, Oweniidae and Ampharetidae (Pearson correlations: 0.80, 0.70 and 0.62, respectively), as was the gastropod *Bittium* spp. (Pearson correlation: 0.60). The taxa with the strongest correlations with coarse sand (500  $\mu\text{m}$ ) were the same 3 families of polychaetes: Ampharetidae, Nephthyidae and Oweniidae (Pearson correlations: 0.64, 0.61 and 0.61, respectively) and the bivalves *Loripes lucinalis* and *Abra* spp. (Pearson correlations: 0.60 and 0.59, respectively). As is apparent from Fig. 4, most taxa were negatively correlated with medium sand (250  $\mu\text{m}$ ), including all 6 taxa mentioned above.

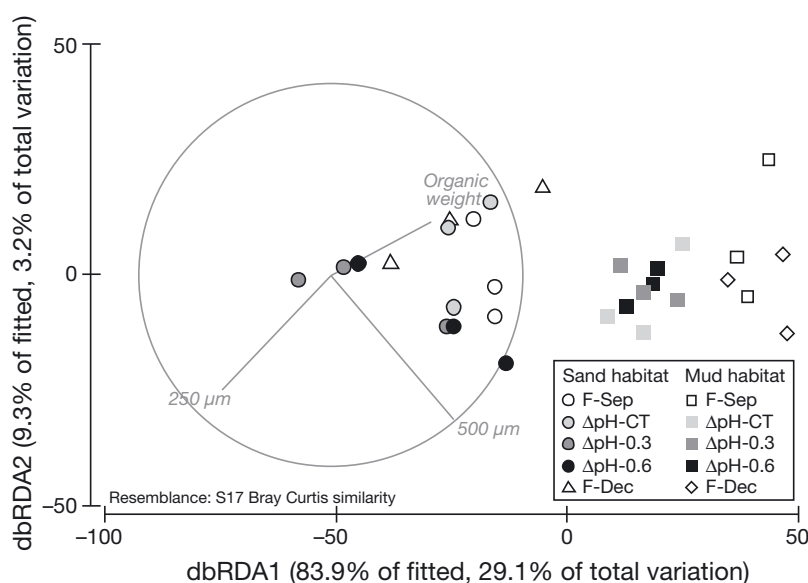


Fig. 4. Diagram of distance-based redundancy analysis (dbRDA) for the best distance-based linear models (DistLM) solution. Vector overlays include the combination of variables selected with the BEST procedure and Akaike's information criterion. Lengths of vectors indicate the relative influence of each variable for the ordination. F: field control; CT: control

## DISCUSSION

### Physical-chemical characteristics of seawater

On average, the planned differences in seawater pH between control and acidified treatments were achieved, although the magnitude of that difference varied among levels of the factor Habitat. The smallest pH values were generally recorded in tanks with Mud, except for the most extreme pH reduction ( $\Delta\text{pH}$ -0.6; Table 1), which reached minimum values in the Blank tanks (without sediment). The latter result does not seem to agree with the consistently smaller TA values in the Mud habitat, which should decrease the CO<sub>2</sub> buffering capacity, allowing larger reductions of pH in that treatment. Carbonate dissolution/precipitation and benthic microbial processes are among the main drivers of alkalinity and pH in coastal ecosystems, through sediment–water fluxes (Duarte et al. 2013). Bacterial sulfate reduction and ammonia production, derived from the anaerobic decomposition of organic matter, can significantly increase TA. On the other hand, oxidation of these constituents generates acid, which contributes to a decrease in TA (Andersson et al. 2007). The fact that no significant differences in TA were detected in our system suggests that the net balance of these processes was unaffected by the increased levels of CO<sub>2</sub>.

All the physical-chemical variables of seawater directly measured in the Ria Formosa lagoon (temperature, TA, pH and salinity) were outside the range observed in our experimental system. The range of TA values in the experimental tanks (3396 to 3861  $\mu\text{mol kg}^{-1}$ ) was within the range previously observed in the same seawater supply (Range et al. 2011, 2012). In contrast to those previous studies, measurements of TA in the lagoon (2607 to 2885  $\mu\text{mol kg}^{-1}$ ) were clearly under the range observed in our experimental system, although still exceeding typical oceanic values (2320  $\mu\text{mol kg}^{-1}$ ; Brewer 1997). The Ria Formosa is a mesotidal shallow lagoon, separated from the Atlantic Ocean (Bay of Cadiz, SW Iberian Peninsula) by a chain of 5 barrier islands and 2 peninsulas. Most of the lagoon is well flushed by tidal exchange and has small freshwater inputs, as most of the incoming streams are ephemeral, with torrential flows during winter and very limited runoff during summer. Five main water bodies have been distinguished in the Ria Formosa lagoon, according to a combination of the natural drainage patterns, human pressures and biological quality elements (Ferreira et al. 2006). For logistical reasons, the field locations sampled during the present study were in the middle part of the lagoon, in a water body to the east of the experimental hatchery. Another recent study in the same water body reported similar values of TA (2622 to 2774  $\mu\text{mol kg}^{-1}$ ; Alexandre et al. 2012).

These combined results denote substantial temporal and spatial variations of seawater carbonate chemistry and suggest the existence of consistent gradients in TA among the main water bodies within the lagoon, which are probably related to freshwater discharges, evaporation and nutrient exchanges with sediments. Given the scarcity of published datasets on the biogeochemistry of coastal marine areas (which are not regularly covered by hydrographic research cruises, buoys and other autonomous monitoring systems), it is difficult to assess if the patterns observed in the Ria Formosa are representative of similar coastal systems in other parts of the world. This issue clearly merits further investigation in future studies.

### Sediment characteristics

Hypotheses s1, s2 and s3 were not supported. Sediment pH was unaffected by the experimental factors. Nevertheless, in both types of habitat, the smallest values of sediment pH were recorded in field controls, suggesting that these intertidal sediments may

be naturally exposed to pH values smaller than the ones imposed in our study. Previous  $\text{CO}_2$  forcing experiments have shown that the pH of sediments can be smaller (Wood et al. 2008) or larger (Dashfield et al. 2008) than in the overlying seawater. Carbonate content and sediment grain size differed significantly between the 2 habitats, but not among the different treatments. Considering the observed reduction of sediment volume (i.e. loss of the deepest layer, 10 to 15 cm) in the *ex situ* treatments, this was a surprising result. We expected this loss of volume to be reflected in sediment composition, at least in the finer grain sizes, which could have been washed away or percolated through the 60  $\mu\text{m}$  mesh at the bottom of the tanks. Instead, sediment composition within each habitat type was largely unaffected during the experiment. There was a decreasing trend for organic content in the Mud habitat, which is probably related to the consumption of seagrass fragments and other detritus that were visibly more abundant on the surface of the sediments at the start than at the end of the experiment. Nevertheless, this pattern was independent of the pH treatments.

Previous observations or predictions of increased dissolution of carbonate sediments (Yates & Halley 2006, Andersson et al. 2007) and decreased grain size (Dove et al. 2013, Wisshak et al. 2013) caused by ocean acidification (OA) originated from studies in coral reefs. Sediments in coral reefs are mainly composed of biogenic  $\text{CaCO}_3$ , derived from the erosion of skeletons of benthic calcifying organisms. The carbonate chemistry of the overlying seawater also differs considerably from the one in our study. Accordingly, the combination of relatively low  $\text{CaCO}_3$  content of sediments (<8%) and the elevated TA of seawater in the Ria Formosa may have contributed to buffer any effect of the increased  $\text{CO}_2$  treatments in terms of sediment pH, dissolution, or grain size.

### Macroinvertebrate assemblages

Hypotheses b1 to b5 were not supported. The assemblages differed significantly between the 2 types of habitat, but not among the 3 acidification levels tested. There were, however, significant differences between the macrofauna in *ex situ* treatments and in field controls. These differences were generally caused by reduced abundances or deletions of those species most sensitive to the stress of confinement in artificial conditions (i.e. experimental stress or experimental artefact), although some crustaceans showed the opposite pattern. Given that the compo-

sition and structure of benthic assemblages are strongly controlled by interactions between species, these different sensitivities may be translated into mechanisms of density compensation (Kroeker et al. 2011), which inevitably increase the difficulty to detect the effects of CO<sub>2</sub> perturbations. Evaluation of the similarity between the results from laboratory and field controls has been acknowledged as an important aspect to consider in ecological experiments (Underwood & Peterson 1988), which has been largely disregarded in OA research.

The type of experimental artefact described above is a common feature of studies done in aquaria or mesocosms, involving the removal of organisms from their natural habitats. Such experimental constraints can, however, have a significant impact on the organisms and influence interpretations of results, by confounding the effects of treatments with the stress of being held in an artificial environment (Russell et al. 2013). This is particularly critical when the experiments involve multiple species, since there is an increased probability that artefacts interact with treatments and potentially weaken causal inferences (Peterson & Black 1994). Accordingly, efforts should be made to reduce experimental stress as much as possible (Widdicombe et al. 2010). In the present study, sediments were collected during an equinoctial low tide, from an area adjacent to the experimental hatchery, which is only exposed during a few days per year. This procedure attempted to minimise variations in tidal regime, ambient temperature, photoperiod, seawater chemistry, or other disturbances resulting from handling and transport. Nevertheless, differences in the physical-chemical characteristics of seawater and other aspects unrelated to carbonate chemistry (i.e. frequency of emersion, depth, flow regime, food supply, recruitment, mortality and reduced emigration and immigration) may have altered the responses of individual species or their interactions, contributing to disguise or amplify the effects of seawater acidification (Riebesell et al. 2010).

Hypotheses b6 and b7 were not supported. Densities ( $N$ ) and number of species ( $S$ ) of gastropods and bivalves were consistently larger in Mud than in Sand, but differences among treatments were only found between field controls and *ex situ* treatments. Despite  $S$  and  $N$  in assemblages of gastropods varying according to the gradient in the *ex situ* treatments, the 3 pH levels tested did not differ significantly. If, however, field controls were excluded from the analyses, the variability among treatments and among replicates (residual variance), as well as

the degrees of freedom of the analyses would be reduced. Under these circumstances, the number of gastropod species would have been found to vary significantly in the Mud habitat ( $\Delta\text{pH-CT} = \Delta\text{pH-0.3} > \Delta\text{pH-0.6}$ , analyses not included). Nonetheless, this apparent increase in the power of the experiment would be achieved by disregarding natural variability in the source populations, compromising any attempt to extrapolate the results to natural systems. This situation clearly illustrates the potential for artefacts interacting with treatments in mesocosm experiments and emphasises the relevance of incorporating field controls in the design, to minimise Type I errors and adequately attribute causal effects.

Gastropod assemblages in the Mud habitat were strongly dominated by *Bittium* spp., which was the strongest overall contributor to dissimilarities among treatments (32.27 %). The large reduction of densities of *Bittium* spp. in the *ex situ* treatments cannot be solely attributed to mortality and was, at least partially, caused by a behavioural response to confinement. In fact, individuals were frequently observed escaping from the tanks during the experiment. Although it would have been interesting to quantify these escapes, this was not feasible. Alternatively, escapes could have been reduced by covering the tanks with fine mesh, but this would also cause undesirable effects (i.e. reduced light and oxygenation, increased epiphytic growth), so it was not pursued. Behavioural responses to OA have previously been reported in various groups or organisms, including gastropods (Bibby et al. 2007, Ellis et al. 2009, Manríquez et al. 2013). *Bittium reticulatum*, which was the dominant gastropod in our samples, has been found to be absent from sites adjacent to CO<sub>2</sub> vents in the Mediterranean (Hall-Spencer et al. 2008), which could be the result of an active avoidance of these highly acidified habitats. Given the very small densities of *Bittium* spp. remaining in the tanks at the end of the experiment, our results were inconclusive regarding the possible avoidance of acidified seawater by these gastropods.

The fact that assemblages of bivalves did not differ, in terms of composition or structure, between *ex situ* treatments and field controls suggests they were well adjusted to the conditions in the experimental tanks. This tolerance was probably related to the microalgal diet supplied. Bivalves are filter-feeders, and the mixture of microalgae used here (*Isochrysis galbana* and *Chaetoceros calcitrans*) has been successfully used to rear different species of bivalves, under similar levels of seawater acidification (Range et al. 2011,



2012). Bivalves were also the only group that did not contribute any species to the list of the largest contributors to dissimilarities among treatments (Table S5 in the Supplement) and consistently showed relatively large values of evenness (Table 3). This is largely explained by the relatively small densities of bivalves in our samples. In fact, the 314 bivalves found represent <10% of the overall number of individuals, which is the smallest contribution among the 4 major groups.

Although the duration of our study (75 d) largely exceeded the median duration of experiments about the effects of OA on the survival of marine organisms (35 d), a recent long-term study on intertidal polychaetes showed that the effects of OA may only become apparent after longer exposures (Godbold & Solan 2013). Furthermore, the authors caution against over emphasising the conclusions from short-term experiments when forecasting the ecological consequences of climatic forcing. As emphasised by Russell et al. (2013), longer term experiments are likely to be more representative of potential future changes to ecosystems, because they will not assess individual acclimation, but rather selection or other population-level effects. Ultimately, a reliable assessment of the ecosystem-level responses to OA will require larger and more realistic experiments, which adequately consider the temporal and spatial variability of natural ecosystems.

Hypotheses b8 and b9 were supported. Polychaetes and crustaceans followed the general pattern described for whole assemblages: their densities were unaffected by the pH manipulations and generally differed between habitat types. One notable exception was the polychaete family Maldanidae, whose large variations among replicates prevented the detection of significant differences (Table S4 in the Supplement). Densities of polychaetes were consistently larger in field samples, relative to *ex situ* treatments, suggesting a high sensitivity to the stress of being held in artificial environments (experimental stress). This pattern is particularly relevant because the perceived tolerance of polychaetes to increased levels of CO<sub>2</sub> is mostly based on *ex situ* studies done in aquaria or small mesocosms, without field controls (Batten & Bamber 1996, Widdicombe & Needham 2007, Ries et al. 2009, Hale et al. 2011), where the responses to experimental stress could not be assessed.

In contrast, some peracarid crustaceans increased their abundances in the *ex situ* treatments, relative to field controls, particularly the amphipods *Melita palmata* and *Apoehyale prevostii*. Both species have been classified as omnivorous, taking advantage of

different resources, but with detritus as the dominant food item (Guerra-García et al. 2014). Accordingly, these amphipods were probably not affected by food quality or quantity in the experimental tanks, and the reduced abundances of other groups may have released them from pressures from competitors or predators. Recruitment was not limited by the experimental setup either. In fact, amphipods are direct developers, lacking free-living larval stages, and the duration of their embryonic development is generally inferior to the duration of the experiment, at least in temperate regions. The few previous studies that have directly tested the effects of increased CO<sub>2</sub> and decreased pH on marine amphipods reported idiosyncratic results on survival, reproduction and growth (Egilsdottir et al. 2009, Hauton et al. 2009, Poore et al. 2013). In field conditions and in multi-species mesocosms, the response of amphipods to OA has also been variable, including reductions in abundance and richness (Hale et al. 2011, Christen et al. 2013), no relationship with pH gradients (Cigliano et al. 2010) and increased abundances (Kroeker et al. 2011). Given their specific life-history traits and the considerable variations previously observed in the responses, amphipods and other peracarid crustaceans seem particularly well suited for investigating population- and assemblage-level response mechanisms to OA.

As emphasised by Hale et al. (2011), even closely related species can show very different responses to OA, so broad generalisations about response patterns of phyla do not seem advisable at the current state of our knowledge. Clearly, the response of assemblages is determined by species-specific tolerances and changes in ecological interactions. Previous work has also shown that other factors, such as nutritional status (Melzner et al. 2011) or source population (Range et al. 2014), can also cause substantial intra-specific variation in the responses (Kroeker et al. 2013). Given that the overwhelming majority of these results originated from *ex situ* studies, typically conducted in aquaria or small mesocosms, without field controls, the response of organisms to confinement in artificial environments (experimental stress) cannot be assessed. As emphasised by our results, the potential for confounding effects caused by interactions of experimental stress with the effects of OA is, therefore, considerable. This underlines the need to increase the realism in this field of research, which can be achieved by a combined approach of controlled mesocosm and laboratory studies with mensurative and manipulative field experiments (Doney et al. 2009, Wernberg et al. 2012).

### Relationship between sediment characteristics and macrofaunal assemblages

Hypothesis r1 was not supported. A significant Habitat × Treatment interaction was found for densities of the 4 dominant groups of organisms, but this pattern was unrelated to the 3 levels of seawater acidification tested. Significant differences among treatments did occur more frequently in Mud than in Sand, but they were always between field controls and *ex situ* treatments. Among the 12 sediment variables considered, only 3 were not significantly related to the densities of macrobenthos (medium gravel, coarse gravel and sediment pH). The coarser grain sizes represented a very small fraction of the total sediments in both habitats, which explains their small contribution to the composition and structure of the assemblages. The presence of bioturbating organisms has previously been shown to affect pH of sediments, particularly in the superficial layer (Dashfield et al. 2008, Widdicombe et al. 2013). In the present study, however, sediment pH was not directly or indirectly affected by the pH of the overlaying seawater and neither of these factors had a structuring effect on macrobenthic assemblages.

The main structuring factors for these assemblages were the organic content and granulometry of sediments, particularly the medium and coarse sand fractions (Fig. 4). Sediments in our samples were composed of a mixture of grain sizes, but predominately fine sand and silt and clay. In general, coarse intertidal sediments drain fast and retain little water and organic matter. In contrast, fine sediments such as muds have the grains tightly packed together, which precludes interstitial circulation of water and oxygen. Medium and fine sands usually have abundant macrofauna, but densities tend to be larger in muds, because there is a larger organic matter content per unit area (Gray 1981). This general pattern was clearly discernible in our samples, particularly in field controls. Most taxa were negatively correlated with medium sand and had larger abundances in the Mud habitat, which was clearly dominated by silt and clay and had significantly larger amounts of organic matter (Table S2 in the Supplement).

The bivalves with the strongest correlations with coarse sand, *Loripes lucinalis* and *Abra* spp. (including *A. alba*, *A. prismatica*, *A. segmentum* and *A. tenuis*) are common species in the mud and muddy sand sediments of the Ria Formosa lagoon (Gamito 2008). *L. lucinalis* and *A. alba* have previously been found to contribute greatly to differentiate muddy sand and sandy mud bottoms in the Ría de Vigo, NW

Spain (Moreira et al. 2005). The strong association of some taxa with organic matter content suggests their abundance may be largely determined by feeding mode. In the Ria Formosa, the dominant species of the polychaete families Oweniidae (*Owenia fusiiformis*) and Ampharetidae (*Amage adspersa*, *Ampharete acutifrons*, *Amphicteis gunneri* and *Melinna palmata*) are surface deposit-feeders, feeding mostly on detritus and microphytobenthos. *Bittium reticulatum* is also classified as a deposit-feeder or grazing herbivore (Gamito 2008). Accordingly, the decrease in the abundance of these taxa in the *ex situ* treatments is consistent with the observed decline of seagrass fragments and other detritus on the surface of the sediments during the experiment, which is well reflected in the organic matter content of sediments. The same mechanism does not apply to the family Nephtyidae, given that it is mainly composed of carnivores (Fauchald & Jumars 1979), including the 2 dominant species in the Ria Formosa, *Nephtys hombergii* and *N. cirrosa* (Gamito 2008). In this case, the reduced abundances in the *ex situ* treatments were probably mediated by the reduction in their prey, which include molluscs, crustaceans and other polychaetes (Fauchald & Jumars 1979).

### Main findings and conclusions

The pH, dissolution rates and grain size of sediments were unaffected by the CO<sub>2</sub> treatments in our experiment. The combination of a relatively low CaCO<sub>3</sub> content of sediments and the elevated total alkalinity of local seawater probably contributed to buffer the effect of the increased CO<sub>2</sub> levels in the composition of the sediments. The results of this and previous studies show substantial temporal and spatial variations of seawater carbonate chemistry in the Ria Formosa and suggest that consistent gradients in total alkalinity may exist among the distinct water bodies defined within the lagoon. Macrofaunal assemblages differed significantly between the 2 types of sedimentary habitat considered (sandy mud and muddy sand), but not among the 3 acidification levels tested. Although there were some changes in composition, the major differences were in terms of the abundance of the dominant taxa. There were also significant differences between *ex situ* treatments and field controls, with densities and number of taxa being generally larger in the latter, relative to the former. Despite their perceived tolerance of CO<sub>2</sub>-induced seawater acidification, polychaetes were found to be particularly sensitive to the experi-

mental stress of being held in artificial environments. In contrast, bivalves and some amphipods were well adjusted to the conditions in the experimental tanks. These distinct sensitivities clearly illustrate the potential for artefacts interacting with treatments in mesocosm studies, emphasising the difficulties of replicating natural or near-natural conditions *ex situ* and the relevance of incorporating adequate field controls in the design of these experiments.

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