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Simulated leakage of high pCO_2 water negatively impacts bivalve dominated infaunal communities from the Western Baltic Sea

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Carbon capture and storage is promoted as a mitigation method counteracting the increase of atmospheric CO_2 levels. However, at this stage, environmental consequences of potential CO_2 leakage from sub-seabed storage sites are still largely unknown. In a 3-month-long mesocosm experiment, this study assessed the impact of elevated pCO_2 levels (1,500 to 24,400 μatm) on *Cerastoderma edule* dominated benthic communities from the Baltic Sea. Mortality of *C. edule* was significantly increased in the highest treatment (24,400 μatm) and exceeded 50%. Furthermore, mortality of small size classes (0–1 cm) was significantly increased in treatment levels ≥6,600 μatm. First signs of external shell dissolution became visible at ≥1,500 μatm, holes were observed at >6,600 μatm. *C. edule* body condition decreased significantly at all treatment levels (1,500–24,400 μatm). Dominant meiofauna taxa remained unaffected in abundance. Densities of calcifying meiofauna taxa (i.e. Gastropoda and Ostracoda) decreased in high CO_2 treatments (>6,600 μatm), while the non-calcifying Gastrotricha significantly increased in abundance at 24,400 μatm. In addition, microbial community composition was altered at the highest pCO_2 level. We conclude that strong CO_2 leakage can alter benthic infauna community composition at multiple trophic levels, likely due to high mortality of the dominant macrofauna species *C. edule*.

Carbon dioxide (CO_2) is one of the most important natural greenhouse gases on Earth, playing a vital role in regulating the global heat budget and, thus, Earth's climate system. Currently, the concentration of atmospheric CO_2 is increasing at an unprecedented rate[1], a process mainly caused by human actions[2]. Anthropogenic CO_2, which enters the ocean, causes ocean acidification through an increase in dissolved inorganic carbon resulting in a decrease in pH. To reduce anthropogenic CO_2 emissions to the atmosphere and mitigate climate change, the World Energy Outlook suggested long-term sequestration of CO_2 (CCS) into sub-seabed geological structures as one suitable strategy[3]. Utilization of this technique, however, depends on the successful management of storage risks. These risks are still poorly constrained and current research is directed to obtain reliable information to establish a legal framework for environmentally safe application of CCS and efficient monitoring techniques[4,5].

One of the main potential risks of CCS is CO_2 leakage through e.g. fracture zones or pipe failure leading to acidification of the pore and bottom water or water column, respectively[6]. The extent of impacts of acidified seawater on the surrounding marine ecosystem will depend on the scale and intensity of leaks, the local hydro-dynamic regime and the duration of exposure[7]. Models that evaluated different potential leakage scenarios in the southern part of the North Sea predict temporary local seawater pH changes ranging from < -0.12 in case

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of a continuous diffuse seepage up to >-1.0 pH units in a continuous point source leakage scenario[7,8]. The latter represents the extreme case of a major geological fault break or an unrecognized pipe failure with outgassing of high amounts of CO_2 into the marine environment[9–12]. Experimental release of ca. 4 tons of CO_2 via sediment injection during the 37 day long QUICS experiment in a Scottish Sea Loch resulted in maximum decreases in sediment pH by >0.8 pH units[12].

Studies that have investigated the influence of elevated seawater pCO_2 on benthic organisms primarily used short- to medium-term exposures, single species experiments and pCO_2 levels relevant for ocean acidification (OA) forecasts. The influence of medium-term exposure to high pCO_2 on communities is not well investigated and different threshold levels need to be identified. The sensitivity of marine organisms to varying pCO_2 levels and accompanying seawater pH variations differs between taxa, resulting in very diverse responses to elevated internal CO_2 concentrations (hypercapnia)[13]. The general response of animals to hypercapnia is related to disturbance and regulation of the intra- and extracellular acid-base balance[13–15]. Those processes are energy consuming and can result in growth reduction, decreased metabolic activity, low reproduction rates, behavioural changes or ultimately death[13,16,17]. Calcifying organisms are particularly susceptible to acidified seawater due to reduced aragonite and calcite saturation of the water which can lead to enhanced dissolution of unprotected skeleton components and reduced availability of inorganic carbon for calcification[18–20]. Non-calcifying, infaunal invertebrates (e.g. meiofauna, and more particular the dominant nematodes) with a low mobility and bacterial communities are naturally exposed to large fluctuations of pore water pH and pCO_2[21,22], and are therefore likely to be more tolerant to high pCO_2 (OA)[13].

Only a handful of studies have exposed sediment communities in their natural composition to elevated seawater pCO_2, primarily due to the great logistic effort necessary to collect and maintain such communities in the laboratory and to control the carbonate system with sufficient accuracy during experiments. Negative effects of medium-term (20 weeks) exposure to high seawater pCO_2 (pH 7.3–5.6) have been reported on macro- and meiofauna diversity and community structure[23]. In addition, Widdicombe and Needham[24] could show that even though seawater acidification did not alter nereid worm burrow size or structure, sediment nutrient fluxes were changed after five weeks of acidification exposure, supposedly due to changes in bacterial communities. Moreover, since the burrowing activity of e.g. echinoderms has a strong influence on the biogeochemistry of sediments and the composition of meiofauna communities[25], it seems likely that changes in macrofauna abundance in response to elevated seawater pCO_2 can have strong repercussions on infaunal ecosystem processes[23,24].

Up to date, no comprehensive study has addressed the impact of elevated seawater pCO_2 and acidification on benthic bivalve dominated communities (e.g. bivalves, meiofauna and bacteria) thriving in shallow, sandy coastal sediments in the North Atlantic region. Bivalves are of high ecological importance as they represent the defining macrobenthic organisms in these habitats, with a key nutritional role for many fish and migratory bird species[26,27]. As they filter large amounts of water, deposit particulate organic matter onto and into the sediment and provide settlement habitat for other organisms, they are irreplaceable key players in the benthic-pelagic coupling[28,29].

A range of recent studies on several (mainly epibenthic) bivalve species could establish that calcification, growth, filtration and metabolism can be negatively impacted by elevated seawater pCO_2, often already at moderately decreased pH >7.5 (ca. $<5,000\,\mu atm$). In addition, increased occurrence of oxidative and acid-base regulatory stress has been observed in bivalves[16,30–32]. Detrimental external and internal shell dissolution occurs in several species when these are exposed to acidified seawater for prolonged time intervals (weeks), with less severe effects observed in species that possess a thick and intact periostracum[33–36]. However, biological impacts such as bioturbation[22,24,25] or interactions between and among different taxa and trophic groups[23,25,37,38] can result in very diverse responses of the biological community when compared to single species/taxon studies. Mesocosm experiments which address the effect of seawater acidification on shallow water benthic species report an overall higher sensitivity of macrofauna while meiofauna, with nematodes as dominant and commonly reported taxon, remain unaffected or even increase in abundance[23,25,37,39]. Laboratory experiments confirm a strong resistance of dominant meiofauna taxa to pH changes as expected to occur as a result of OA by the year 2300[39] but a reduced survival of nematodes has been observed when the pH is further reduced to values that could potentially occur in response to a severe CCS leakage scenario ($<$pH 7)[40]. Microbial communities are important for the coastal ecosystem as they are primarily responsible for organic matter remineralisation, nutrient cycling and function as food source for grazing marine organisms[41–44]. Investigations about the direct effects of lowered pH are still inconsistent[41,45]. While some studies observed differences in abundance of a few genera or rare taxa, others found a change in bacterial biomass production with increasing pCO_2[46–49].

In this study, we conducted a mesocosm experiment using natural *C. edule* dominated sandy communities from the Western Baltic Sea utilizing a flow-through seawater design with optimized food supply. Three of the dominant infauna bivalve species from the western Baltic and North Sea are the cockle *Cerastoderma edule*, the soft-shell clam *Mya arenaria,* and the Baltic tellin *Macoma balthica*[50]. *C. edule* and *M. balthica* live within the top two to five centimetres of the sediment, while *M. arenaria* occurs to sediment depths of up to 50 cm[51]. Generally, all three species are widespread in tidal flats and shallow coastal areas and serve as an important link between primary producers and consumers. Owing to their very high densities in the sediment (often $>1,000$ individuals m^{-2}) and considerable mobility and activity[52] they exert a strong influence on infaunal communities and biogeochemical processes, e.g. by increasing primary production and fertilization of microphytobenthos through NH_4^+ excretion[52,53]. To predict marine benthic ecosystem vulnerability to potential chronic leakages from CCS storage sites, experiments need to utilize a high pCO_2 range, realize a multispecies approach and intermediate exposure durations. In search for an indicator species useable in future CCS site assessments, experiments focused on the most abundant bivalve *C. edule*. We hypothesized that *C. edule* would be very sensitive to elevated pCO_2, as it lives close to the sediment surface. *C. edule* mainly occurs in relatively unpolluted areas while e.g. *M. balthica* can also be found in polluted and hypoxic areas[54], suggesting a higher resistance of *M. balthica* to unfavourable environmental conditions. To extend the common single-species approach to an ecosystem investigation and to test

for the role of *C. edule* as an important ecosystem engineer, the impact of high pCO_2 on benthic meiofauna and bacterial communities was also assessed. We hypothesized that elevated pCO_2 and changes in bivalve abundance and fitness would impact meiofaunal and microbial communities.

Materials and Methods

Experimental setup.   Sandy communities were exposed to six different seawater pCO_2 regimes for a total of three months (17.12.2011–06.03.2012) in a climate - controlled room. Six header tanks were continuously supplied with filtered seawater from Kiel Fjord, each one connected to six experimental units (EU) ensuring continuous seawater supply (Supplementary Figure S5). This design is a randomized design (B4) according to Cornwell and Hurd[55]. Each EU consisted of a round plastic container with a volume of 12.5 L containing ca. 9.5 L of sediment and an overlying water column of ca. 3 L. The lower 10 cm of the sediment consisted of sieved sand taken from a local beach (Kiel, Falckenstein: 54°23,66 N; 10°11.56 E) while the upper 10 cm consisted of surface sediment from the station at which the experimental animals were sampled to resemble natural conditions as well as to provide naturally occurring microbial and meiofauna communities. Bivalves and sediment were sampled in Kiel Fjord at Falckenstein with a Van Veen grab in 1–2 m depth using the vessel FK Polarfuchs on November 21st 2011 and kept in holding basins at 9 °C before being placed in EUs. Density of infauna bivalves was determined during the sampling process. 1 m^2 of sediment at Falckenstein was found to contain 146 *M. arenaria*, 9 *M. balthica,* and 1,040 *C. edule*. In order to simulate a natural size distribution in our laboratory experiment, we sieved the collected sediment (1 mm mesh width). All bivalves were taken out and replaced by a defined number per EU: 5 *M. arenaria* (size classes: 0.5–1 cm: 2 animals; 1–1.5 cm: 2 animals; 2–2.5 cm: 1 animal), 1 *M. balthica*, and 40 *C. edule* (size classes: 0–0.5 cm: 3 animals; 0.5–1 cm: 18 animals; 1–1.5 cm: 11 animals; 1.5–2 cm: 7 animals; 2–2.5 cm: 1 animal). Small gastropods (exclusively *Hydrobia* spp.) were abundant with ~10 individuals per EU. Due to their small size (<0.5 mm) they were randomly distributed within all EUs with the sieved sediment. Due to the natural low diversity of the Baltic, the density of other macrofauna individuals was <1 individuals per m^2. These low abundant species (e.g. nereid polychaetes, pharid bivalve species) were excluded from the experiment. The experimental units were kept in a seawater flow-through system for two weeks under control conditions prior to the experiment to allow proper acclimatization of biogeochemistry and the faunal community. Seawater pH was maintained in the header tanks using a pH feedback system (IKS Aquastar, iksComputersysteme GmbH, Karlsbad, Germany). Treatment levels were achieved through continuous addition of acidified water from the header tanks into the overlaying seawater of each EU and included levels of 900 µatm (control, pH 7.8 NBS scale), 1,500 µatm (pH 7.7), 2,900 µatm (pH 7.4), 6,600 µatm (pH 7.0), 12,800 µatm (pH 6.7), and 24,400 µatm (pH 6.4) (Supplementary Table S1). 900 µatm was used as a control due to the high background pCO_2 in Kiel Fjord[31,35]. To support the bivalve nutritional needs unicellular algae (*Rhodomonas* sp.) were cultured as described in Thomsen *et al.*[35] and added continuously into the header tanks via a peristaltic pump, thus maintaining a stable concentration of 3,500–4,000 (Supplementary Table S1) cells ml^{-1} within header tanks (Supplementary Table S1).

A flow rate of 100 ml min^{-1} was provided to each EU from the respective header tank via gravity feed. Throughout the experiment, pH, salinity, temperature, and flow rate were measured daily in each replicate. Salinity and temperature fluctuated in accordance with naturally occurring changes in Kiel Fjord seawater (14.6–20.5 psu and 4.3–8.9 °C, respectively). Light conditions were similar for all EUs. Dead animals were removed daily and behaviour of bivalves (presence/absence on the sediment surface) was noted every other day starting in the third experimental week. Carbonate chemistry and algae concentration in the EUs were measured weekly. Dissolved inorganic carbon (C_T) was measured using an Automated Infrared Inorganic Carbon Analyzer (AIRICA, Marianda, Kiel, Germany). Seawater chemistry (pCO_2 and calcium carbonate saturation state) was then calculated according to the guide to best practices for ocean CO_2 measurements[56], using CO2SYS[57] with pH (NBS scale) and C_T, temperature, salinity, and first and second dissociation constants of carbonic acid in seawater[47].

Bivalve sampling and processing.   At the end of the experiment, all but four *C. edule* specimen per EU were frozen at −20 °C. Shell-free dry mass was measured according to Thomsen *et al.*[31]. To survey shell dissolution, five randomly selected *C. edule* from each treatment were analyzed using a stereomicroscope (40-fold magnification). In addition, scanning electron microscopy (SEM) was used to examine external shell dissolution at a higher resolution. For that purpose, three shells of the 900–6,600 µatm CO_2 treatments (as signs of dissolution were obvious at higher levels) were mounted separately on SEM pedestal stubs, coated with gold-palladium and examined using scanning electron microscopy (Nanolab 7, Zeiss, Oberkochen, Germany and HitachiS4800, Hitachi High - Technologies Europe, Krefeld, Germany).

The extent of oxidative stress in the lipid fraction of the whole body tissue was estimated by analysing the malondialdehyde (MDA, a marker of lipid peroxidation) content in four *C. edule* specimens per EU, which were stored frozen at −80 °C prior to measurement. The entire tissue mass of each bivalve was separately processed via grinding in liquid nitrogen. Fifty milligrams of ground tissue powder of each of the four specimens from the same EU were pooled together to obtain sufficient tissue amounts for the analysis (one pool per EU). MDA concentration of each replicate was determined following the protocol of Uchiyama and Mihara[58]. Approximately 100 mg tissue-powder was homogenized with 0.2% phosphoric acid in a 1:5 ratio (sample/phosphoric acid). In the next step an equivalent volume of 2% phosphoric acid was added. One blank (homogenate + 0.2 ml 3 mM hydrogen chloride) and two samples (homogenate + 0.2 ml TBA solution) were adjusted to a pH of 1.6 (with HCl or NaOH) and were incubated at 100 °C for one hour. After cooling, 0.5 ml of butanol was added, samples were vortexed for 40 seconds and centrifuged at 1,000 g for 5 minutes. The supernatant was collected into fresh vials and centrifuged for 5 minutes at 14,000 g. Samples and blanks were transferred to a 96 well plate and the extinction was measured in a plate reader (Plate Chameleon, Hidex, Turku, Finland) at 532 and 600 nm. Tissue concentration of MDA was calculated following equation (1).

$$C_{tiss} = \frac{C_{MDA} \times V_{But} \times V_{Extr}}{V_{aliq} \times W}$$

(1)

C_{MDA}: MDA concentration
V_{But}: volume of butanol [ml]
V_{Extr}: extraction volume [ml]
V_{aliq}: volume of homogenate [ml]
W: weight of tissue [g]

Meiofauna sampling and processing. The uppermost centimetre of each EU was sampled after six and twelve weeks using a small corer (Ø 2.5 cm) and stored in 4% buffered paraformaldehyde until further extraction of the meiofauna. For this purpose, samples were washed on two stacked sieves in order to separate the macrofauna fraction (on a 1 mm sieve) from the meiofauna fraction (on a 38 μm sieve). Subsequently, meiofauna was extracted by triple density gradient centrifugation (3000 rpm) with colloidal silica gel LUDOX HS40 Dupont (specific gravity 1.18) as a flotation medium[59], fixed in 4% buffered formaldehyde solution and stained with Rose Bengal. Meiofauna was counted under a stereo microscope (50x magnification) and identified to higher taxonomic level consulting e.g. Higgins and Thiel[60]. Only nematodes that were in a good shape and showed no clear signs of degradation (loose cuticle, lack or damage of internal structures, biofilm, flattened or dehydrated habitus) were counted. For nematode identification, 100 nematodes were picked out per sample, transferred to glycerine following the protocol of De Grisse[61] and subsequently mounted on paraffin-ring glass slides. Nematodes were identified to genus level following a pictorial key[62] and the NeMys database[63] under a microscope (Leica DMR, 10x–100x magnification).

Microbial community sampling and processing. After six and twelve weeks, one corer sample (Ø 2.5 cm, 1 cm depth) per EU was collected, transferred into 50 ml plastic tubes and stored frozen at −20 °C for bacterial community analysis. The samples were subjected to total community DNA extraction using the FastDNA SPIN Kit for Soil (Qbiogene, Carlsbad, CA) including an additional heating step to increase yield and final elution of the DNA in TE-buffer. Benthic bacterial community structures were determined by means of the high-throughput fingerprinting technique ARISA, following a previously published procedure by Ramette[43] with slight modifications: Final concentrations of PCR ingredients within 50 μl-reactions were 0.4 μM of each primer, 0.1 mg ml^{-1} BSA, 250 μM of each dNTP (peqGOLD Kit; Peqlab, Erlangen, Germany), 1x Buffer S with 1.5 mM MgCl$_2$(Peqlab), 1.0 mM extra MgCl$_2$ (Peqlab) and 2.5 U peqGOLD *Taq*-DNA-Polymerase (Peqlab). The forward primer was labelled with FAM at its 5′-end. For each sample (EU), three PCR replicates were prepared. For two samples (EUs) a successful amplification could not be validated, they were therefore excluded from further analyses.

Statistical analyses. Univariate, parametric and non-parametric analyses were performed using the program R version 3.0.2.[64]. Multivariate, non-parametric analyses were performed with the program Primer version 6.1.11 with the add-on software Permanova+ version 1.0.1[65,66]. Regression analysis (ANCOVA) was performed using Prism 6 software (GraphPad Software, Inc.). A significance level of α = 5% was chosen for all statistical tests. Significant test results are reported in the results, statistical information can be found in the supplementary tables.

To test for differences in *C. edule* behaviour, a permutational ANOVA (PERMANOVA), with the factor EU nested in treatment, was used as a non-parametric solution to a repeated measures analysis. *C. edule* mortality between treatments and the fraction of dissolved shells between treatments was tested using a Kruskal-Wallis test combined with a Kruskal multiple comparisons test since assumptions of normality (Shapiro-Wilk test) and homogeneity of variances (Levene's test) were not met. Mortality between different size classes within each treatment was tested with a Kruskal-Wallis test. For this, animals were pooled in two groups, large (1–2, 5 cm shell width) and small (0–1 cm shell width). The influence of pCO_2 on malondialdehyde (MDA) content was tested using a one-way analysis of variance (ANOVA) and a Tukey HSD post hoc test. The difference in shell-free dry mass between treatments was tested with an analysis of covariance (ANCOVA), using log shell - free dry mass and log shell width. As no significant interaction was found for replicate and treatment (nested ANOVA, EU nested in CO_2 treatment, p-value = 0.8411), all measured animals (n = 479) of each treatment were used in the regression analysis.

Meiofauna community analysis was based on calculated densities (individuals 10 cm^{-2}) while nematode community analysis was based on relative abundances and calculated densities. Due to an unbalanced and non-normal distributed dataset, Permanova was chosen as an ANOVA approach for repeated measures to test for differences in total meiofauna densities and meiofauna and nematode community structure between treatments, time and interaction of both, with EU nested in treatment. When the number of unique permutations was lower than ten a Monte Carlo (MC) test was applied to calculate the p-value. The data were square root transformed and Bray-Curtis resemblance matrices were calculated. A pairwise-comparison test was executed for factors that differed significantly. The PermDisp tests always assured homogeneity of data dispersion unless mentioned otherwise. A multidimensional scaling (MDS) plot was created to visualize the results. Permanova was repeated for the meiofauna dataset after excluding Nematoda and a SIMPER analysis was performed to reveal the taxa that contributed most to the differences between treatments. The taxa with the most impact on dissimilarities were tested each by a univariate Permanova analysis as described above. For taxa with empty samples a dummy variable was added to avoid undefined values when calculating the Bray-Curtis resemblance matrix as suggested by
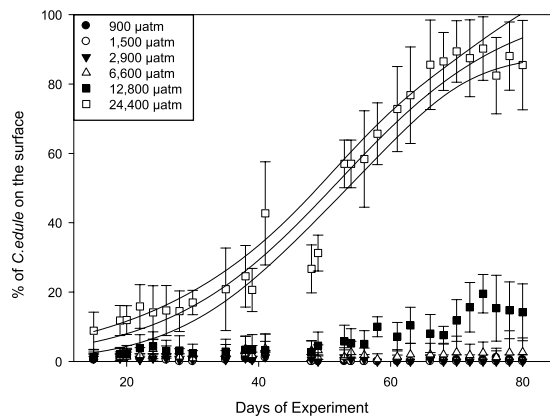
Figure 1. *C. edule* behaviour. Average abundance of non-buried *C. edule* over the complete experimental phase in % of total, curve fitted for 24,400 μatm including 95% confidence interval.

Clarke *et al.*[67]. Meiofauna diversity indices (Shannon-Wiener index, Simpson index and Pielou's evenness index) were calculated and tested for significant differences between treatments with Permanova.

Quality assessment of raw bacterial community profiles and binning (2–3 replicates per sample) were done as previously reported[43]. Merged community profiles were generated in R (v.2.13.2[64]); by using a custom script and considering Operational Taxonomic Units (OTUs) that occurred at least twice[43]. Non-metric Multidimensional Scaling (NMDS) was used to represent dissimilarity matrices based on Bray-Curtis or Jaccard coefficients into a reduced space[68]. Analysis of similarity (ANOSIM) was conducted with the PAST software (Version 1.76[69]). To test for the effect of pCO_2 treatment and time on bacterial community composition, multivariate ANOVA and variation partitioning were conducted in R.

Results

Bivalve community response.   Out of the three investigated bivalve species, the cockle *C. edule* was the most abundant and most sensitive to acidified seawater. As the abundance of the other two bivalve species was very low and no mortality was recorded, we focus on *C. edule* in the following sections. Throughout the experiment, cockles were either buried with open siphos or lying on the sediment surface. Under control conditions and at 1,500 μatm, cockles were mostly buried (Fig. 1). In the highest CO_2 treatment (24,400 μatm), cockles migrated towards the surface (Fig. 1). At 12,800 μatm, some cockles were observed on the surface, however always at a lower abundance than in the highest treatment. The distribution of cockles on the surface was significantly influenced by CO_2 treatment (p(perm) = 0.0001; Supplementary Table S2). Although time and the interaction between time and treatment were significant (p(perm) = 0.0001), 63% of the variance were explained by the factor treatment. In the 24,400 μatm treatment, 50% of cockles were located on the sediment surface on day 50 (Fig. 1).

Mortality of *C. edule* increased with seawater pCO_2 with 50% mortality in the highest treatment already after 68 days (Fig. 2). A significantly elevated mortality could be shown for the 24,400 μatm group compared to the control, 1,500 μatm, and 2,900 μatm groups (Kruskal-Wallis multiple comparison test, $p < 0.05$, Supplementary Table S3, S3a). Mortality, when averaged over all size classes, tended to increase in 12,800 μatm as well (total mortality ca. 15%). However, this increase was not significant by the end of the experiment. Smaller individuals reacted more sensitively towards high pCO_2 (Fig. 3). There were no differences in mortality between size classes in the control, 1,500 μatm, and 2,900 μatm treatments. At 6,600, 12,800 and 24,400 μatm, mortality in the smaller size class (0–1 cm) was significantly higher than mortality of cockles in the larger size class (1–2.5 cm) (Kruskal-Wallis multiple comparison test, $p < 0.05$, Supplementary Table S4).

The comparison of intact shells against shells with signs of dissolution showed an increase in shell corrosion of *C. edule* with increasing pCO_2 (Figs 2 and 4). Shells from the control treatment had an intact periostracum and were not characterized by shell dissolution (Fig. 4, N = 3 of 3 observations). Shells from ≥1,500 μatm were characterized by signs of external dissolution (SEM analysis, N = 3 of 3 observations). Stereo microscopic images demonstrated visible signs of shell dissolution for all treatments above 2,900 μatm, with increasing severity in higher pCO_2 treatments (Fig. 4). For some cockles (<5%), exclusively small animals (<0.7 cm) holes were already visible at 2,900 μatm. Cockles maintained under high pCO_2 (6,600, 12,800 and 24,400 μatm) were characterized by severe shell damage. In the 6,600 μatm treatment, 72% of dead cockles, mostly small individuals, contained holes in their shells. In the two highest treatments (12,800 μatm and 24,400 μatm) >85% of deceased cockles were characterized by holes in their shells. This rate of shell dissolution was significantly higher when compared to the control and 1,500 μatm treatment (Kruskal-Wallis, $p < 0.05$, Supplementary Table S3, S3b).

Whole body malondialdehyde (MDA) concentrations, an indicator for oxidative stress, were significantly lower in the 6,600 μatm, 12,800 μatm and 24,400 μatm groups when compared to the control (Fig. 5a, Tukey HSD, $p < 0.05$). Additionally, MDA values of the 24,400 μatm treatment were significantly lower than values measured in 1,500 μatm and 2,900 μatm animals.

Slopes of log shell free dry mass vs. log shell width linear regressions (900–24,400 μatm) were not significantly different between treatments ($F_{(5,467)} = 0.49$, $p = 0.7851$, Fig. 5b), while y-intercepts were significantly different ($F_{(5,472)} = 32.5$, $p < 0.0001$). It appears that y-intercepts of all treatment levels (1,500–24,400 μatm) were
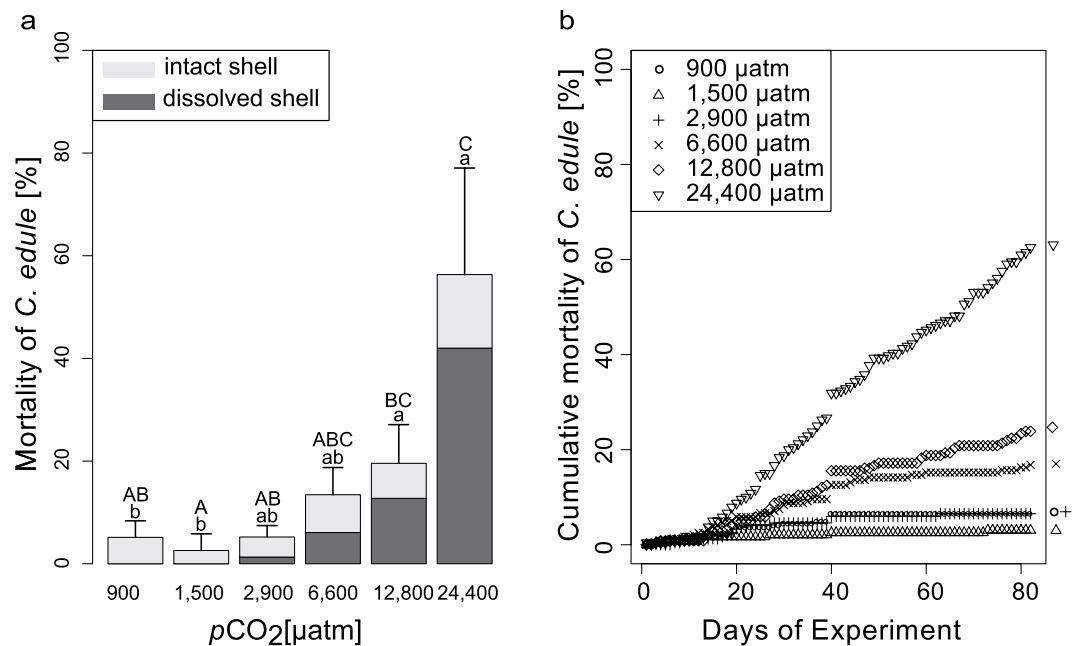
Figure 2. (**a**) Bars showing *C. edule* mortality over the entire experimental duration (mean ± SD); colour coding showing fraction of corroded vs. intact shell (white = intact, grey = corroded); letters indicate significant differences between treatments. (**b**) Cumulative mortality plotted over the duration of the experiment. 50% mortality at 24,400 μatm was reached at day 68.
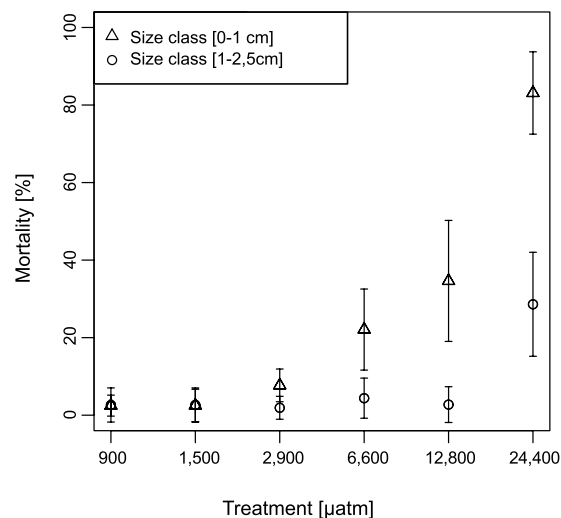

Figure 3. Cumulative mortality of different *C. edule* size classes during the experiment plotted for each treatment.

significantly lower (no overlap in 95% confidence intervals) than that of the control group (900 μatm), indicating reduced body condition at elevated seawater pCO_2 (Fig. 5c). Slopes of control (900 μatm) and field animals collected just prior to the experiment were similar ($F_{(1,110)} = 0.15$, p = 0.7021). Y-intercepts were significantly different between these two groups ($F_{(1,111)} = 17.94$, p < 0.0001), with slightly reduced condition in the control animals vs. field collected animals (Supplementary Table S5).

M. arenaria and *M. balthica* remained burrowed during the entire experimental duration in all treatments and no mortality was observed for these two species.

Meiofauna community response. A total of twelve meiofauna groups were found. The four most abundant taxa were Nematoda (72.5 ± 7.5%), Gastrotricha (11.0 ± 7.1%), Copepoda (5.9 ± 4.5%), and crustacean nauplii (2.6 ± 3.2%). Total meiofauna densities ranged from 218 to 988 ind. 10 cm⁻². Towards the end of the experiment, significantly higher total meiofauna densities were found in the 24,000 μatm treatment compared to all other treatments, except for the 1,500 and 2,900 μatm treatment (Pairwise tests (Treatment * Time),
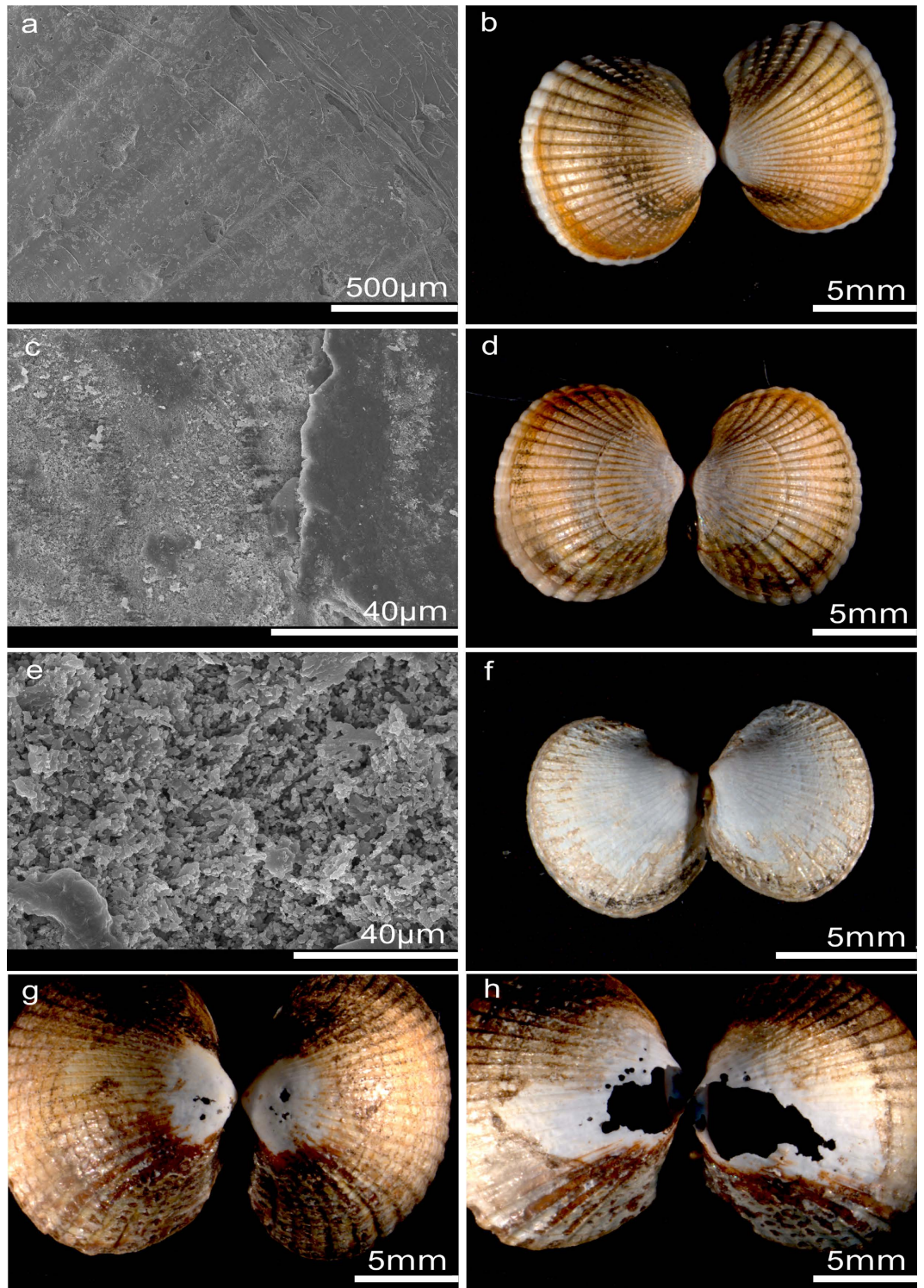
Figure 4. Shell corrosion in *C. edule*. (a,b) Control (900 μatm), no shell corrosion visible on the outside of the shell. **(c,d)** 1,500 μatm, shell corrosion on the outside of the shell. **(e,f)** 6,600 μatm, shell dissolution on the outside of the shell. **(g,h)** 24,400 μatm, strong dissolution signs, holes.

p(MC) $\leq$ 0.0495, Supplementary Table S6a). Meiofauna community composition significantly differed based on the factors treatment (Main test, p(perm) = 0.0004, PermDisp < 0.05) and time (Main test, p(perm) = 0.0022). The meiofauna community of the 24,400 μatm treatment had a significantly different composition compared to the assemblages in all other treatments (p(perm) $\leq$ 0.0201, Fig. 6, Supplementary Table S6a). Evenness and diversity of the total meiofauna community decreased with time (p(perm) $\leq$ 0.0275, PermDisp < 0.05) but did not differ between treatments.
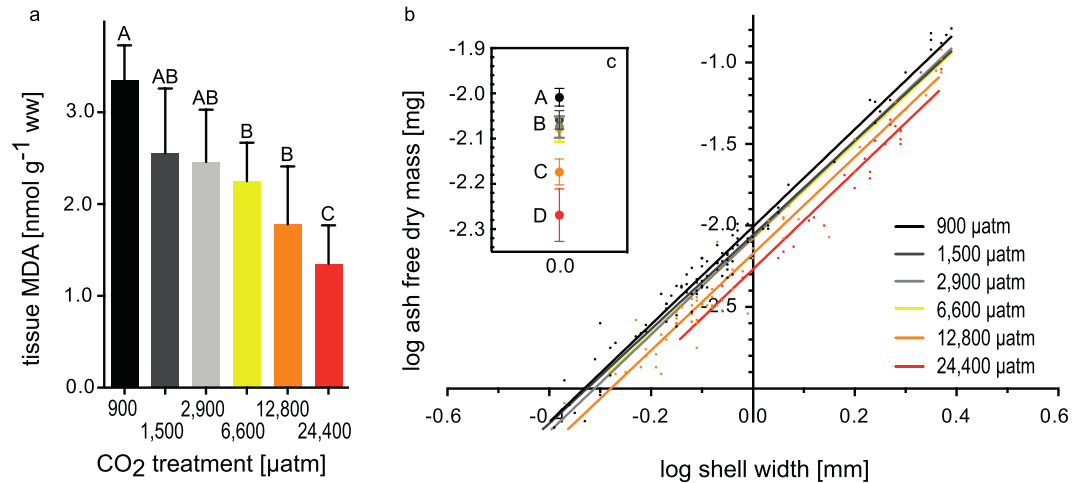
Figure 5. *C. edule* condition and MDA accumulation. (a) Average of tissue MDA content [nmol g^{-1}ww] for the different treatments. Means and standard deviation, letters indicate significant differences between treatments. (**b**) Regressions of log shell-free dry mass plotted against log shell width, single data points plotted for the 900, 12,800 and 24,400 μatm treatment (equations in Supplementary Table S5) (**c**) y intercepts of regressions of shell free dry mass and 95% confidence interval.
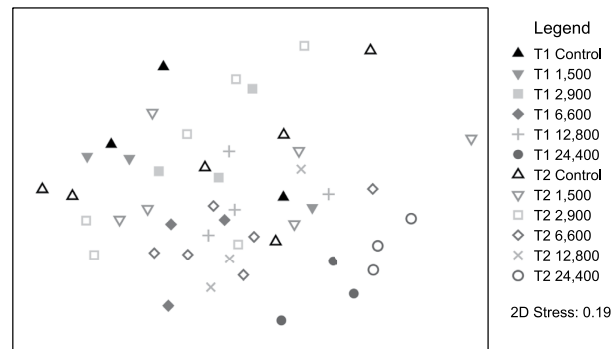

Figure 6. MDS plot representing the meiofauna community composition. Data based on square root transformed densities. Symbols indicate the factors "time" (T1 = after six weeks and T2 = after twelve weeks) and "treatment". (Control, 1,500 μatm–24,400 μatm).

The densities of nematodes (352.43–1733.64 ind. 10cm^{-2}), did not consistently differ over time or between treatments. It only occasionally differed between the 6,600 μam and 1,500 μatm treatment after six weeks, and between the 24,400 μatm and 12,800 μatm treatment after twelve weeks (see Supplementary Table S6a). A total of 36 nematode genera were found. The overall most abundant genera (>5%) were *Ascolaimus* (34.7% ± 2.2%), *Metachromadora* (13.2% ± 1.0%), *Hypodontolaimus* (9.4% ± 1.0%), *Microlaimus* (5.9% ± 0.5%) and *Enoplolaimus* (5.6% ± 0.6%). Multivariate analyses of relative and total densities of nematode genera did not reveal any significant difference between samples according to the pCO_2 level, time or a combination of both. Samples exhibited an overall high evenness (Pielou's evenness index) ranging from 0.60 to 0.89 but univariate measures based on densities and relative abundances (genus richness, Shannon-Wiener index, Simpson index, Pielou's evenness index) did not differ between samples based on the factors treatment, time or a combination of both.

When excluding Nematoda from the community composition analysis, Permanova revealed significant differences between treatments (p(perm) = 0.0002, PermDisp < 0.05) and over time (p(perm) = 0.0023). Differences occurred between the 24,400 μatm treatment and all other treatments (p(perm) ≤ 0.0298). According to the SIMPER analysis, Gastrotricha contributed most to these differences (25.53–41.08%), followed by Copepoda (24.19–11.25%), Ostracoda (14.98–9.88%) and Gastropoda (11.73–7.40%). Gastrotricha, Copepoda, Nauplii and Ostracoda contributed most to the difference over time (22.40%, 18.70%, 15.47% and 11.77%, respectively). Univariate analyses of the densities of the taxa contributing most to the differences (Fig. 7) revealed diverse responses. Copepoda densities were not impacted by treatment and time, whereas nauplii densities decreased significantly from the first to the second sampling time point (p(perm) = 0.0246). Gastrotricha densities were increased in the 24,400 μatm treatment compared to the control, 2,900 μatm and 6,600 μatm treatment (Pairwise test, p(perm) ≤ 0.0305) and densities of the 12,800 μatm treatment were increased compared to the control and 2,900 μatm group (p(perm) = 0,0171 and p(perm) = 0,034, respectively) but did not differ from the 1,500 μatm and 6,600 μatm treatment. Ostracoda densities increased over time (Main test, p(perm) = 0.0385)
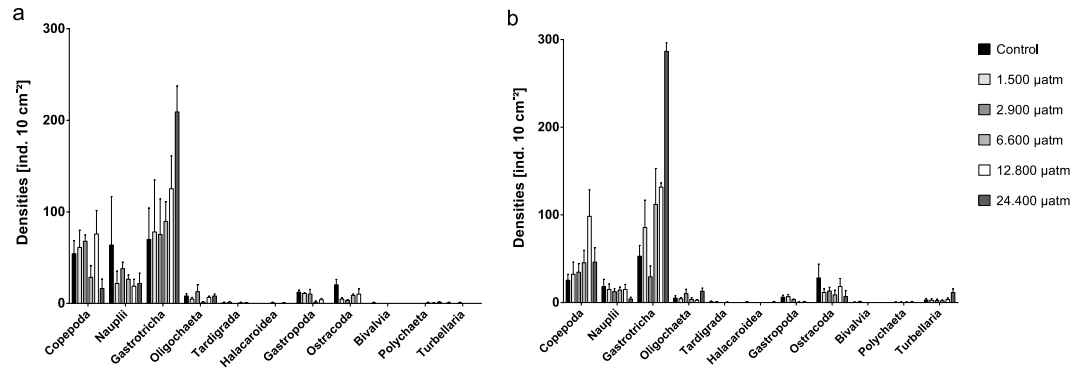
Figure 7. Average meiofauna densities excluding nematodes. Sampled after (**a**) six weeks and (**b**) twelve weeks plotted per treatment in number of individuals per 10 cm^2 (mean ± standard error).

but were significantly lower in the 24,400 µatm treatment compared to the control, 1,500 µatm and 6,600 µatm treatment (p(perm) ≤ 0.0163). Gastropoda densities decreased over time (p(perm) = 0.0001, permDisp < 0.05) and differed between treatments (p(perm) = 0.0097) with decreased densities in all treatments ≥6,600 µatm (p(perm) ≤ 0.0269) when compared to treatments ≤ 2,900 µatm. At six weeks, Turbellaria were absent in >80% of the samples, while after twelve weeks densities ranged between 1 and 9 individuals per sample with a presence in >50% of all samples.

Bacterial community.   Community fingerprinting of benthic bacterial communities by ARISA indicated similar average OTU numbers for all treatments and time points, i.e. for 6 and 12 weeks respectively 196 ± 9 and 179 ± 14 (900 µatm), 191 ± 13 and 196 ± 8 (1,500 µatm), 190 ± 15 and 174 ± 17 (2,900 µatm), 188 ± 12 and 179 ± 19 (6,600 µatm), 184 ± 17 and 183 ± 13 (12,800 µatm), and 184 ± 11 and 189 ± 12 (24,400 µatm). The percentage of shared OTUs among the different EUs for a given treatment and time could be as low as 53–70% (max. 60–78%), and varied between 70–83% for joint OTU profiles (one per treatment and time point). NMDS did not show a separation of bacterial communities according to CO_2 treatment and/or time (data not shown). However, PAST analyses indicated a significant difference between bacterial communities thriving at 900 µatm (control) and 24,400 µatm (abundance data only, Bonferroni-corrected $p < 0.05$ after 6 and 12 weeks, group separation R < 0.5, Supplementary Table S7), as well as between 1,500 µatm and 24,400 µatm (abundance and presence-absence data, Bonferroni-corrected $p < 0.05$ after 6 and 12 weeks, group separation R < 0.5). Variation partitioning demonstrated that pCO_2 treatment and time had both significantly influenced bacterial community composition, together explaining 11.7% of the observed variation ($p < 0.001$). Time alone explained 5% ($p < 0.001$) of the community changes and pCO_2 treatment 6.9% ($p < 0.011$). The interaction between the two factors was not significant ($p > 0.05$).

Discussion

To our knowledge, this is the first experiment to examine the impact of elevated seawater pCO_2 on benthic infaunal communities in coastal sandy sediments from the Baltic Sea under near-natural conditions. Our study indicates that persistent (3 month) strong leakage from a sub-seabed CCS site and the subsequent distribution of a high pCO_2 bottom water plume could lead to massive accumulation of *C. edule* on the seafloor and strong reductions in survival, with a high sensitivity especially for smaller bivalve size classes. Changes in microbial and meiofaunal community composition at the highest pCO_2 level indicate a direct or cascading effect of elevated CO_2 concentration on the entire benthic assemblage in a coastal sandy sediment ecosystem. However, we also demonstrate pronounced sub-lethal effects at lower treatment levels that warrant further research attention.

High pCO_2 changes coastal bivalve communities and bivalve behaviour.   The observed behavioural changes of *C. edule* as a result of exposure to high seawater CO_2 correspond well with responses observed for the same species during exposure to hypoxia[70,71]. With increasing pCO_2, moribund or weakened *C. edule* accumulated on the sediment surface. The fraction of *C. edule* on the surface of the sediment increased with duration of the experiment. Behavioural responses such as gathering of bivalves on the seafloor could be used as cost-efficient tool for future monitoring of sub-seabed CCS storage sites, e.g. by towing camera systems across large sea floor areas, when appropriate software processing and detection tools are being developed. Quite similarly, Widdicombe *et al.*[23] observed emersion of infauna echinoderms from the sediment during a high – CO_2 mesocosm incubation when pH values dropped below 6.5.

We observed substantial mortality of smaller size classes of *C. edule* in all treatments >6,000 µatm. This corresponds with previous work on other bivalve species where smaller juveniles appeared to be more sensitive to elevated seawater pCO_2[72,73]. This effect might be related to less favourable area to volume ratios, as smaller animals have to protect a relatively larger surface area from acid-base disturbance and relatively larger shell area from (internal) dissolution. Shell production costs and inorganic carbon demand for calcification are also much higher in smaller bivalves[31,74]. Size-dependent mortality implies that leakage could lead to an alteration of the demographic structure of bivalve communities.

In our study, shell corrosion was evident from 1,500 μatm to 24,400 μatm and signs of severe dissolution and presence of holes were found in most animals exposed to 12,800 μatm and 24,400 μatm. These findings indicate that even moderate degrees of acidification can already lead to non-reversible shell damage. Outer shell corrosion has been observed in a number of gastropod and bivalve molluscs[34,75] and has often been linked to absence of a thick and intact periostracum[34]. *C. edule* is characterized by a very thin periostracum (ca. 2 μm)[76] and a shell that is exclusively composed of aragonite, the polymorph of calcium carbonate that is most prone to dissolution[50,77]. In contrast, mytilids are protected by a periostracum of >20 μm[76] and can live in seawater which is strongly undersaturated for calcium carbonate ($\Omega_{arag} < 0.2$) as long as their periostracum is intact. Corrosion starts to occur when the periostracum is mechanically damaged[35], leading to complete dissolution of the shell in extreme cases, e.g. in deep-sea hydrothermal vent mussels that live in strongly acidic waters with pH <6[33]. While bivalves are able to repair holes and fractures in their shells[78], it is apparent from our results that *C. edule* in the highly acidified treatments were unable to allocate sufficient resources to shell repair, or that the rate of dissolution simply overwhelmed their repair capacity. Holes in their shells probably lead to massive and energy intensive stimulation of the immune system due to invasion of foreign microorganisms and loss of valuable proteins from the extrapallial fluid, the fluid which is in contact with the inner side of the shell[79]. In summary, progressive shell corrosion, even in the lower treatment levels (i.e. 1,500 μatm and higher), constitutes a large problem as shell integrity is essential for fitness of bivalve species.

One of the toxic effects of elevated oxyradical formation in cells is an increase in lipid peroxidation levels[80]. A commonly employed mode of detection is the reaction of lipid peroxidation intermediates with thiobarbituric acid in the so called TBARs assay. One of the major products of lipid peroxidation, but by far not the only one, is malondialdehyde (MDA), which serves as a marker for oxidative stress[81]. Oxidative stress is frequently also related to metabolism[82,83]. Previous studies on bivalves suggest a good relationship between high MDA accumulation and elevated metabolic rate[84,85]. MDA concentration was low in bivalves exposed to pCO$_2$ >6,600 μatm suggesting that animals exposed to such high CO$_2$ concentrations suffer from metabolic depression. While intermediate levels of acidification (i.e. <4,000 μatm) have repeatedly been shown to cause elevated metabolic rates[86,87] and increased oxidative stress[32] in a number of bivalve species, studies using treatment levels of >4,000 μatm have generally found metabolic reduction[86,88].

When employed as a strategy in the long run, metabolic suppression will lead to consumption of endogenous energy stores and reduced fitness. It has been demonstrated in a range of marine invertebrate species that acidification primarily impacts energy allocation processes and that ultimately, sensitivity is defined by depletion of available energy (scope for growth) by basal metabolism or reduced energy uptake[17,89]. In support of this view, all high-CO$_2$ exposed *C. edule* in our experiment (1,500–24,400 μatm) were characterized by a reduction in body condition, as indicated by reductions in shell-free dry mass in relation to shell width. This effect becomes more pronounced at the higher treatment levels (Fig. 5b,c) and correlates with reduced MDA accumulation. These findings indicate a negative scope for growth, which is unsustainable in the long - run. In a next step, it will be important to investigate aerobic metabolism and energy uptake in this species to better understand the underlying processes leading to energy budget disturbance.

M. arenaria and *M. balthica* survived the complete experimental duration. Even though abundance of these two species was low, the observations confirm our original hypothesis that *C. edule*, the species most sensitive to hypoxic stress, also is the most vulnerable to ocean acidification[90]. *M. balthica* is a species which is generally quite tolerant towards stressful conditions. It occurs deep in the sediment where oxygen availability is low[91] and tolerates polluted sediments[54]. Resistance of *M. arenaria* could be due to a thick, protective periostracum (20 μm)[76]. Additionally, a greater burrowing depth[92] might render this species less sensitive to acidified seawater. All bivalves used in this study have a shell consisting of aragonite, the more soluble calcium carbonate polymorph[50,76]. While the mineralogy is an important factor in defining susceptibility of bivalves[34], there is a high variation in vulnerability between species with the same shell mineralogy[30]. Crystal size and the proportion of organic matrix within the shell play an additional factor in resistance against environmental stress such as acidified seawater[76].

Very high pCO$_2$ induces shifts in meiofaunal community structure.

The findings of this experiment indicate that when very high seawater carbon dioxide levels occur, meiofauna community structure at higher taxon level can change as a result of differential sensitivity. The changes in densities were most severe in some of the less abundant meiofauna taxa that are not often reported. While most acidification studies focused on the dominant taxa such as Copepoda and Nematoda[23,25,37,39], only few other studies emphasized the importance of changes in taxon composition, particularly including less abundant taxa as a proxy for the status of benthic environments[93–95].

In this study, densities of three meiofauna taxa were affected by changing pCO$_2$ (i.e. Gastrotricha, Gastropoda, and Ostracoda). In accordance with the results of the macrofauna analysis, the calcifying Gastropoda and Ostracoda suffered from decreased densities in treatments with high pCO$_2$ (at >6,600 μatm and >24,000 μatm, respectively). Densities of Gastrotricha on the other hand increased significantly at the highest treatment level. It is, however, impossible to differentiate whether this is the direct result of the seawater acidification having a positive effect on their physiology (growth and reproduction) or whether this group was favoured by indirect factors, such as changes in food availability or space occupation by the disappearance of macrofauna or other taxa. Gastrotricha, as well as many other meiofauna taxa, feed on bacteria and protozoa[96]. The divergent microbial community in the highest acidification treatment could be the driver of the change in meiofaunal community structure with a strong increase in Gastrotrich densities. Despite all uncertainties, our study shows that Gastrotricha act as a physiologically tolerant, opportunistic taxon, apparently benefitting from severe seawater acidification.

In a laboratory experiment without macrofauna, Kurihara *et al.*[39] found no differences in the abundance and biomass of nematodes, harpacticoid copepods or harpacticoid nauplii between treatments with 380 ppm CO$_2$

(control) and 2,400 ppm CO_2 (acidified) over a time frame of 56 days. Several single-species studies reported decreased reproduction and slowed larval development of copepods when seawater pCO_2 is elevated[97–99]. Interestingly, the decline in densities of nauplius larvae over time in our mesocosm experiment occurred both in the control and acidified treatment indicating that the reproductive success of the copepods was not influenced by increased pCO_2.

Nematodes, the most abundant taxon in our experiment, seemed to be unaffected by the different treatments in terms of community composition and abundance with no significant changes over time. Several experimental studies on nematodes indicate that a certain threshold exists above which nematodes do not suffer from pH reduction in short- to medium-term (days to weeks) experiments. Once pH drops below this threshold (~pH 6), a decline of nematode densities has been observed that can be assigned to direct physiological responses[40]. Widdicombe et al.[23] found significant changes in nematode community structure only after a 20 weeks exposure and only at pH values lower than 6.0, while macrofauna decreased in abundance at much less severe pH changes. Similarly, in a different mesocosm experiment, nematodes remained unaffected following a seven week exposure to seawater pH of 7.5[25]. In the study of Takeushi et al.[40], nematode survival was monitored over a time frame of one week and only in the most severe treatments with a pH of 5.4 and 5.1 survival rates were significantly decreased with a species specific response related to the activity of the nematodes. Sub-lethal measures such as reproductive success and scope for growth might be more appropriate variables to assess sensitivity during medium-term exposure[39]. In our case nematode densities remained stable in all treatments and between time steps, suggesting that reproduction had most likely taken place. This was supported by the occurrence of juveniles and gravid nematodes.

High pCO_2 impact on bacterial communities.

Significant differences between bacterial communities became evident when comparing the highest treatment (24,400 μatm, severe leakage scenario) to the control (900 μatm) and slightly increased CO_2 level (1,500 μatm). This suggests the presence of bacteria adapted to a wide range of natural fluctuations in pCO_2, but also the risk of substantially influencing ecosystem functionality at very high pCO_2. Part of the potentially occurring changes in bacterial community structure might have been masked by the high spatial variability of the bacterial communities, as suggested by low R-values (PAST analyses) and a low percentage of shared OTUs among the EUs of a given treatment and time. The overall average number of OTUs in this study (186 ± 14 OTUs, 0–1 cm sediment depth, all treatments and time points) was slightly higher than the average value obtained from samples taken during a previous study on shallow subtidal sands in the North Frisian Wadden Sea[100] (145 ± 45.7 OTUs, 0–5 cm sediment depth, different seasons). Both pCO_2 level and time played a significant role in the observed community shifts, but as the two factors did not significantly interact, at this point, the prolonged pCO_2 treatment can neither be confirmed nor disregarded as a causal factor. Overall, influential factors might, besides a direct CO_2 influence, include a change in sediment nutrient composition or a change in sediment bio-irrigation rates mediated by dying cockles. While C. edule has previously been shown to significantly influence the microphytobenthic primary production due to release of NH_4^{+}[53], bacterial abundance was not significantly influenced by bio-diffusing activities of C. edule. The comparatively minor effects of substantial decrease in C. edule density at the highest CO_2 treatment level on the microbial community structure could be explained by a less pronounced impact of C. edule on sediment oxygen and nutrient fluxes compared to polychaetes (Nereis diversicolor) that construct elaborate burrows and strongly shape microbial communities[101]. However, the loss of an important ecosystem engineer poses unknown risks. A progressive loss of C. edule could affect settlement success of other macrobenthic species (e.g. polychaetes) that are otherwise competitively impacted by C. edule[52]. These species could more strongly impact microbial and meiofauna communities or alter ecosystem functionality during long-term CO_2 leakage. Similar to findings in our experiment, Kerfahi et al.[46] demonstrated shifts in sediment microbial communities (top 2 cm of the sediment) along CO_2 clines (600–1,600 μatm) in the Mediterranean. The authors found abundances of most dominant genera to be unaffected by CO_2, while 5% of genera differed in abundance along the CO_2 cline. Whether these changes can alter the biogeochemical functions of marine sediments still needs to be investigated in future experiments.

We can conclude that experimental medium-term exposure to high seawater pCO_2, which mimics strong leakage from sub-seabed CCS sites, can cause high mortality of C. edule and shifts in the composition of meiofauna and microbiota in a shallow sandy sediment environment. The response of bivalves was strongly species-specific and size-dependent. C. edule was found to be sensitive to most treatment levels with signs of shell corrosion at $pCO_2 \geq 1,500$ μatm and reduced body condition at $CO_2 \geq 1,500$ μatm, which points at a negative, unsustainable energy budget. This is supported by reduced tissue MDA accumulation at $\geq 6,600$ μatm. Thus, even lower levels of leakage resulting in seawater pCO_2 values of $>1,500$ μatm could strongly impact C. edule dominated sediments in the long-run as they might lead to reduced growth, enhanced mortality and reduced reproductive output. Longer-term ecological studies on C. edule are needed to investigate these processes in more detail. The dominant meiofauna taxa tolerated the highest acidification levels. Rare taxa on the other hand were affected by very high pCO_2 illustrating a decrease in calcifying organism abundance and an increase in other, more opportunistic taxa. While the calcifying organisms where likely directly affected by seawater acidification, it is unclear whether the response of other taxa is directly related to acidification or rather a result of cascading effects due to high C. edule mortality. Severe leakage from storage sites that result in very high seawater pCO_2 ($>20,000$ μatm) on a longer time scale could thus lead to significant changes in the overall benthic community structure, with unforeseen consequences for ecosystem health and functions. Lower levels of leakage ($<5,000$ μatm) have sub-lethal impacts on the dominant macrofauna species and may impact benthic ecosystem function in the long-term. Clearly, more research effort needs to be devoted into studying longer term consequences of exposure to lower seawater and sediment pCO_2 levels to better constrain the risks that are associated with sub-lethal species responses, particularly with respect to potential impacts on sediment meiofauna and bacterial communities.

References

1. Field, C. B. *et al.* Climate Change 2014: Impacts, Adaptation, and Vulnerability. Part A: Global and Sectoral Aspects. Contribution of Working Group II to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. (Cambridge, UK/New York, NY: Cambridge University Press, 2014).
2. Le Quéré, C. *et al.* Global carbon budget 2014. *Earth Syst. Sci. Data Discuss.* **7,** 521–610 (2014).
3. IEA. *World Energy Outlook 2010.* (Organisation for Economic Co-operation and Development, 2010).
4. Hawkins, D. G. No exit: thinking about leakage from geologic carbon storage sites. *Energy* **29,** 1571–1578 (2004).
5. Keating, G. N. *et al.* Mesoscale Carbon Sequestration Site Screening and CCS Infrastructure Analysis. *Environ. Sci. Technol.* **45,** 215–222 (2010).
6. Blackford, J. C. & Gilbert, F. J. pH variability and CO_2 induced acidification in the North Sea. *J. Mar. Syst.* **64,** 229–241 (2007).
7. Blackford, J. *et al.* An initial assessment of the potential environmental impact of CO_2 escape from marine carbon capture and storage systems. *Proc. Inst. Mech. Eng. Part -J. Power Energy* **223,** 269–280 (2009).
8. Blackford, J. C., Jones, N., Proctor, R. & Holt, J. Regional scale impacts of distinct CO_2 additions in the North Sea. *Mar. Pollut. Bull.* **56,** 1461–1468 (2008).
9. Widdicombe, S. *et al.* Impact of sub-seabed CO_2 leakage on macrobenthic community structure and diversity. *Int. J. Greenh. Gas Control.* **38,** 182–192 (2015).
10. Taylor, P. *et al.* Impact and recovery of pH in marine sediments subject to a temporary carbon dioxide leak. *Int. J. Greenh. Gas Control.* **38,** 93–101 (2014).
11. Blackford, J. *et al.* Detection and impacts of leakage from sub-seafloor deep geological carbon dioxide storage. *Nat. Clim. Change.* **4,** 1011–1016 (2014).
12. Blackford, J., Haeckel, M. & Wallmann, K. Report on range of long-term scenarios to be simulated. Deliverable Number D12.2, WP12/CCT2; lead beneficiary number 2 (2012).
13. Pörtner, H. O., Langenbuch, M. & Reipschlager, A. Biological impact of elevated ocean CO_2 concentrations: Lessons from animal physiology and earth history. *J. Oceanogr.* **60,** 705–718 (2004).
14. Seibel, B. A. & Walsh, P. J. Biological impacts of deep-sea carbon dioxide injection inferred from indices of physiological performance. *J. Exp. Biol.* **206,** 641–650 (2003).
15. Seibel, B. A. & Walsh, P. J. Carbon cycle - Potential, impacts of CO_2 injection on deep-sea biota. *Science* **294,** 319–320 (2001).
16. Melzner, F. *et al.* Future ocean acidification will be amplified by hypoxia in coastal habitats. *Mar. Biol.* **160,** 1875–1888 (2013).
17. Stumpp, M., Wren, J., Melzner, F., Thorndyke, M. C. & Dupont, S. T. CO_2 induced seawater acidification impacts sea urchin larval development I: Elevated metabolic rates decrease scope for growth and induce developmental delay. *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* **160,** 331–340 (2011).
18. Andersson, A. J., Mackenzie, F. T. & Gattuso, J.-P. Effects of ocean acidification on benthic processes, organisms, and ecosystems. in *Ocean Acidification* (eds Gattuso, J. P. & Hansson, L.) 122–153 (Oxford University Press, 2011).
19. Dupont, S., Ortega-martínez, O. & Thorndyke, M. Impact of near-future ocean acidification on echinoderms. *Ecotoxicology* **19,** 449–462 (2010).
20. Orr, J. C. *et al.* Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. *Nature* **437,** 681–686 (2005).
21. Widdicombe, S., Spicer, J. I. & Kitidis, V. Effects of ocean acidification on sediment fauna in *Ocean acidification* (eds Gattuso, J. P. & Hansson, L.) 176–191 (Oxford University Press, 2011).
22. Zhu, Q., Aller, R. C. & Fan, Y. Two-dimensional pH distributions and dynamics in bioturbated marine sediments. *Geochim. Cosmochim. Acta* **70,** 4933–4949 (2006).
23. Widdicombe, S. *et al.* Effects of CO_2 induced seawater acidification on infaunal diversity and sediment nutrient fluxes. *Mar. Ecol. Prog. Ser.* **379,** 59–75 (2009).
24. Widdicombe, S. & Needham, H. R. Impact of CO_2-induced seawater acidification on the burrowing activity of *Nereis virens* and sediment nutrient flux. *Mar. Ecol. Prog. Ser.* **341,** 111–122 (2007).
25. Dashfield, S. L., Somerfield, P. J., Widdicombe, S., Austen, M. C. & Nimmo, M. Impacts of ocean acidification and burrowing urchins on within-sediment pH profiles and subtidal nematode communities. *J. Exp. Mar. Biol. Ecol.* **365,** 46–52 (2008).
26. Beukema, J. J., Dekker, R. & Philippart, C. J. M. Long-term variability in bivalve recruitment, mortality, and growth and their contribution to fluctuations in food stocks of shellfish-eating birds. *Mar. Ecol. Prog. Ser.* **414,** 117–130 (2010).
27. van Gils, J. A., Piersma, T., Dekinga, A., Spaans, B. & Kraan, C. Shellfish dredging pushes a flexible avian top predator out of a marine protected area. *PLoS Biol.* **4,** e376 (2006).
28. Riisgård, H. U. *et al.* The exhalant jet of mussels *Mytilus edulis*. *Mar. Ecol. Prog. Ser.* **437,** 147–164 (2011).
29. Graf, G. Benthic-Pelagic Coupling - a Benthic View. *Oceanogr. Mar. Biol.* **30,** 149–190 (1992).
30. Gazeau, F. *et al.* Impacts of ocean acidification on marine shelled molluscs. *Mar. Biol.* **160,** 2207–2245 (2013).
31. Thomsen, J., Casties, I., Pansch, C., Körtzinger, A. & Melzner, F. Food availability outweighs ocean acidification effects in juvenile *Mytilus edulis*: laboratory and field experiments. *Glob. Change Biol.* **19,** 1017–1027 (2013).
32. Tomanek, L., Zuzow, M. J., Ivanina, A. V., Beniash, E. & Sokolova, I. M. Proteomic response to elevated PCO_2 level in eastern oysters, *Crassostrea virginica*: evidence for oxidative stress. *J. Exp. Biol.* **214,** 1836–1844 (2011).
33. Tunnicliffe, V. *et al.* Survival of mussels in extremely acidic waters on a submarine volcano. *Nat. Geosci.* **2,** 344–348 (2009).
34. Ries, J. B., Cohen, A. L. & McCorkle, D. C. Marine calcifiers exhibit mixed responses to CO_2-induced ocean acidification. *Geology* **37,** 1131–1134 (2009).
35. Thomsen, J. *et al.* Calcifying invertebrates succeed in a naturally CO_2-rich coastal habitat but are threatened by high levels of future acidification. *Biogeosciences* **7,** 3879–3891 (2010).
36. Melzner, F. *et al.* Food supply and seawater pCO_2 impact calcification and internal shell dissolution in the blue mussel *Mytilus edulis*. *PLoS One* **6,** e24223 (2011).
37. Hale, R., Calosi, P., McNeill, L., Mieszkowska, N. & Widdicombe, S. Predicted levels of future ocean acidification and temperature rise could alter community structure and biodiversity in marine benthic communities. *Oikos* **120,** 661–674 (2011).
38. Ishida, H. *et al.* Effects of CO_2 on benthic biota: An *in situ* benthic chamber experiment in Storfjorden (Norway). *Mar. Pollut. Bull.* **73,** 443–451 (2013).
39. Kurihara, H., Ishimatsu, A. & Shirayama, Y. Effects of elevated seawater CO_2 concentration on the meiofauna. *J. Mar. Sci. Technol.-Taiwan* **15,** 17–22 (2007).
40. Takeuchi, K., Fujioka, Y., Kawasaki, Y. & Shirayama, Y. Impacts of high concentration of CO_2 on marine organisms; a modification of CO_2 ocean sequestration. *Energy Convers. Manag.* **38,** Supplement, S337–S341 (1997).
41. Liu, J., Weinbauer, M. G., Maier, C., Dai, M. & Gattuso, J.-P. Effect of ocean acidification on microbial diversity and on microbe-driven biogeochemistry and ecosystem functioning. *Aquat. Microb. Ecol.* **61,** 291–305 (2010).
42. Marinelli, R. L. & Waldbusser, G. G. In *Interactions Between Macro- and Microorganisms in Marine Sediments* (eds Kristensen, E., Haese, R. R. & Kostka, J. E.) 233–249 (American Geophysical Union, 2005).
43. Ramette, A. Quantitative Community Fingerprinting Methods for Estimating the Abundance of Operational Taxonomic Units in Natural Microbial Communities. *Appl. Environ. Microbiol.* **75,** 2495–2505 (2009).
44. Thompson, R. C., Norton, T. A. & Hawkins, S. J. Physical stress and biological control regulate the producer–consumer balance in intertidal biofilms. *Ecology* **85,** 1372–1382 (2004).

45. Joint, I., Doney, S. C. & Karl, D. M. Will ocean acidification affect marine microbes? *ISME J*. **5,** 1–7 (2011).
46. Kerfahi, D. *et al.* Shallow Water Marine Sediment Bacterial Community Shifts Along a Natural CO2 Gradient in the Mediterranean Sea Off Vulcano, Italy. *Microb. Ecol.* **67,** 819–828 (2014).
47. Roy, R. *et al.* The Dissociation-Constants of Carbonic-Acid in Seawater at Salinities 5. *Mar. Chem.* **44,** 249–267 (1993).
48. Coffin, R. B., Montgomery, M. T., Boyd, T. J. & Masutani, S. M. Influence of ocean CO2 sequestration on bacterial production. *Energy* **29,** 1511–1520 (2004).
49. Tait, K., Stahl, H., Taylor, P. & Widdicombe, S. Rapid response of the active microbial community to CO2 exposure from a controlled sub-seabed CO2 leak in Ardmucknish Bay (Oban, Scotland). *Int. J. Greenh. Gas Control.* **38,** 171–181 (2014).
50. Taylor, J. D., Kennedy, W. J. & Hall, A. The shell structure and mineralogy of the bivalvia. 2. Lucinacea, Clavagellacea conclusions. (Trustees of the British *Museum*, 1973).
51. Möller, P., Pihl, L. & Rosenberg, R. Benthic faunal energy flow and biological interaction in some shallow marine soft bottom habitats. *Mar Ecol Prog Ser* **27,** 109–121 (1985).
52. Flach, E. C. The Influence of the Cockle, *Cerastoderma edule*, on the Macrozoobenthic Community of Tidal Flats in the Wadden Sea. *Mar. Ecol.* **17,** 87–98 (1996).
53. Swanberg, L. I. The influence of the filter-feeding bivalve *Cerastoderma edule* L. on microphytobenthos: a laboratory study. *J. Exp. Mar. Biol. Ecol.* **151,** 93–111 (1991).
54. McGreer, E. R. Factors affecting the distribution of the bivalve, *Macoma balthica* (L.) on a mudflat receiving sewage effluent, Fraser river estuary, British Columbia. *Mar. Environ. Res.* **7,** 131–149 (1982).
55. Cornwall, C. E. & Hurd, C. L. Experimental design in ocean acidification research: problems and solutions. *ICES J. Mar. Sci. J. Cons.* doi: 10.1093.icesjms.fsv118 (2015).
56. Dickson, A. G., Sabine, C. L. & Christian, J. R. *Guide to best practices for ocean CO2 measurements*. (North Pacific Marine Science Organization, 2007).
57. Lewis, E. & Wallace, D. W. R. CO2SYS—Program developed for the CO2 system calculations Report ORNL. *CDIAC-105 Oak Ridge TN Carbon Dioxide Inf. Anal. Cent. ORNL* (1998).
58. Uchiyama, M. & Mihara, M. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. *Anal. Biochem.* **86,** 271–278 (1978).
59. Heip, C., Vincx, M. & Vranken, G. *The ecology of marine nematodes*. (Aberdeen University Press, 1985).
60. Higgins, R. P. & Thiel, H. *Introduction to the study of meiofauna*. (Smithson. Inst. Press Wash. DC, 1988).
61. De Grisse, A. Redescription ou modifications de quelques technique utilisés dan l'etude des nématodes phytoparasitaires. *Meded. Rijksfak. LandbWet. Gent.* **34,** 251–369 (1969).
62. Warwick, R. M., Platt, H. M. & Somerfield, P. J. *Free-living marine nematodes. Part III: monhysterids.* (Field Studies Council, for the Linnean Society of London and the Estuarine and Coastal Sciences Association, 1998).
63. Vanaverbeke, J. *et al.* NeMys: World Database of Free-Living Marine Nematodes. (2014). Available at: www.nemys.ugent.be. (Accessed: 1th September 2014).
64. R. Core Team. *R: A language and environment for statistical computing.* (R Foundation for Statistical Computing, 2013).
65. Anderson, M., Gorley, R. N. & Clarke, R. K. Permanova+ for Primer: Guide to Software and Statistical Methods. (PRlMER-E Ltd Plymouth, 2008).
66. Clarke, K. R. & Gorley, R. N. *User manual/tutorial.* (PRIMER-E Ltd Plymouth, 2006).
67. Clarke, K. R., Somerfield, P. J. & Chapman, M. G. On resemblance measures for ecological studies, including taxonomic dissimilarities and a zero-adjusted Bray–Curtis coefficient for denuded assemblages. *J. Exp. Mar. Biol. Ecol.* **330,** 55–80 (2006).
68. Legendre, P. & Legendre, L. *Numerical ecology.* (Elsevier, 1998).
69. Hammer, Ø., Harper, D. A. T. & Ryan, P. D. Past: Paleontological Statistics Software Package for education and data analysis. *Paleontol. Electrónica* **4,** 9 pp. (2001).
70. Diaz, R. J. & Rosenberg, R. Marine benthic hypoxia: a review of its ecological effects and the behavioural responses of benthic macrofauna. *Oceanogr. Mar. Biol. Annu. Rev.* **33,** 245–03 (1995).
71. Rosenberg, R., Hellman, B. & Johansson, B. Hypoxic tolerance of marine benthic fauna. *Mar. Ecol. Prog. Ser. Oldendorf* **79,** 127–131 (1991).
72. Waldbusser, G. G., Bergschneider, H. & Green, M. A. Size-dependent pH effect on calcification in post-larval hard clam *Mercenaria* spp. *Mar. Ecol. Prog. Ser.* **417,** 171–182 (2010).
73. Green, M. A., Jones, M. E., Boudreau, C. L., Moore, R. L. & Westman, B. A. Dissolution mortality of juvenile bivalves in coastal marine deposits. *Limnol. Oceanogr.* **49,** 727–734 (2004).
74. Waldbusser, G. G. *et al.* A developmental and energetic basis linking larval oyster shell formation to acidification sensitivity. *Geophys. Res. Lett.* **40,** 2171–2176 (2013).
75. Hall-Spencer, J. M. *et al.* Volcanic carbon dioxide vents show ecosystem effects of ocean acidification. *Nature* **454,** 96–99 (2008).
76. Harper, E. M. The molluscan periostracum: an important constraint in bivalve evolution. *Palaeontology* **40,** 71–98 (1997).
77. Glover, C. P. & Kidwell, S. M. Influence of organic matrix on the post-mortem destruction of molluscan shells. *J. Geol.* **101,** 729–747 (1993).
78. Mount, A. S., Wheeler, A. P., Paradkar, R. P. & Snider, D. Hemocyte-Mediated Shell Mineralization in the Eastern Oyster. *Science* **304,** 297–300 (2004).
79. Kádár, E. Haemocyte response associated with induction of shell regeneration in the deep-sea vent mussel *Bathymodiolus azoricus* (Bivalvia: Mytilidae). *J. Exp. Mar. Biol. Ecol.* **362,** 71–78 (2008).
80. Maritim, A. C., Sanders, R. A. & Watkins, J. B. Diabetes, oxidative stress, and antioxidants: A review. *J. Biochem. Mol. Toxicol.* **17,** 24–38 (2003).
81. Valavanidis, A., Vlahogianni, T., Dassenakis, M. & Scoullos, M. Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. *Ecotoxicol. Environ. Saf.* **64,** 178–189 (2006).
82. Finkel, T. & Holbrook, N. J. Oxidants, oxidative stress and the biology of ageing. *Nature* **408,** 239–247 (2000).
83. Del Rio, D., Stewart, A. J. & Pellegrini, N. A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. *Nutr. Metab. Cardiovasc. Dis.* **15,** 316–328 (2005).
84. Abele, D., Tesch, C., Wencke, P. & Pörtner, H. O. How does oxidative stress relate to thermal tolerance in the Antarctic bivalve *Yoldia eightsi*? *Antarct. Sci.* **13,** 111–118 (2001).
85. McArthur, M. C. & Sohal, R. S. Relationship between Metabolic Rate, Aging, Lipid Peroxidation, and Fluorescent Age Pigment in Milkweed Bug, *Oncopeltus fasciatus* (Hemiptera). *J. Gerontol.* **37,** 268–274 (1982).
86. Thomsen, J. & Melzner, F. Moderate seawater acidification does not elicit long-term metabolic depression in the blue mussel *Mytilus edulis*. *Mar. Biol.* **157,** 2667–2676 (2010).
87. Beniash, E., Ivanina, A., Lieb, N. S., Kurochkin, I. & Sokolova, I. M. Elevated level of carbon dioxide affects metabolism and shell formation in oysters *Crassostrea virginica*. *Mar. Ecol. Prog. Ser.* **419,** 95–108 (2010).
88. Michaelidis, B., Ouzounis, C., Paleras, A. & Pörtner, H. O. Effects of long-term moderate hypercapnia on acid-base balance and growth rate in marine mussels *Mytilus galloprovincialis*. *Mar. Ecol. Prog. Ser.* **293,** 109–118 (2005).
89. Dorey, N., Lançon, P., Thorndyke, M. & Dupont, S. Assessing physiological tipping point of sea urchin larvae exposed to a broad range of pH. *Glob. Change Biol.* **19,** 3355–3367 (2013).

90. Dries, R.-R. & Theede, H. Sauerstoffmangelresistenz mariner Bodenvertebraten aus der Westlichen Ostsee. *Mar. Biol.* **25**, 327–333 (1974).
91. Darr, A., Gogina, M. & Zettler, M. L. Detecting hot-spots of bivalve biomass in the south-western Baltic Sea. *J. Mar. Syst.* **134**, 69–80 (2014).
92. Baker, P. K. & Mann, R. *Soft Shell Clam, Mya Arenaria.* (Living Resources Subcommittee, Chesapeake Bay Program, 1991).
93. Bianchelli, S., Gambi, C., Zeppilli, D. & Danovaro, R. Metazoan meiofauna in deep-sea canyons and adjacent open slopes: A large-scale comparison with focus on the rare taxa. *Deep Sea Res. Part Oceanogr. Res. Pap.* **57**, 420–433 (2010).
94. Gambi, C., Lampadariou, N. & Danovaro, R. Latitudinal, longitudinal and bathymetric patterns of abundance, biomass of metazoan meiofauna: importance of the rare taxa and anomalies in the deep Mediterranean Sea. *Adv. Oceanogr. Limnol.* **1**, 167–197 (2010).
95. Pusceddu, A., Bianchelli, S., Gambi, C. & Danovaro, R. Assessment of benthic trophic status of marine coastal ecosystems: Significance of meiofaunal rare taxa. *Estuar. Coast. Shelf Sci.* **93**, 420–430 (2011).
96. Giere, O. *Meiobenthology: the microscopic motile fauna of aquatic sediments* (Springer, 2009).
97. Fitzer, S. C. *et al.* Ocean acidification induces multi-generational decline in copepod naupliar production with possible conflict for reproductive resource allocation. *J. Exp. Mar. Biol. Ecol.* **418–419**, 30–36 (2012).
98. Kurihara, H. & Ishimatsu, A. Effects of high CO2 seawater on the copepod (Acartia tsuensis) through all life stages and subsequent generations. *Mar. Pollut. Bull.* **56**, 1086–1090 (2008).
99. Kurihara, H., Shimode, S. & Shirayama, Y. Effects of raised CO2 concentration on the egg production rate and early development of two marine copepods (Acartia steueri and Acartia erythraea). *Mar. Pollut. Bull.* **49**, 721–727 (2004).
100. Böer, S. I. *et al.* Time- and sediment depth-related variations in bacterial diversity and community structure in subtidal sands. *ISME J.* **3**, 780–791 (2009).
101. Mermillod-Blondin, F., Rosenberg, R., Francois-Carcaillet, F., Norling, K. & Mauclaire, L. Influence of bioturbation by three benthic infaunal species on microbial communities and biogeochemical processes in marine sediment. *Aquat. Microb. Ecol.* **36**, 271–284 (2004).

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H.S. and F.M. designed research; H.S., L.M., S.M., F.M. and K.G. performed experiments; H.S., L.M., K.G., S.M., S.G., D.A., A.V. and F.M. analyzed data; K.G., S.M., S.G., A.V. and F.M. contributed reagents/analytic tools; H.S., L.M., K.G. and F.M. wrote the manuscript text. All authors commented on and discussed several draft versions of the manuscript.

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