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The response and potential adaptation of marine species to CO_2 exposure associated with different potential CO_2 leakage scenarios

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1.0 Executive summary

Within ECO2, the aim of work package 4.2 was to investigate the response and potential adaptation of marine species to CO_2 exposure associated with different potential CO_2 leakage scenarios. The result was a combination of experimental investigations from whole organism to community level responses to a range of elevated pCO_2 conditions, considering different ontogenetic stages from larvae through to adult.

Understanding the response of adult infauna organisms to elevated pCO_2 conditions was investigated in model species of bivalves and urchins. Medium term (3 month) exposures of an assembled marine infauna community from the Western Baltic Sea was investigated to six different pCO_2 levels in a mesocosm experiment. The response of bivalves *Cerastoderma edule*, *Mya arenaria* and *Macoma balthica*, as well as bacterial community composition and meiofauna community abundance and composition were analysed. **Increasing pCO_2 resulted in higher mortality and shell corrosion, with smaller organisms demonstrating greater vulnerability (>1500 µatm)**. While *C. edule* showed high sensitivity towards acidification, no mortality occurred in *M. arenaria* and *M. balthica*, indicating **responses to CCS leakage will be species specific**. Microbial communities and meiofauna composition changed significantly, yet subtly, at the highest treatment level.

Echinoderms comprise key ecosystem engineers of the soft-sediment shelf sea benthos. They have been identified as potentially vulnerable to acidified conditions because of their calcareous skeletons and typically poor acid base buffering capacity. The blood gas and acid base status of *Paracentrotus lividus* was determined during two chronic hypercapnic exposure investigations, including a short term (seven days) and medium term (65 days) exposure. *P. lividus,* though lacking a significant buffer, **were shown to tolerate chronic hypercapnia (20,000 ppm) for up to two months although substantial spine dissolution was identified during exposure to pH < 6.52 for 56 days**

compared to controls. This highlights the ability of *P. lividus* to demonstrate a short term buffering capacity, which was not detected over a medium term exposure. Nevertheless long duration exposure to acidification through CCS leaks would lead to high rates of mortality.

Many benthic organisms create micro-habitats allowing control and manipulation of their environment. pO_2 and pCO_2 was measured using micro-electrodes in the burrows of the infaunal brittlestar *Amphiura filiformis* under control conditions and scenarios relevant for CO_2 leakage. It was found that the condition within the burrow in control condition was hypoxic and hypercapnic but any increases of environmental pCO_2 were additive. **Elevated pCO_2 not only impacted the environment of *A. filiformis* but had a negative effect on physiology**. An array of methods including qPCR of candidate genes and measurements of activity, expression, feeding, respiration and acid-base regulation indicated an uncompensated acidosis leading to metabolic depression and decreased performance. The acoel worm *Symsagittifera roscoffensis* was used to understand the effect of elevated pCO_2 on symbiotic organisms such as corals. **This species was found to be resistant to extreme high pCO_2** and that observed impacts identified in other photosymbiotic species, such as foraminifera or corals, could occur via indirect impacts (e.g. calcification or feeding).

In general, **larvae were found to be more susceptible than adults to elevated pCO_2/reduced pH** but that different sub-lethal effects are produced at different critical pH thresholds.

In conclusion, this research contributing to this WP has demonstrated that responses to acute exposure to elevated pCO_2 from CCS leakage are **species-**

specific. Some benthic species exhibit extreme tolerance to elevated pCO_2 in the short and medium term. However, other species – including burrowing infauna – might be more susceptible to elevated pCO_2 in sediments. **Of concern are the larval stages of key ecosystem engineers** that, at key times of the year, might be susceptible to the impacts of a CCS reservoir failure.

2.0 General introduction

Identifying the effects of elevated seawater pCO_2 (hypercapnia) on marine organisms has risen up the research agenda because of the realization that rising atmospheric CO_2 concentrations have led to a decrease in ocean average surface pH by 0.1 units since industrialization and are expected to decline further by 0.3 to 0.5 units until the end of the century, a phenomenon known as ocean acidification (Caldeira and Wickett, 2003, Dupont and Pörtner, 2013). In response, the long-term sequestration of carbon dioxide into sub-seabed geological structures (carbon capture and storage; CCS) in sub-sea bed reservoirs has been advocated as a potential mitigation strategy by the World Energy Outlook (IEA, 2010). It is argued that this technique will permit the continued combustion of fossil fuels for energy whilst preventing further additions of CO_2 to the atmosphere from this source (Haugen and Eide, 1996).

The potential of this strategy for mitigation of climate change impacts is scientifically well recognized (Widdicombe et al. 2009; Hoegh-Guldberg and Bruno, 2010). For example, the Skagerrak and Kattegat region has been identified as a suitable area for CCS (Haugen et al., 2011). However, before CCS can be relied upon as a safe development to mitigate global climate change, assessments on the stability of storage sites needs to be ascertained. Leakages from CCS sites pose a threat to marine life, as CO_2 stored under the seabed could enter the overlying water column through fracture zones and lead to acidification of the sediment pore waters and the overlying water column (Blackford & Gilbert 2007). The potential risks of seepage of pure CO_2 may represent an enormous local challenge to benthic and infaunal organisms due to strong local pH fluctuations (IPCC, 2005). Benthic habitats are often already confronted with strong fluctuations in pO_2 und pCO_2, leading to naturally acidified conditions, and these may be amplified by CCS leakage or by ocean acidification (Melzner et al., 2012).

These risks have been inadequately investigated to date and within the remit of the *E*CO_2 Project a key aim has been to produce robust data from which to establish a legal framework for environmentally safe application of CCS and potential monitoring techniques (Hawkins 2004; Keating et al. 2011).

Water breathing animals exchange CO_2 across epithelia by maintaining a diffusion gradient with approximately 0.2-0.4 kPa higher pCO_2 values in tissues compared to the surrounding water (Evans et al., 2005, Melzner et al., 2009). In order to maintain this diffusion gradient, the increase of seawater pCO_2 will result in an increase of pCO_2 in body tissues and fluids. Such hypercapnic conditions can cause an extracellular acidosis if not actively compensated by hydrogen ion (H^+) secretion or/and bicarbonate (HCO_3^-) accumulation in body fluids (Heisler, 1989). Earlier studies using *Sipunculus nudus* as a marine model organism demonstrated that an uncompensated extracellular acidosis can trigger metabolic depression (Reipschläger and Pörtner, 1996, Reipschläger et al., 1997, Pörtner et al., 1998). CO_2 induced acid-base disturbances have been demonstrated to alter the physiology and developmental features of marine invertebrates (Thomsen and Melzner, 2010, Hu et al., 2011, Stumpp et al., 2011b, Stumpp et al., 2012). For example, echinoderms, crustaceans and molluscs have been shown to alter growth/developmental rates, oxygen consumption and gene expression in response to hypercapnia (Kurihara et al., 2007, Dupont et al., 2010, Lannig et al., 2010, Walther et al., 2010, Hu et al., 2011, Stumpp et al., 2011a, Stumpp et al., 2011b, Stumpp et al., 2012, Dupont and Thorndyke, 2014).

Infauna organisms could be especially affected by long term / chronic leakage of acidified seawater, as motility of sediment dwelling macrofauna is reduced compared to many epibenthic species. Furthermore, because of the very low O_2 partial pressures (Vopel et al., 2003) in burrow habitats, very likely accompanied by high CO_2 partial pressures and low pH, burrowing species already experience higher levels of acidity compared to other benthic epifauna.

It can be expected that increases in seawater pCO_2 will strongly affect CO_2 and pH gradients within sediment burrows, leading to strong acid-base challenges to infaunal organisms. There is a lack of detailed understanding into the types of stresses imposed on infauna organisms from CO_2 gradients that would formulate in the sediment as CO_2 leaks from sub seabed storage sites, i.e. bubbles causing sediment disturbance, CO_2 gradients in sediments and how this might impact on movement/behavior and feeding in sediment dwellers.

No studies have addressed the impacts of elevated seawater pCO_2 on the ecologically very important infaunal bivalve communities of shallow, sandy coastal sediments in the North Atlantic region so far. Bivalve molluscs are the defining macrobenthic organisms in these habitats, with a key role for many fish and migratory bird species: they constitute 60-70% of the benthic biomass and a similar fraction of the diet of e.g. Wadden Sea birds (Beukema et al. 2010). Changes in bivalve abundance, either due to natural fluctuation or due to human harvesting activities, have been demonstrated to directly influence bird stocks (Van Van Gils et al. 2006). 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* (Taylor et al., 1973). *C. edule* and *M. balthica* live within the top 2-5 cm of the sediment surface, while *M. arenaria* occurs to sediment depths of up to 50 cm (Möller et al. 1985). *C. edule* can occur at densities up to 60,000 ind. m^{-2} in the field (Jensen, 1992). Abundance varies with sediment type and area. Möller and Rosenberg (1983) found abundances of *M. arenaria* of 13,000 to 458,000 ind. m^{-2} in sandy areas of Western Sweden and 2,000 to 4,000 ind. m^{-2} in soft bottom areas. Abundances of *C. edule* were lower with 5,000 to 59,000 ind. m^{-2} and 600 to 1,400 ind. m^{-2} in the same sediment types. *M. balthica* occurs in much lower densities compared to *C. edule* or *M. arenaria* with 30 ind. m^{-2} in the Dutch Wadden Sea (Beukema 1976). 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 and considerable mobility and activity (Flach 1996), they exert a strong influence on infauna communities and on biogeochemical processes, e.g. fertilization of microphytobenthos through NH_4^+ excretion (Flach 1996, Swanberg 1991). The shell of all three species consists of aragonite, the polymorph of calcium carbonate that is most prone to dissolution (Taylor et al., 1973; Glover and Kidwell, 1993). The thickness of the periostracum, the organic cover that protects the shell from the outside, varies with species: *C. edule* (2 µm) and *M. balthica* (5 µm) have a thin periostracum, while the periostracum of *M. arenaria* is thicker (20 µm, Harper, 1997).

Owing to the difficulties associated with maintaining such communities in a natural-like state in the laboratory, primarily with respect to their enormous filter feeding capacity, past research efforts have focused on infauna bivalves in single species experiments and without providing sediment. Green et al. (2004) demonstrated the importance of sediments being provided for meaningful assessment of bivalve vulnerability to seawater acidification: these authors could show that severe dissolution mortality of freshly settled juvenile hard clams *Mercenaria mercenaria* can occur in the first few cm of the sediment, which they found to be undersaturated with calcium carbonate during certain periods of the year. Larval *M. mercenaria* were later identified to be vulnerable to ocean acidification as well, with reduced rates of calcification, fitness and increased mortality observed at seawater pCO_2 lower than 1,600 µatm (Talmage & Gobler 2010). A range of recent studies on several (mainly epibenthic) bivalves 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 (<5,000 µatm). In addition, increased occurrence of oxidative and acid-base regulatory stress has been observed in bivalves (see review by Gazeau et al. 2013, Melzner et al. 2013, Thomsen et al. 2013, Tomanek et al. 2011). 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 (Tunnicliffe et al. 2009, Ries et. al. 2009, Thomsen et al. 2010, Melzner et al. 2011).

It has also been demonstrated that the burrowing activity of infauna (e.g. echinoderms) has a strong influence on the biogeochemistry of sediments and the composition of meiofauna communities (Dashfield et al. 2008). Changes in macrofauna abundance in response to elevated seawater pCO_2 could thus have strong repercussions on infauna ecosystem processes (Widdicombe et al. 2009, Widdicombe & Needham 2007). However, these strong effects of CO_2-enriched seawater on infauna communities in the above studies were observed only at pH values < 7.0 (pCO_2 >10,000 μatm).

Only a handful of studies have exposed sediment communities in their natural (or approximately 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 sufficiently accurately during experiments. Widdicombe & Needham (2007) found in a five-week experiment that seawater acidification did not alter nereid worm burrow size and structure. However, they found significant changes in sediment nutrient fluxes, which they attributed to changes in bacterial communities. In one of the most comprehensive studies so far, Widdicombe et al. (2009) could demonstrate that 20-week exposure to elevated seawater pCO_2 significantly altered community structure and reduced macrofauna and nematode species diversity, with stronger negative effects observed for macrofauna communities. Sediment type also had a strong influence on CO_2 effects, with sandy sediment communities impacted more negatively by acidification. In addition, community compositional changes were accompanied by changes in nutrient fluxes.

Comprehensively, species sensitivity to elevated pCO_2 should be considered in

terms of the success over the whole life-cycle. Larvae are also often considered as a bottleneck in benthic species life-history cycle. Larvae of the green sea urchin (*Strongylocentrotus droebachiensis)* have been used as a model to better understand the effect of elevated pCO_2. Larvae were cultured from fertilization to metamorphic competence under a range of pCO_2. Despite sub-lethal negative impact on growth rate (with potential consequences for fitness), *S. droebachiensis* expressed remarkable plasticity to pCO_2 and were able to develop up to 5000 µatm. At highest pCO_2 abnormal development and high mortality were observed. These negative effects were associated with perturbation in acid-base regulation and consequences for the larval digestion (Stumpp et al. 2013). Exposure to elevated pCO_2 led to a drop in gastric pH of the larvae stomach, which decreased enzymatic digestive efficiency and triggered compensatory feeding. When larvae of the purple sea urchin *Strongylocentrotus purpuratus* were exposed to pCO_2 levels above their natural variability range, they underwent high-frequency budding (release of blastula-like particles; Chan et al. 2012). This was interpreted as an attempt to reduce larval size and metabolic costs during transient environmental challenges. In conclusion, elevated pCO_2 also has the potential to impact larval physiology and translate into significant consequences to fitness and long-term population sustainability (Chan et al. 2012; Dorey et al. 2013; Stumpp et al. 2012).

The few studies that have investigated the influence of elevated seawater pCO_2 on benthic communities up to now were primarily short term exposures and single species experiments that utilized pCO_2 levels corresponding to ocean acidification scenarios and often did not incorporate natural sediments that the species are associated with (see e.g. Doney et al. 2009, Kroeker et al. 2010, 2011 for review). To predict benthic marine ecosystem vulnerability to potential chronic leakages from CCS storage sites, meaningful experiments need to (i)

utilize a pCO_2 range that incorporates higher pCO_2 levels than expected to occur through ongoing ocean acidification, (ii) perform analyses on the community level, (iii) and use long-term exposures.

3.0 Experimental studies in *E*CO_2

3.1 Simulated leakage of high *p*CO_2 water significantly impacts bivalve dominated infauna communities from the Western Baltic Sea *[Schade et al., in prep.]*

Owing to the high sensitivity of bivalves and of sandy sediment communities to acidified seawater in general, and the high abundance of infauna bivalves in valuable coastal habitats, a mesocosm experiment was conducted using natural-like bivalve dominated sandy communities from the Western Baltic Sea as a model system using a flow though seawater design with optimized food supply. Mortality, growth, fitness and shell integrity of the dominant bivalve, the cockle *C. edule* was studied. In addition, malondialdehyde (MDA) concentrations in *C. edule* tissues were assessed as a marker of oxidative stress and metabolism. We also monitored survival of the bivalves *M. balthica* and *M. arenaria*, species that occur at much lower densities in the same habitat, as well as microbial and meiofauna community structure. It was hypothesised (i) that *C. edule* would be very sensitive to seawater acidification, reacting with increased mortality and shell dissolution. The species has a very thin periostracum and has previously been found to react much more sensitively to abiotic stress (e.g. hypoxia, Dries & Theede 1974) than the other two species. In addition, (ii) it was hypothesized that changes in *C. edule* abundance and fitness would impact microbial and meiofauna composition.

3.1.1 Materials and Methods

Experimental setup.

The mesocosm experiment took place during December 17th 2011 to March 6th 2012. Sandy communities were exposed to 6 different seawater *p*CO_2 regimes (6 replicates each, 36 experimental units) for a total of 3 months in a climate

controlled room. Six header tanks were continuously supplied with filtered seawater from Kiel Fjord (Fig. 1). pH was maintained in the header tanks using a pH feedback system (IKS Aquastar, iks Computersysteme GmbH, Karlsbad, Germany). Treatment levels were achieved through addition of gaseous CO_2. Calculated pH (NBS scale) ranged from 6.4 to 8. The corresponding pCO_2 levels were 900 (control), 1,500 (treatment 1), 2,900 (treatment 2), 6,600 (treatment 3), 12,800 (treatment 4) and 24,000 µatm (treatment 5). Algae (*Rhodomonas* sp.) were cultured as previously described (Thomsen et al. 2010) and added continuously into the header tanks to maintain a stable concentration of ca. 4,000 cells ml^{-1} in the header tanks via a peristaltic pump (MCP, ISMATEC, IDEX Health & Science GmbH, Wertheim-Mondfeld, Germany). Each header tank continuously supplied six replicate experimental units (EU) of a size of 11.5 l.

Each EU consisted of a round plastic bucket containing sediment (20 cm deep) and a free water column (10 cm, Fig. 1). 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); the upper 10 cm consisted of surface sediment from the station at which the experimental animals were sampled to resemble the natural conditions, as well as provide naturally occurring microbial communities and meiofauna. Bivalves and sediment were sampled in Kiel Fjord at Falckenstein with a Van Veen grab in ca. 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. $1m^2$ of sediment at Falckenstein was found to contain 136 *M. arenaria*, 9 *M. balthica* and 1,010 *C. edule* (average values of 3 Van Veen grabs). In order to simulate the density and size distribution observed in the natural habitat in our laboratory experiment, five *M. arenaria* (size classes: 0.5-1 cm: two animals; 1-1.5 cm: two animals; 2-2.5 cm: one animal); one *M. balthica* and 40 *C. edule* (size classes: 0-0.5 cm: three animals; 0.5-1 cm: 18 animals; 1-1.5 cm: 11 animals; 1.5-2 cm: seven animals; 2-2.5 cm: 1 animal) were added to each EU.

A flow rate of 100 ml min^{-1} was provided to each EU from the respective header tank via gravity feed. A pH meter equipped with a pH electrode (SenTix 81 Plus, WTW Wissenschaftlich Technische Werkstätten GmbH, Weilheim, Germany) was used to measure temperature and pH; a salinometer (Cond315i instrument, WTW Wissenschaftlich Technische Werkstätten GmbH, Weilheim, Germany) was used to measure salinity. Throughout the experiment pH, salinity, temperature and flow rate were controlled daily. The salinity ranged between 14.6 and 20.5 psu and temperatures ranged between 8.9°C in December and 4.3°C in March. Temperature and salinity and temperature in the EUs fluctuated with natural occurring changes in Kiel Bay seawater. Light conditions were similar for all basins. The light intensity ranged from 5.53 to 7 µmol $s^{-1}m^{-2}$, with light hours between 8:00 to 17:00. Dead animals were removed daily from the EUs. Behavior 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) were then calculated according to the Guide to best practices for Ocean CO_2 measurements (Dickson et al., 2007), using CO2SYS (Lewis and Wallace, 1998) using pH (NBS scale) and C_T, temperature, salinity, and first and second dissociation constants of carbonic acid in seawater (according to Roy et al., 1993).

Bivalve sampling, meiofauna and microbial community analysis

At the end of the experiment four *C. edule* were frozen at -80°C for oxidative stress analysis. For the measurement of tissue malondialdehyde (MDA) content, the frozen bivalve tissues were ground in liquid nitrogen using mortar and pestle. The four bivalves taken from each replicate were ground separately. From each individual, 50 mg were taken to mix all four bivalves of each replicate

in one pool. This was due to the requirement for sufficient amounts of bivalve tissue for the measurements. Each replicate pool was then measured separately. All samples were constantly kept frozen at -80 °C or in liquid nitrogen. MDA concentration was determined following the protocol of Mihara and Uchiyama (1978). Tissue was homogenized with phosphoric acid (0.2%) in relation 1:5 and the same amount of phosphoric acid (2%) was added. One blank (homogenate and 3 mM hydrogen chloride) and two samples (homogenate and TBA solution) were incubated at 100°C for one hour for each treatment. 0.5 ml of butanol was then added. After several vortexing procedures all samples and blanks were measured in a plate reader (Plate Chameleon, Hidex, Turku, Finland) at 532 and 600 nm. A difference between the two extinctions was calculated to then assess MDA concentration with a standard curve (buffer solution containing 1.01 mM MDA in 1.1 % H3PO4). Tissue concentration of MDA was calculated followed equation 1:

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

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]

All other bivalves were frozen at -20°C for measurements of shell free dry mass according to Thomsen et al. (2013). In addition, SEM and stereomicroscope analysis were carried out using *C. edule* shells to study the degree of shell dissolution. There were 5 randomly selected *C. edule* from each treatment were analyzed using a stereo - microscope (40-fold magnification) for signs of external shell dissolution and presence of holes. SEM was used to examine

external shell dissolution in the lower CO_2 treatments (control – treatment 3, 3 shells each). Shells were mounted on SEM pedestal stubs. Sections were coated with gold-palladium and examined using scanning electron microscopes (Nanolab 7, Zeiss, Oberkochen, Germany and Hitachi S4800, Hitachi High - Technologies Europe, Krefeld, Germany).

Samples for analysis of bacterial and meiofauna community structure were taken after 6 and after 12 weeks with a corer of 2.5 cm diameter and 1 cm depth. Sediment samples for bacterial community analysis were transferred into Eppendorf tubes and kept frozen at -20°C. One sample per EU was analyzed. For each CO_2 treatment level, 6 replicate sediment samples were obtained and subjected to total community DNA extraction by 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 (Promega Corporation, Madison, WI). Benthic bacterial community structures were determined by means of the high-throughput fingerprinting technique ARISA, following a previously published procedure with slight modifications (Ramette, 2009): Final concentrations of PCR ingredients within 50 µl-reactions were 0.4 µM of each primer (Biomers, Ulm, Germany), 250 µM of each dNTP (peqGOLD Kit; Peqlab, Erlangen, Germany), 0.1 mg ml^{-1} BSA (Sigma-Aldrich Biochemie GmbH, Hamburg, Germany), 1 × 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, three PCR replicates were prepared. Quality assessment of 2-3 raw profiles and binning were done as previously reported by (Ramette, 2009). Samples 23 (after 12 weeks) and 15 (after 12 weeks) did not show any content and were excluded from the analysis.

Meiofauna samples were stored in 4% PFA until analysis. All samples were sieved on a 1-mm and 32-µm mesh. The fractions retained on the 32-µm mesh sieve were centrifuged three times with the colloidal silica polymer LUDOX 40

(Heip et al. 1985) and rinsed with tap water. The extracted fraction was preserved in 4% buffered formalin and stained with Rose Bengal. All metazoan meiobenthic organisms were classified at higher taxon level and counted under a stereoscopic microscope (Guilini et al. 2012).

Statistical analysis

For most of the statistical analysis the program R (v.2.13.2; The R Foundation for Statistical Computing; http://www.R-project.org) was used. Mortality of different size classes within each treatment was tested with a Kruskal Wallis test as normal distribution and homogeneity of variances could not be achieved. Kruskal mc was used as a post hoc test. Mortality in all size classes and fraction of dissolved shells were tested the same way. The influence of pCO_2 on malondialdehyde (MDA) content and shell free dry mass was tested using an ANOVA and a Tukey HSD post hoc test. MDA values were normally distributed (p-value >0.05) and homogeneity of variances was given (p-value >0.05). Normal distribution could not be achieved for shell free dry mass. However, as the histogram showed a near normal distribution of values the parametric test was used. Homogeneity of variances was achieved after box-cox transformation of values. Behavior was not normal distributed and thus tested using PERMANOVA as a non-parametric solution to a repeated measures analysis. Merged bacterial community profiles were generated in R by using a custom script and considering Operational Taxonomic Units (OTUs) that occurred at least twice (Ramette 2009). Variation partitioning and a multivariate ANOVA were conducted in PAST. Meiofauna compostion, as well as Gastrotricha abundance was tested using PERMANOVA. Total meiofauna density and abundance of the most abundant meiofauna groups were tested in R with an ANOVA. For all graphs, standard deviation (SD) is given.

3.1.2 Results

Mya arenaria and *Macoma balthica* remained burrowed during the entire experimental duration in all treatments. Mortality and behavior were observed during the experiment. No mortality was observed during the entire experimental duration for these two species. In the following, we will therefore focus on *C. edule*, which reacted much more sensitively to acidified seawater than the other two species.

Cerastoderma edule *mortality*

Mortality of *C. edule* was significantly impacted by high seawater pCO_2. A significantly elevated mortality could be shown for treatment 5 compared to the control and treatments 1 and 2. 50% mortality for *C. edule* in the highest treatment was reached after 68 days (Fig. 2, Tab. 1). Mortality, when averaged over all size classes, tended to increase in treatment 3 and 4 as well (total mortality 10-20%). However, this increase was not significant yet by the end of the experiment.

Smaller individuals reacted more sensitively towards acidification (Fig. 3, Tab. 1). There were no differences in mortality between size classes in the control, treatment 1 and treatment 2. Mortality in the smallest size class (0-0.5 cm) was significantly higher than mortality of cockles of a size 1-1.5 cm in treatment 3. Mortality of the smallest cockles (0-0.5 cm) in treatment 4 was significantly higher than that of the three largest size classes (1-1.5 cm, 1.5-2 cm, 2-2.5 cm). In the highest treatment, mortality was significantly higher in the smallest size class in comparison to cockles sized 1-2.5 cm. Unnoticed mortality did not occur in any EU, no empty shells were found at the termination of the experiment.

C. edule *shell integrity*

The comparison of intact shells against shells with holes showed an increase in shell dissolution of *C. edule* with increasing pCO_2 (Fig. 4; Tab. 1). Significantly higher rates of shell dissolution were found in treatments 4 and 5 (Tab. 1). Stereo - microscope images (at 40-fold magnification) demonstrated visible signs of shell dissolution for all treatments above 1,000 µatm (treatments 1-5), with the severity of dissolution increasing in higher treatments (Fig. 4). SEM analysis confirmed that shells from the control treatment possessed an intact periostracum and were not characterized by shell dissolution (Fig. 4, n=3 of 3 observations). Shells from treatment 1 (1,500 µatm) were characterized by signs of external dissolution (n=3 of 3 observations). However, these subtle signs of corrosion could not be resolved with the stereomicroscope (Fig. 4). SEM images were not obtained for the highest two treatments, as dissolution was obvious in the stereomicroscopic images already (Fig. 4). Cockles maintained under high pCO_2 (treatments 3, 4 and 5, >6,600 µatm) were additionally characterized by holes in the shell. Shells of freshly, i.e. <12h, deceased animals were observed post-mortem during the experiment for presence or absence of holes. Holes were visible at 6,600 µatm, however, only in very few individuals (less than 5%) and exclusively in smaller size classes (< 0.7 cm).

Cerastoderma edule *behaviour*

Different types of behavior could be observed: (1) cockles were burrowed with open siphons, (2) cockles were burrowed with closed siphons (not visible) and (3) cockles were lying on the sediment surface. At the control and treatment 1, clams were mostly displaying type 1 behavior (Fig. 5). In the highest CO_2 treatments (24,400 µatm), cockles migrated towards the surface, displaying type 3 behavior (Fig. 5). In 12,800 µatm, some clams were observed on the surface, however always at a lower abundance than in the highest treatment. Fig. 5 shows the amount of *C. edule* on the surface for experimental duration. PERMANOVA results ($F=2.80$, $p<0.01$) demonstrated that significantly more

cockles were located on the sediment in treatment 5 than in all other treatments at the end of the experimental period. In treatment 5, 50% of clams were located on the sediment surface on day 50 (Fig. 5).

C. edule *MDA content and shell - free dry mass (condition index)*

Whole body malondialdehyde concentrations (MDA) were significantly lower in treatment 3, 4 and 5 when compared to the control (Fig. 6). Additionally, MDA values of treatment 5 were significantly lower than values measured in treatment 1 and 2. Significant differences in shell - free dry mass were shown for treatment 5 when compared to the control, treatment 2, 3 and 4 (Tukey HSD, $p<0.05$). A significant difference was also found for treatment 4 in comparison to treatment 1. In all cases with significant differences between treatments, shell free dry mass at a particular shell width, an indicator for condition, was lower for high pCO_2 treatments (Fig. 6).

Bacterial community

Bacterial communities were significantly different when comparing our highest treatment and treatment 1 and the control ($p<0.05$, Table 2). Comparing OTUs using ARISA indicated a high variability between replicates within one treatment and between treatments. Groups were not well separated at both sampling time points (6 and 12 weeks). The similarity between different replicates of treatments was 50-80%, while the similarity in OTUs between different treatments was 70-80%. ANOVA analysis demonstrated that 5% change in bacterial communities could be explained by time, while CO_2 treatment explained 6.9% of the variation. Time and treatment together explained 11.7% of the variation. No interaction between time and CO_2 treatment was found. NMDS plots (not shown) do not suggest separation of the CO_2 treatment groups in terms of bacterial community composition.

Meiofauna community

Meiofauna community composition was found to differ between treatments and time. Using PERMANOVA, a time and treatment effect was detected for community composition (time: $p<0.01$; $F=5.25$; CO_2 treatment: $p<0.01$; $F=3.4$), as well as a treatment effect on abundance of gastrotricha ($p<0.01$, $F=5.9$). Abundance of gastrotricha was found to increase with pH (Fig. 7). Copepod abundance was also significantly impacted by CO_2 treatment (ANOVA; $p<0.02$, $F=3.72$) (Fig. 7).

3.1.3 Discussion

This is one of the few experiments to date to examine the impact of elevated seawater pCO_2 on a benthic infauna community and the first to investigate bivalve dominated communities under near – natural conditions by carefully transferring natural communities into the laboratory. Carbonate chemistry could be maintained constant by using a seawater – flow through experimental design and sufficiently high food concentrations could be maintained to support the very high filtration activity of the experimental species. Our study indicates strongly reduced survival of *Cerastoderma edule* at the highest treatment level, while other bivalve species were less severely impacted. Mortality of *C. edule* went along with significant changes in microbial and meiofauna community composition at the highest CO_2 level, indicating that loss of a key macrofauna species can lead to significant alteration of coastal sandy communities.

Response of C. edule *to increased* pCO_2

Significant mortality was observed in higher pCO_2 treatments for *C. edule*. Mortality decreased significantly with size. *C. edule* maintained at 24,400 µatm were characterized by continuous and high mortality (averaged over all size

classes) after an initial period of one to two weeks. High seawater pCO_2 >6,600 µatm resulted in increasing mortality for *C. edule*, although this trend was not statistically significant by the end of the experiment when averaged over all size classes (Fig. 2). The results of this study support findings of earlier studies that found increased mortality in adult bivalve species only at very high pCO_2 (>10,000 µatm), whereas elevated mortality was found in larval and juvenile live stages at lower pCO_2 levels (1,000-5,000 µatm) already (see reviews by Kurihara 2008; Kurihara et al. 2008; Gazeau et al. 2013). Significant mortality in this study was observed in the highest experimental treatment for all size classes. While larger cockles could still be observed at the termination of the experiment (29% mortality in size class 2-2.5 cm in treatment 5), smaller size classes suffered from high mortality that approached 100% (Fig. 3). In all treatments >6,000 µatm a difference in mortality between size classes was observed, with the smallest size class being the most sensitive (Fig. 3). This corresponds to previous work on other bivalve species where smaller juvenile bivalve individuals also reacted much more susceptible towards higher seawater pCO_2 (Green et al. 2004; Waldbusser et al. 2010). Such effects might be related to less favorable area – volume ratios, as smaller animals have to protect a larger surface area from acid-base disturbance and relatively larger shell areas from (internal) dissolution. Shell production costs are also much higher in smaller bivalves (Thomsen et al. 2013, Waldbusser et al. 2013).

In our study, we screened shells for signs of dissolution following termination of the experiment. Increased corrosion of the shell was evident from treatment 1 (1,500 µatm) to treatment 5, with the degree of severity increasing progressively. Signs of severe dissolution and holes were present in most animals in treatments 4 and 5. These findings indicate, that even moderate degrees of acidification can already lead to non – reversible reductions in shell integrity.

In order to learn more about the impacts of elevated seawater pCO_2 on *C. edule* physiological processes and to develop markers for stress induced damage, we measured whole – body accumulation of malondialdehyde (MDA) as a marker for oxidative stress. Lipids are the most vulnerable class of molecules towards oxidative stress (Nyska & Kohen 2002, Del Rio et al. 2005). One of the toxic effects of oxyradical formation within cells is an increase in lipid peroxidation (Maritim et al. 2003). The most studied product formed, and an important marker of lipid peroxidation, is MDA. MDA is highly toxic and potentially mutagenic; it can impair mechanisms involved in cell functionality (Del Rio et al. 2005). As oxidative stress is often directly related to metabolism (Finkel and Holbrook 2000; Del Rio et al. 2005), we used MDA concentrations as a proxy for metabolic rate. Previous studies on bivalves suggest a good relationship between high MDA accumulation and high metabolic rate (McArthur & Sohal 1982; Abele et al. 2001; Heise et al. 2002). We found significant decreases in MDA concentration in bivalves exposed to pCO_2 >6600 µatm, which suggests that these experimental animals were suffering from metabolic depression. Behavioral changes of *C. edule* were observed during exposure to high CO_2 that correspond well to responses observed during exposure to hypoxia (Rosenberg et al., 1991; Diaz and Rosenberg, 1995). With increasing pCO_2, moribund or weakened *C. edule* accumulated on the sediment surface. The proportion of *C. edule* on the surface of the sediment increased with duration of the experiment. Behavioral responses such as massive accumulation of bivalves on the seafloor could be used as a cheap and efficient monitoring tool for future monitoring of sub-seabed CCS storage sites, e.g. by towing camera systems across large sea floor areas. Avoidance behavioural in relation to environmental stress of hypercapnia or hypoxia has also been noted by (Dupre and Wood, 1988), as organisms try and move away from a negative stimulus. Quite similarly, Widdicombe et al. (2009) observed emersion of infauna echinoderms from the sediment during high – CO_2 incubation. .

Response of Mya arenaria *and* Macoma balthica *to potential leakage*

Interestingly, *Mya arenaria* and *Macoma balthica* survived the complete experimental duration at all treatment levels. Resistance of *M. arenaria* could be due to a thicker periostracum (20 µm) (Harper 1997). Additionally, a greater burrowing depth (Baker and Mann, 1991, Willmann 1989, Koie et al. 2001) 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 (Taylor et al. 1973; Harper 2000).

Microbial communities

Differences in bacterial communities were found only for the highest treatment compared to treatment 1 and the control. Even though differences were found, different treatments shared a large amount of OTUs. The amount of shared OTUs was similar between replicates and within treatments suggesting a very high spatial variability. The effect of elevated pCO_2 could only explain 6.9% of the change in community composition, while time explained 5%. Influential factors might include a change in sediment nutrient composition or a change in sediment bioirrigation rates mediated by dying cockles. While *C. edule* was shown to significantly influence the microphytobenthic primary production due to release of metabolic NH_4^+ (Swanberg 1991), bacterial abundance was not significantly influenced by bio diffusing activities of *C. edule* at a density of 250 ind. m^{-2}, likely because movement of *C. edule* did not increase oxygen flux into the sediment – unlike the action of polychaetes (*Nereis diversicolor*) that construct elaborate burrows and strongly shape microbial communities (Mermillod-Blondin et al. 2004). Thus, the comparatively minor effects of substantial decreases in *C. edule* density at the highest CO_2 treatment level on microbial community structure could be explained by the less pronounced impact *C. edule* has on sediment oxygen and nutrient fluxes (Mermillod-Blondin et al. 2004). However, a progressive loss of *C. edule* could positively affect

settlement success of other macrobenthic species (e.g. ploychaetes) that are otherwise competitively excluded by *C. edule* (Flach 1996), and these species could more strongly impact microbial communities during long-term CO_2 leakage.

Meiofauna community

Meiofauna organisms are very important for remineralization of organic matter in sediments and can significantly influence the composition of microbial communities (Nascimento et al. 2012). In our experiment, the composition of the meiofauna community was significantly altered by high CO_2. Total abundance of meiofauna did not change significantly, nor did Nematoda abundance, suggesting a generally high resistance of meiofauna organisms to increased seawater pCO_2. A significant change in abundance could be detected for Gastrotricha and Copepoda. Gastrotricha abundance significantly increased in treatment 5. An increase in abundance could be explained by more favorable conditions with decreasing pH, either as a direct effect or indirectly through higher food availability. As Gastrotricha are detritus feeders (Giere 2010), increased availability of organic compounds, potentially through increased mortality of *C. edule*, may have enabled this increase in abundance. Further fine scale determination of meiofauna taxonomic composition of the experimental samples from this experiment (Guilini et al. work in progress) may resolve this issue.

3.2 The respiratory and acid base response of echinoderms to chronic hypercapnia *[Morgan and Hauton, in prep.]*

Understanding the degree of tolerance that marine organisms may exhibit to chronic hypercapnia driven by a CCS leakage scenario, requires investigation into the tiered response, quantifying capacity for compensation or acid base buffering. This respiratory response was investigated using a selection of echinoderm species under chronic hypercapnic exposure ranges with varying temporal scales. In addition the immune response of the organism was investigated for signs of impaired immune activity signalled by changes in immune receptive cells. This would help to elucidate the secondary effects impacting the physiology of an organism under chronic acidification.

Experimental design was adapted for epi and eufauna species of urchins, but in all cases water acidity was manipulated using CO_2 gas, injected directly or indirectly (using header tanks), into the experimental tanks. The pH of seawater was controlled using pH electrodes connected to solenoid valves, which automatically shut off the gas flow when the required pH is achieved. This ensured that acidification and the required pH was maintained constant throughout the duration of experimentation. pCO_2 in the water above the sediment in experimental tanks was controlled at approximately 1000, 2000, 5000 and 20,000 µatm.

3.2.1 Materials and methods

The medium term (56 days) buffering capacity of *Paracentrotus lividus* was established to identify its tolerance to chronic hypercapnia. A subsequent experiment with higher temporal resolution was then used to determine the early response to hypercapnia over a seven day exposure.

Urchin origin and acclimation for medium term (67-day) investigation

P. lividus were supplied by Dunmanus Seafoods (West Cork, Ireland) and transported to Plymouth Marine Laboratory in aerated containers, kept moist with sea water-soaked towels to prevent desiccation (May 2012). On arrival, urchins were placed in recovery tanks to monitor spawning for 48 hours before being introduced into the main aquaria. *P. lividus* were allowed to acclimate to normoxic/normocapnic conditions (14.5 ˚C, Salinity = 34) for two weeks prior to sea water acidification. During acclimation, mortality and general health status were monitored as indicated by spine loss and lack of feeding. *P. lividus* were fed twice weekly fresh *Laminaria digitata* collected from the shores of Plymouth Sound. Any remaining algae were removed 24 hours after introduction into the tanks. No urchins were fed in the immediate 24 hours before sampling. The seawater physicochemical parameters including pH, temperature, salinity and ammonia, were monitored and controlled weekly.

Urchin origin and acclimation for short term (7-day) investigation

P. lividus were again obtained from Dunmanus Seafoods (May 2013) and transported to National Oceanography Centre Southampton as before. Urchins were allowed to acclimate to normoxic/normocapnic conditions (O_2 = >10.50 mg/L, T= 13.5 ˚C, Salinity = 34) for two weeks prior to seawater acidification. *P. lividus* were fed twice weekly herbivore algae gel (Nutrazu, Aquatic Herbivore Gel, Brogaarden, Denmark) with any remaining algae gel being removed 24 hours after each feed. As before, urchins were not fed within 24 hours of sampling. Sea water physiochemistry were again monitored and controlled daily.

Experimental treatments and coelomic fluid sampling procedure

Both investigations followed the same experimental arrangement and sampling protocol based on the recent description by Findlay (2013). The mesocosm system consisted of six tanks, incorporating two replicates for each of three treatment levels. The three treatment levels comprised the Control exposure (~400 ppm (pH 7.98), in addition to nominal treatment levels of 5000 ppm pCO_2 (7.11 pH) and 20000 ppm pCO_2 (6.52 pH). Briefly, exposure treatments were created and controlled using 100% CO_2 gas additions to a mixing tank, regulated via a 'pH controller' (pH controller 201, Aqua Digital, Germany), which was connected to a solenoid valve (M- ventil standard, Aqua Medic, Germany). Tank pH was detected using a pH electrode (Aqua Medic, Germany) connected to the pH controller. The pH electrode was calibrated using standard NIST buffers (Radiometer Analytical, Denmark). The aquarium tanks were also supplied independently with air to maintain normoxic conditions. Weekly measurements of p_wO_2, pH_w, salinity, nutrients, DIC and TA were collected from each tank. Temperature was monitored daily and maintained at 14.0˚C +/- 0.5˚C. A 12 h:12 h light:dark cycle was maintained in the aquaria.

In the 56-day experiment coelomic fluid was sampled from individual urchins for blood gas analysis, ion content and determination of L-lactate concentration. Urchins were sampled immediately upon removal from mesocosm to obtain a clear, bubble free fluid sample that was collected using a chilled glass 1 mL syringe (Susuki, USA) and 23 gauge needle. A subsample of the coelomic fluid was transferred to a gas tight Hamilton syringe (Hamilton, gas tight, Switzerland, vol = 50 µL) and inserted into a pH flow-through and reference microelectrode (Microelectrodes Inc, USA). Following this 10 µL of fluid was inserted into the Tucker and Cameron chambers respectively (see below). The remaining coelomic fluid was transferred to a micro centrifuge tube and frozen until further analysis of cation and L-lactate concentration was determined.

3.2.3 Coelomic fluid gas responses

Coelomic fluid O_2 content was determined using the Tucker chamber (TC500 chamber; Strathkelvin, UK) following the method of Tucker (1967). The Tucker chamber thermostat-controlled water jacket housed an O_2 electrode (1302 electrode, Strathkelvin) connected to O_2 meter (782 strathkelvin), total content of O_2 (Co_2) was calculated as,

$$\frac{\Delta PO2}{760} \times \left(\frac{100}{0.01}\right) \times 0.976 \text{ X } \alpha_k \,/\, R = O_2 \text{ mmol.L}^{-1}$$

A correction factor of 0.976 was applied to compensate for the small amount of KCN that over flowed from the Tucker chamber on insertion of coelomic fluid sample (Cameron, 1971). The solubility coefficient for O_2 in potassium cyanide (α_k) was taken from Tucker (1967) as 0.0252 at 32°C. The ideal gas constant R (2.2414) at standard temperature (0°C) and pressure (760 mm Hg).

CO_2 content was determined using a Cameron chamber in a similar arrangement to that of the Tucker chamber (Cameron, 1971). A CO_2 electrode (E5037 electrode; Radiometer, Denmark), housed within a custom built water jacket glass 'Cameron' chamber (Loligo systems; Denmark) was connected to a blood gas analyser (PHM73, Radiometer). The resulting change in partial pressure from the Cameron chamber was recorded and the meter deflections converted into CO_2 (mmol.L^{-1}) via a two-step calculation:

$$Pcal = P_f - (P_i * 0.947) = 10 \text{ mmol.L}^{-1}$$

Where: Pcal represents the ΔpCO_2 with every 10 mmol.L^{-1} CO_2.
The total content of CO_2 in the coelomic fluid sample (Cco_2) was then be calculated by:

$$((pCO_{2f}\,(pCO_{2i} * 0.947))/Pcal) * 10 = Cco_2 \text{ mmol.L}^{-1}$$

Where pCO_{2i} is the initial meter reading on insertion of degassed hydrochloric acid, pCO_{2f} is the final meter reading and the insertion of coelomic fluid. To compensate for the small amount of acid that overflowed from the chamber on insertion of the sample a correction factor of 0.947 was again applied to the calculation.

Non bicarbonate buffer lines were constructed for the urchin *Paracentrotus lividus* using the method of Spicer *et al*., (1988) in which individual coelomic fluid samples of 300 µL were equilibrated using gas mixing pumps (M303 a/f, Wöstoff, Germany) using known pCO_2 tensions (0.1 -1.0 mm Hg, 0.013 – 0.133 kPa) and measuring the TCO_2 *in vitro* at 1 mmHg pCO_2 (Mettler Toledo CO_2 analyzer 965D, U.K). Coelomic fluid pH (Mettler Toledo Seven multi pH meter, Switzerland) was measured at each equilibrated pCO_2 tension inside a purpose built glass chamber, thermostat controlled to 14°C (Lauda proline RP845). The coelomic fluid was gently mixed for at least 20 minutes to ensure complete equilibration for each change in pCO_2 tension, noted as stable pH reading. Bicarbonate was calculated using the following equation,

$$(pCO_2 \times \alpha CO_2) \times 10 \text{ ^ } (pH - pK_1) = [HCO_3^-] \text{ mmol.L}^{-1}$$

Where αCO_2 is the CO_2 solubility coefficient in seawater (0.0468 mmol.L^{-1} mm Hg^{-1}, at 10°C), taken from Spicer et al., (1988), the dissociation constant (pK_1) was calculated as follows,

$$pH(Log_{10}([HCO_3^-]/(\alpha CO_2 * pCO_2) = pK_1$$

Coelomic fluid L-lactate concentration

P. lividus coelomic fluid L-lactate concentration was determined using the methods of Bergmeyer (1985) modified for use in a microplate reader and commercially available as a test kit (Lactate assay kit no. 735, Trinity Biotech,

Ireland). Coelomic fluid samples were first deproteinized by adding an equal volume of 0.6M perchloric acid before neutralization with a stepwise addition of 2.5M potassium carbonate. Denatured sample was centrifuged at 10,000 g for 20 minutes at 4˚C and the supernatant removed for use in the L-lactate assay at an absorbance of 552 nm (FLUOstar OPTIMA, BMG Labtech, Germany).

Coelomic fluid ion content analysis

Following digestion in concentrated sub boiled nitric acid the samples were diluted in 0.4M sub boiled nitric acid. The samples were filtered through 0.2μm syringe filters before analyses on a Perkin Elmer Optima 4300DV ICP-OES using synthetic standards to calibrate. Instrument drift was monitored and corrected for using a drift monitor solution analysed every 10 samples.

Preparation of spines for scanning electron microscopy (SEM) and chemical analysis

Urchin spines were removed from the test upon sampling and stored in 70% ethanol at 4˚C. In preparation for SEM analysis the spines were washed in 70% ethanol and dried in an oven at 50˚C for two hours. Spines were either mounted longitudinally onto aluminium stubs and sputter coated with carbon, ready for analysis, or mounted in resin for cross-sectional analysis. For cross sectional analysis, the base of the spines (attached to the test) were glued to a glass slide, the glass slide was then inserted into a mould which was filled with epofix resin (EPO-FIX, USA), with hardener, air bubbles were removed under vacuum. The resin was left to set for 24 hours at room temperature. Thereafter, the base of the resin mount (i.e. the glass slide) was sanded off, and the surface was ground using progressively finer grades of diamond polish (Wendt Diamond Polish, Sheffield, U.K). The resin mounts were carbon coated, in preparation for SEM analysis.

Electron microscopy was undertaken using a Leo 1450VP (variable pressure) SEM with a PGT light element energy dispersive spectroscopy (EDS) system. Mineral standards (Micro-Analysis Consultants Ltd, Cambridge, UK) were used to calibrate the SEM for quantitative X-ray microanalysis of the *P. lividus* spines. Operating conditions were 20kV, with a probe current of 700 pA and a working distance of 19 mm. A take-off angle of 35° was utilized. Spectra were collected for 120 s using EDS spot analysis. The spectra were processed with the Phi-Rho-Z technique using imix© software, formerly supplied by PGT.

Differential coelomocyte counts

20 µl of coelomic fluid was diluted 1:1 with echinoderm anticoagulant (Matranga et al., 2000). Differential coelomocyte counts were made using a 0.1mm deep Improved Neubauer haemocytometer under bright field. Populations of phagocytes, red and colourless spherule cells and vibratile cells were enumerated according to the descriptions of Smith (1981) and Matranga et al. (2005).

Statistical analysis

All data were tested for homoscedacity of variance and normality. To investigate the effect of time and exposure pCO_2 including the interaction between time and pCO_2 exposure, on blood gas variables O_2, pCO_2, HCO_3^-, pH and lactate, two way ANOVAs were employed. This was followed by *post hoc* Bonferroni tests (SPSS vs 21). Comparisons between pCO_2 exposures and time for coelomic fluid cation concentration were conducted using 2 way ANOVA. Spine length between control and the 20,000 ppm exposure group were conducted using 1 way ANOVA. Comparison between non bicarbonate buffer curves and pK1 for each treatment group were conducted using 2-way ANOVA and Holm-Šídák *post hoc* testing. Coelomocyte counts failed tests for normality and equal variance and as a result were analysed using a Kruskal Wallis one way ANOVA on Ranks,

3.2.2 Results

Coelomic fluid gas analysis

The O_2 content of the coelomic fluid initially decreased between day one and day seven of the 67-day exposure, after which Co_2 remained relatively constant throughout the two month experiment. The extracellular pH (pH_e) of urchins nominally exposed to 5000 and 20,000 ppm pCO_2 was significantly lower than in the Control treatment (P = 0.014), and remained so throughout the two-month duration (data from day 67 shown in Figure 8a).

The coelomic fluid total oxygen (Co_2) measured in the short term pCO_2 investigation was greater than that found during the medium term investigation, regardless of pCO_2 exposure treatment analysed (Fig 8b.). However there was no effect of pCO_2 treatment on the coelomic fluid Co_2 (P = 0.055), or effect of time between day one and seven between the pCO_2 treatments groups (P = 0.135). Coelomic fluid pH decreased significantly with an increase in p_wCO_2 exposure (P < 0.001), however there was no effect of time observed (P = 0.480).

Coelomic fluid bicarbonate buffering

Temporal changes in the acid base status of *P. lividus* indicated that the coelomic fluid was not buffered by an increase in bicarbonate (HCO_3^-), despite a significant reduction in coelomic fluid pH_e between the pCO_2 exposures (P = 0.003; Fig. 9.A). The acid base status of *P. lividus* remained well below the non-bicarbonate buffer line constructed from the same coelomic fluid samples, indicating a respiratory acidosis occurred in all treatments, including the Control. As the magnitude and duration of high pCO_2 exposure increased the absence of HCO_3^- buffering resulted in metabolic acidosis (Fig 10) which, coupled with the persistent low CO_2, indicated that the *P. lividus* were not able to match their metabolic demands.

Acid base disturbance of *P. lividus* during the short-term investigation demonstrated a significant increase in coelomic fluid CCO_2 in exposure 3 treatment group (pH 6.52) ($P < 0.001$). There was however no effect of time observed ($P = 0.065$) (Fig 9 B.). Coelomic fluid CCO_2 remained elevated throughout the duration of the hypercapnic exposure; however, no compensatory increase in HCO_3^- was observed. The respiratory acidosis remained uncompensated in both treatment groups 2 and 3 throughout hypercapnic exposure.

Coelomic fluid ionic content and spine chemical analysis – 67-day investigation

There was a significant difference between the Mg^{2+} and Na^{2+} concentrations in the coelomic fluid between the controls and CO_2-exposed urchins ($P = 0.04$; Fig. 11 &12); however, there was no significant effect of time on the concentration of these two cations ($P = 0.199$). There was no significant difference between the calcium Ca^{2+} ($P = 0.083$) (Fig. 11), strontium Sr^{2+} ($P = 0.067$) (Fig 12) or potassium K^+ ($P = 0.060$) concentrations of control vs 20,000 ppm and the effect of time (1 and 2 month time point) was not significant.

Scanning electron microscopy of P. lividus spines, spine length and chemical analysis of spine content – 67-day investigation

Spine dissolution is evident in the cross sectional SEM images of *P. lividus* spines from control and nominal 20,000ppm group (Fig. 13) compared to the controls. After two months exposure at this high pCO_2 the outer ring of the spine increased its porosity and the spindles that create the structure of the spine were reduced to the core of the spine. As a result of spine dissolution weakening the integrity of the spine, there was a significant reduction in spine length (Fig. 14).

Interestingly chemical analysis of *P. lividus* spines indicted a small but significant ($P = 0.001$) increases in % Mg content in spines from urchins exposed to 20,000

ppm for two months (Fig 15). There was no other increase in cation content found between urchin spines from different treatments.

Coelomocyte counts.

Differential coelomocyte counts were extremely variable throughout the entire experiment and there were no consistent changes with treatment or time (Fig. 16). The circulating coelomocyte population was dominated by phagocytic cells followed by red spherule cells in the majority of urchins, irrespective of treatment condition. Smaller numbers of colourless spherule cells were seen in all urchins and vibratile cells were only occasionally seen. There was no evidence for an effect of elevated pCO_2 on the circulating coelomocyte population.

3.2.3. Discussion

Environmental conditions

Echinoderms are particularly vulnerable to environmental acidification due to their lack of buffering capacity in the coelomic fluid due to the absence of respiratory proteins (Holtmann et al., 2013; Miles et al., 2007; Spicer et al., 1988; Spicer and Widdicombe, 2012). Furthermore, the echinoderm test is composed of a calcareous skeleton, which is susceptible to dissolution due to the change in chemical parameters in acidified sea water.

Blood gas response

Maintenance of a constant extracellular pH is critical for preserving intercellular processes, and as such poikilothermic animals regulate pH, to create a constant relative alkalinity (Heisler et al., 1976). Disturbances in acid base status therefore compromise the ability of marine organisms to adapt to changing conditions if unable to compensate via acid base buffering capacity. Echinoderms are particularly vulnerable to acid base disturbances, because of a

reliance on diffusive respiratory gas exchange, lack of a respiratory pigment or substantial protein concentration in the coelomic fluid, weak ionic regulatory ability, combined with a vulnerable calcareous test. Under the chronic, under chronic acidified conditions it is no surprise they face difficulty compensating in acidified environmental. The concentration of pCO_2 they could be potentially exposed to in the event of a CCS leak pose a serious threat to their survival, however the duration of exposure is a critical threshold.

Spine dissolutions and coelomic fluid ions, chemical analysis

Reports on the ionic regulatory ability of Echinoderms suggest there is no uniform regulation of cations but instead they display varying degrees of ionic regulatory capacity (Freire et al., 2011). Holtmann *et al*., (2013) investigated the role of body cavity epithelia to maintaining coelmic fluid pH and found the intestine of *S. droebachiensis* formed a barrier to HCO_3^- and was selective to cation diffusion, aiding the retention of bicarbonate during acid base buffering. In contrast the peritoneal epithelium of *S. droebachiensis* was not cation selective, however this did aid buffering by allowing diffusion of carbonate via test dissolution (Holtmann et al., 2013).When the osmo and ionic regulatory capacity of the intertidal sea urchin *Echinometra lucunter* was investigated in response to elevated Mg^{2+} and K^+, spine dropping was observed in addition to a reduction in mobility of the ambulacral feet. An increase in either cations was thought to induce muscle relaxation and depress neuromusculature transmission and excess salt has also been linked to a decrease in activity of metabolic enzymes (Freire et al., 2011).

Previous studies have highlighted the complex and highly variable calcification rates found in marine calcifiers in response to ocean acidification (Courtney et al., 2013). When exposed to high pCO_2 conditions in combination with a low (20°C) and high (30°C) temperature exposures the calcification rates of the reef urchin *Echinometra viridis* declined, with the most marked response exhibited in

the winter exposure of 20°C (Courtney et al., 2013). It was predicted that upper summer exposure would enhance calefaction rates despite the decrease due to ocean acidification and this would balance out the decrease experienced in the winter months. How this would impact on spine retention and growth is unclear.

The magnesium calcite exoskeleton of echinoderms is highly susceptible to dissolution through acidification as seawater pH decreases (Shirayama and Thornton, 2005). The evidence of spine dissolution and corresponding reduction in spine length in *P. lividus* after 1 month exposure to acidified seawater of 20,000 ppm, highlights the fact. In addition, Holtmann et al., (2013) discovered spine dissolution in *S. drobachiensis* to be more severe then that of the test, predicting increased predation pressure as a result. Test dissolution was also thought to have occurred in *P. miliaris* during ocean acidification exposure for 8 days (pH < 7.44) (Miles et al., 2007), however no SEM images were reported for either test of spine. The growing spines of *Eucidaris tribuloides*, were reported to be covered with a epidermis layer (Markel and Roser, 1983), which may provide protection to acidified waters.

Behavioural observations

It was noted in both short and long-term investigation that where possible the urchins would try and migrate vertically through the tanks. It was interesting that *P. lividus* displayed this behaviour as the injection of CO_2 gas into the tanks was well mixed and careful measures were taken to ensure no pH/pCO_2 gradients formed in each tank, therefore no one source point existed as a particular site of acidification. The direction of movement may have been driven by other factors, such as temperature, although the tank temperature was constant and all tanks housed in the same temperature controlled rooms. Furthermore these observation reflect behavioural observations of urchins recorded in a field experiment conducted as part of the UK QICS Project (unpubl.

obs.; www.bgs.ac.uk/qics/). The driving factors behind this behavioural still need to be determined but may support those of Melzner et al. (GEOMAR; see above) and may provide a means for remote and in real time monitoring the biological impact of even a small and chronic CO_2 leak through surficial sediments from a CCS reservoir.

Mortality rates observed

Surprisingly we experienced very low mortality in exposure group 3 urchins during the medium term exposure investigation, with a loss of 20% of after the 2 month investigation, however after 100% mortality in the exposure group 2 group after the same period suggests others factors may have influenced to mortality in the group. In contrast during the short-term investigation, after 7 days exposure to pH 6.5, 100% mortality of exposure group 3 urchins was found, whilst zero mortality of the control group 1 and group 2 was observed. High mortality has also been observed in various species exposed to chronic hypercapnia, for example below pH 6.16, 100% mortality was observed in *P. miliaris* after 8 days, which agrees with the findings of our short-term investigation (Miles et al., 2007). The mortality rates of the clam *Ruditapes philippinarum* and early life stages of gilthead seabream *Sparus aurata* were investigated in response to chronic acidification resulting in 100 % mortality observed below pH 6 (Basallote et al., 2012). The main contributing factor was the speed at which the acidification developed, prohibiting both species from employing any compensatory ability or time to adjust. Thus the conditions under which biota may be exposed to CCS leakage scenarios must consider that the speed at which environmental pH declines will significantly affect the mortality rates observed and ability for compensation (Basallote et al., 2012).

3.2.4 Conclusion

In the absence of respiratory pigments, proteins influence the buffering capacity of the coelomic fluid almost entirely; a low protein concentration will reduce the

CO_2 capacity of the coelomic fluid agreeing with the findings of the present study. Low protein concentration may be typical in echinoderms body fluid in general. *P. lividus* exhibited low protein and low HCO_3^- concentration resulting in an inability to buffering chronic acidification exhibited by a significant reduction in pHcf, in addition to a decline in $CcfO_2$. However no appreciable increase in L-lactate suggests *P. Lividus* was still operating with an aerobic metabolism, albeit at a reduced rate. Metabolic rate under chronic acidification still needs to be quantified in *P. lividus*, however it is predicted that in the surviving urchins this would be heavily suppressed in order to aid survival. We found no change in cation concentration in the coelomic fluid providing no evidence of test dissolution, however further SEM imaging of the test would be required to validate this especially in light of the evidence of spine dissolution. Spine dissolution will affect the integrity of the spine, and evidence of spine dissolution resulting in weakening of the spine have been found in other urchins (Holtmann et al., 2013). Behavioral and community interaction studies have yet to investigate the impact reduced spine integrity will have on the urchin functioning, however it is predicted that weak spines will reduced mobility, increase vulnerability to prey, and negativity impact on feeding. Therefore in the event of a CCS leakage scenario through direct and indirect impacts, *P. lividus* should be classed as a highly vulnerable species.

3.3 Energy metabolism and regeneration impaired by seawater acidification in the infaunal brittlestar, *Amphiura filiformis [Hu et al., submitted]*

The infaunal brittlestar *Amphiura filiformis* is an important species in many polar and temperate marine benthic habitats with densities of up to 3500 individuals per square meter (Rosenberg et al., 1997). *A. filiformis* lives in semI-permanent sediment burrows and feeds on particulate organic matter (POM) by extending 2-3 arms into the water column (Loo et al., 1996). This species is an important prey for many predators like crustaceans and fish leading to sub-lethal injury (e.g. loss of exposed arms) (Duineveld and Van Noort, 1986). Since arms are essential for suspension feeding (Woodley, 1975), respiration (Ockelmann, 1978) and ventilation of the burrow (Nilsson, 1999), long term selection pressure on *A. filiformis* has led to the ability to autotomize their arms in case of an attack by a predator, and to a high potential of regenerating these lost tissues (Dupont and Thorndyke, 2006). The process itself and the physiological properties of regeneration have been investigated in earlier studies, suggesting that energetic costs for the regeneration of arms are significant (Fielmann et al., 1991, Pomory and Lawrence, 1999). Moreover, depending on the position of autotomy the available energy can be either favored for growth or differentiation of the regenerating arm piece (Dupont and Thorndyke, 2006). Previous studies demonstrated differential responses of regeneration rates in brittlestars exposed to seawater acidification (Wood et al., 2008, Wood et al., 2011). The Arctic brittlestar *Ophiocten sericeum* decreased regeneration rates under acidified conditions whereas *A. filiformis* increased regeneration rates under acidified conditions of pH 7.3. However, in both species reduced seawater pH led to an increase in metabolic rates which has been hypothesized to support increased energetic demands to maintain calcification. The present investigated whether elevated seawater pCO_2 levels, relevant for ocean acidification and potential CO_2 seepage from CCS sites, may impact energy metabolism and regeneration capacities of the infaunal brittlestar

A. filiformis. To test how changes in seawater pCO_2 affect the micro-environment surrounding *A. filiformis* we determined abiotic factors (e.g. pO_2, pH and pCO_2) within their burrows. This information is crucial in order to estimate the actual pCO_2 levels seen by the animal, and helps to understand how elevated seawater pCO_2 could affect the physiology of infaunal organisms. Furthermore, it can be assumed that already under control conditions *A. filiformis* experiences increased hypercapnic and hypoxic conditions within their burrows due to respiration and metabolic release of CO_2. This would probably lead to an additive effect of increased seawater pCO_2 to the naturally increased pCO_2 levels within burrows. We hypothesize that decreased seawater pH imposes significant challenge to the energy metabolism of these animals due to low acid-base regulatory abilities. According to earlier studies conducted on other invertebrate species (Reipschläger and Pörtner, 1996, Michaelidis et al., 2007, Thomsen and Melzner, 2010, Stumpp et al., 2012) we expect that also *A. filiformis* may tolerate moderate acidification but aerobic metabolism cannot support energetic demands during severe acidification over longer periods leading to the onset of metabolic depression. This may particularly affect the regeneration process as it is believed to be associated with high energetic costs.

3.3.1 Material and Methods

Animals and sampling site

Sediment containing *Amphiura filiformis* was collected at 30-35 m depth, using a box corer, in the vicinity of The Sven Loven Centre for Marine Sciences (SLC), Kristineberg, Sweden, in September 2011. Individuals were immediately collected from sediment cores by gentle rinsing to avoid breaking of arms and maintained in natural flowing deep seawater (NFDS) at 12 ^{o}C, pH 8.0 and a salinity of 31. Animals were acclimated to lab facilities 3 weeks prior to the start of experiments. In total we conducted 4 separate experiments to determine behavior, respiration and ammonia excretion rates, growth, extracellular acid-

base status and regeneration capacity in *A. filiformis* and abiotic conditions within the sediment burrows. Depending on the experiment, we let the animals bury in their natural sediment (exp 1 and 3) or not (exp 2 and 4). We applied between two (exp 3 and 4) and three (exp 1 and 2) pH treatments based on following assumptions. Natural seawater pH of 8.1 was used as control condition. Medium pH drops down to pH 7.6 and 7.3 can be expected to occur within the next centuries due to rising atmospheric pCO_2 conditions and were used as simulated ocean acidification scenarios. Low pH treatments (pH 7.0) were applied as potential carbon capture storage scenario or can be expected to occur in sediment burrows at predicted ocean acidification scenarios (in experiments without sediment).

All experiments were in accordance with the Swedish law for animal welfare and were approved by the animal welfare officers of the University of Gothenburg.

Experiment 1: Determination of abiotic parameters within burrows.
Intact specimens with a disc diameter of 5-6 mm and fresh mass ranging from 216 to 275 mg that showed no evidence of recent regeneration events and no apparent gonads were used in all experiments. O_2 and pH in *A. filiformis* burrows were measured using fiber optic O_2 and pH sensors (PreSens oxygen micro optode, type PSt1; PreSens pH microsensors NTH-HP5-L5) mounted on a micromanipulator and connected to a fiber optic oxygen and pH transmitter, respectively (Oxy4 Micro and pH Micro, PreSens, Regensburg, Germany). The sensors were calibrated according to the manufacturer's instructions.

"Thin aquaria" filled with sieved mud (dimensions 8 cm x 18 cm x 1 cm) as described in Rosenberg et al. (1991) were equipped with 10 -12 animals each, allowing us to observe the vertical position and burrows within the sediment. Experimental "thin aquaria" were supplied with seawater from independent header tanks at a flow rate of 40-45 ml min^{-1} which were adjusted to the

respective pH using a computerized feedback system as described above (pH 8.1 and 7.0). All experiments were set up as 2 controls (pH 8.1; > 90 % O_2 sat.) and 2 parallels where pH was lowered to pH 7 (0.63 kPa CO_2). Experiments were repeated 2 to 3 times resulting in a total independent replicate number of n= 4-6. O_2 and pH in different depth levels (0, 5, 10, 15, 20 20 and 30 mm) of burrows were measured visually (accuracy ± 100 µm). In total, at least 10 different burrows, containing live animals for each depth level were recorded. Seawater was sampled from the burrows using a gastight syringe (Hamilton) mounted on a micromanipulator. Alkalinity from seawater samples was essentially determined spectrophotometrically (ND-2000, NanoDrop Technol, Wilmington, DE) according to Sarazin et al. (1999) in a volume of 1.5 µl.

Experiment 2. 4 week low pH exposure within natural sediments

Animals were maintained in a flow through seawater system consisting of 12 PVC aquaria (5 L volume) in a 10 ^{o}C climate chamber at the SLC. A light regime with a 12 h : 12 h light/dark-cycle was chosen. NFDS with a salinity of 31 ± 0.07 was distributed to the experimental aquaria at a rate of 50 ml min^{-1}. Before the start of the experiments the animals were maintained in these tanks under control conditions for 10 days. Each of the experimental tanks contained 15 individuals that were allowed to burry in 4 cm of sieved (0.7 mm) sediment from the collection site. Three pH levels (control: pH 8.1, medium: pH 7.3 and low: pH 7.0) with 4 replicates each were continuously maintained in the respective aquarium using a computerized feedback system (AquaMedic) that regulates pH (NBS scale) by addition of pure gaseous CO_2 directly into the seawater (+/−0.05 pH units). pH was controlled independently in each aquarium and aquaria were randomly arranged with respect to pH level. pH, temperature and salinity was monitored daily and total alkalinity (AT) was determined once a week along the incubation time and was essentially measured following Sarazin et al. (1999). pH was monitored using a Metrohm

(827 pH lab) pHNBS meter. The carbonate system speciation was calculated from pHNBS and alkalinity using CO2SYS (Lewis and Wallace, 1998) (Table 3) with dissociation constants from Mehrbach et al. (1973) refitted by Dickson and Millero (1987). The measured carbonate chemistry is in accordance to field conditions with pHNBS levels of 8.0 to 8.1 at 10 ^{o}C and an alkalinity ranging from 2.15 to 2.3 mmol.L^{-1}. The total duration of the experiment was 4 weeks and behavioral observations, oxygen consumption, NH_4^+ excretion measurements (see below for methods) and tissue sampling were performed once every 7 days after the start of the experiment (four times in total). Before each sampling time point, the total number of visible arms that were extended into the water column or laying on the sediment was determined for each tank. Three specimens from each aquarium (3 pH levels, 4 replicates, 4 time points, 3 animals per time point and aquarium) were gently taken from the sediment and rinsed with seawater from the respective pCO_2 treatment to remove particles from the animals. The 2 animals were used for respiration and NH_4^+ excretion measurements and the remaining animal was used for gene expression analysis. Metabolic and NH_4^+ excretion rates from each replicate aquarium and sampling time point were averaged over the time course of 4 weeks by keeping the replicates (n = 4). For gene expression analyses arms were removed from the body using forceps and both body parts (arms and body) were shock frozen in liquid nitrogen and stored at -80˚C.

Experiment 3: Determination of extracellular (perivisceral coelomic fluid) acid base status

To investigate the effects of CO_2 induced seawater acidification on extracellular pH (pHe) homeostasis, specimens were maintained in a flow through seawater system consisting of 9 PVC aquaria (5 L volume; for more details see experiment 1). Before the start of the experiments the animals were acclimated in these tanks 1 under control conditions for 5 days. Each of the experimental tanks contained 48 individuals. For practical reasons and to avoid sediment effects on

pH exposure animals were kept without sediment. Three pH levels (control: pH 8.1, medium: pH 7.6 and low: pH 7.3) with 3 replicates each were continuously maintained in the respective aquarium as described above. Seawater physiochemical parameters were measured and calculated as in experiment 1 and are given in table 3. The total duration of the experiment was 15 days and extracellular pH measurements were performed at 5 time points: 0, 2, 6, 10 and 15 days. In total, two specimens (one for pH and one for alkalinity measurements) from each aquarium (3 pH levels, 3 replicates, 5 time points, 3 animals per time point and aquarium) were gently taken from the aquaria and excess water was removed on a paper tissue. pHe was determined within 30 seconds at 10˚C by insertion of fiber optic pH sensors (PreSens pH microsensors NTH-HP5-L5; tip diameter 150 µm) into the coelomic cavity. The sensors were calibrated according to the manufacturer's instructions at the same ambient temperature of 10˚C. For total alkalinity (AT) measurements, 5 µl of coelomic fluid were quickly sampled from the coelomic cavity using a gastight syringe with a 21 gauge needle. The entire sampling of coelomic fluid was completed in less than 1 min. The coelomic fluid was transferred to an eppendorf tube and centrifuged for 60 s (6000 rpm; 4300 x g) using a minifuge (Capsulefuge PMC-880 Gilson). 3 µl of the supernatant were used for further analyses. Alkalinity from coelomic fluid samples was determined spectrophotometrically (ND-2000, NanoDrop Technol, Wilmington, DE) according to Sarazin et al. (1999) in a volume of 1.5 µl. The carbonate system was calculated as described in the previous section.

Experiment 4: effects of seawater hypercapnia on arm regeneration

To determine the impact of low pH and regeneration on metabolic rate, 48 brittlestars were divided into two groups for the hypercapnia treatment (pH 8.1 and pH 7.0) consisting of 12 control (intact) and 12 amputated animals each. Regeneration rates are dependent on the position of amputation (arm loss; (Dupont and Thorndyke, 2006)) and three arms were amputated at 1 cm from

the disc to favor fast regeneration. Intact and amputated animals were placed in the same aquaria of the respective pCO_2 treatment and continuously supplied with a flow of fresh seawater (40-45 ml min^{-1}). For practical reasons and to avoid sediment effects on pH exposure animals were kept without sediment and were separated individually in 6-well plates (each well had a volume of 15 ml) covered with a net (mesh size 1.5 mm) to avoid interaction between individuals and to 1 be able to track individuals along the experimental period. Respiration rates were determined (see section below) as described before at 0, 1, 2, 4, 3 6, 12 and 18 days post amputation (dpa) in both regenerating and intact animals. The fresh mass of each individual was determined before the start of the experiment. In three timely separated experiments, with the same experimental design, amputated brittlestars maintained under both pH (8.1 and 7.0) levels were removed from the aquaria every 4, 6, 12 and 18 days, anesthetized with iso-osmotic 4% $MgCl_2$, and the total length of the regenerate (RL) and the length of the differentiated part of the regenerate (fully formed segments with clearly developed ossicles, podia and spines; DL) were measured using a dissecting microscope (Leica MZ 16 A) (see Dupont and Thorndyke, 2006).

Determination of oxygen consumption and NH_4^+ excretion rates

Animals were placed in glass respiration chambers with a volume of 26 ml containing 0.45 µm filtered seawater equilibrated with the appropriate pCO_2 level. Respiration chambers were closed, submerged in a water bath at 10˚C and O_2 saturation was measured continuously (once every 30 seconds) for 2-3 hours using fiber optic oxygen sensors (PreSens oxygen micro optode, type PSt1) placed in the lid of respiration chambers that were connected to an OXY-4 mini multichannel fiber optic oxygen transmitter (PreSens, Regensburg, Germany). The sensors were calibrated according to the manufacturer's instructions. Preliminary experiments demonstrated that the brittlestars could not sufficiently mix the water volume. Therefore, two syringes were gas tightly connected to the respiration chambers to gently mix the water every 20 min.

This resulted in a linear decrease of oxygen concentrations during the incubation period. O_2 concentrations never decreased below the 70% air saturation level. After the respiration measurement animals were removed from the chambers and the missing water volume was refilled with 0.45 µm filtered seawater, respiration chambers were closed and measured for 6 to 12 hours for detection of background respiration. Bacterial respiration never exceeded 10% of animal respiration. Prior to the start of the incubation, animals were acclimated for 30 min in the respiration chambers. For calculation of O_2 consumption rates, the linear decrease in O_2 concentration between start and the end of the measurement period was considered. Subsequently, body weights of individuals were measured. O_2 consumption rates (MO_2) are expressed as µmol O_2 gFM^{-1} h^{-1}.

Ammonium excretion rates were determined from NH_4^+ concentration measurements prior to and following incubation of brittlestars for respiration measurements. Before closing and after opening of respiration chambers, a 1 ml seawater sample was removed for NH_4^+. NH_4^+ concentration in seawater samples was determined using the indophenol method and measurements were conducted using an automated spectrophotometer (TRAACS 2000, Bran&Luebbe, Norderstedt, Germany) calibrated with a standard series of known NH_4^+ concentrations. Additionally, a separate glass chamber without animal was also incubated to determine background readings of filtered seawater for ammonium excretion and respiration rates. Ammonia (NH_3) was not measured as NH_3 concentrations at pH values of 8 to 7.1 are negligible (0.2-2% of total ammonium/ammonia (Korner et al., 2001). Ammonium excretion rates are expressed as µmol NH_4^+ gFM^{-1} h^{-1}. The O_2 to nitrogen ratio which serves as an indicator for protein metabolism was calculated according to the following equation:

$$O:N = 2\ MO_2\ (NH_4^+ \text{ excretion})^{-1} \quad \text{(Equ. 1)}$$

Biometric analyses

Biometric parameters of animals from the CO_2 pertubation experiment (experiment 2) were analyzed using whole animal fresh mass (FM), dry mass (DM), ash - free dry mass (AFDM) and ash content (ash dry mass) of several body parts (body and arms). Masses were determined using a precision scale (AT261 Deltarange, Mettler, Giessen, Germany). After determination of wet mass by gently drying animals on a paper towel, body parts were dried at 60˚C for 20 hours (Modell 400 Memmert, Schwabach, Germany) and masses were determined again. Ash dry mass was determined by placing arms and body parts in a drying oven at 550˚C for 20 hours (B170, Nabertherm, Bremen, Germany) prior to mass determination. AFDM was then calculated by subtracting ash dry mass from dry mass of the arms and bodies, respectively. Biometric measurements including determination of disc diameter, wet mass, dry mass and ash dry mass were conducted with brittlestars that were used for respiration and ammonium excretion trials after the physiological measurements were completed. For statistical analyses, values of single individuals (two per replicate) were averaged and the n=4 experimental aquaria per pH level were considered the unit of replication.

Preparation of mRNA

Frozen arm and disc tissues from *A. filiformis* were homogenized in Tri reagent (Sigma-Aldrich, T-9424). Samples were centrifuged for 10 min at 12,000 g and the pellet was discarded to remove all calcium carbonate from tissues. Total RNA was purified using the Ribopure. column system (Ambion, Austin, USA) following the manufacturer's protocol. DNA contamination was removed with DNase I. The amount of mRNA was determined by spectrophotometry (ND-2000, NanoDrop Technol, Wilmington, DE). All mRNA pellets were stored at -20˚C.

Real-time quantitative PCR (qPCR)

The mRNA expressions of target genes were measured by qPCR using the Applied Biosystems 7300 Real Time PCR System. *A. filiformis* sequences were obtained from a 454 sequencing library of regenerating arm tissues (Ortega-Martinez unpublished data). Sequences used for gene expression studies were translated to amino acid sequences, blasted and aligned with sequences from other echinoderms. Primers were designed (Table 4) using primer analysis software Primer Express version 2.0 (Applied Biosystems) with the default parameters of the TaqMan MGB Probe and Primer design procedure. PCRs contained 2 ng of cDNA, 50 nM of each primer, and the SYBR. Green MasterMix (Applied Biosystems) in a final volume of 10 µl. All qPCRs were performed as follows: 1 cycle of 50˚C for 2 min and 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 s and 60˚C for 1 min (the standard annealing temperature of all primers). PCR products were subjected to a melting-curve analysis to verify that only a single product was present. Control reactions were conducted with DNAse treated RNA samples to determine levels of background and genomic DNA contamination. The standard curve of each gene was confirmed to be in a linear range with ubiquitin conjugated enzyme (UCE) as reference gene. The expression of this reference gene was checked for stability and has been demonstrated to be stable also in other marine species among ontogenetic stages and during CO_2 treatments (Hu et al., 2011, Hu et al., 2013, Tseng et al., 2013).

Statistical analyses

For metabolic and NH_4^+ excretion rates, each of the four experimental replicates per treatment were used and averaged over the four time points. Normality of distributions was assessed via the Kolmogorov–Smirnov test. Differences between hypercapnia treatment groups were analyzed using one-factorial ANOVA followed by *post hoc* Tukey tests using Sigma Stat (SystatSoftware INC.). A two-way ANOVA was performed for biometric and extracellular pH HCO_3^- data

with time and pH as variables followed by *post hoc* Holm-Sidak test. Percentage values of visible arms in relation to all arms were arcsine transformed prior to statistical analysis (one way ANOVA). The threshold for significance was $p < 0.05$. Student t-test was used to compare metabolic rates of amputated and intact animals. The data in the text and figures are presented as the mean ± standard error (SE).

3.3.2 Results

CO_2 perturbation experiments

In order to investigate the effects of seawater acidification on physiology, behavior and abiotic parameters inside of sediment burrows we performed four pH perturbation experiments (table 3). The first experiment (experiment 1) addressing the effects of acidification on metabolic rates, NH_4^+ excretion, gene expression and composition of body parts used seawater pH values of 8.0, 7.3 and 7.0 corresponding to pCO_2 levels of 526, 3396 and 6644 µatm in the seawater above the sediment. pH in the benthic layer of the Gullmarsfjord were estimated using data from SMHI Database Svenskt Havrarkiv (from March to September, period of 1959-1987) and varies between 7.8 in Autumn and Winter and 8.1 in Spring. To address the extra-cellular acid-base status of *A. filiformis* exposed to different pH conditions (experiment 2) we used pH values of 8.0, 7.6 and 7.3 corresponding to pCO_2 levels of 492, 1473 and 3213 µatm. The pH levels used in these two experiments simulate potential scenarios in the context of ocean acidification in benthic habitats (e.g. pH 8.0, 7.6 and 7.3) as well as a more extreme pH level of 7.0 which simulates acidification by leakage of pure CO_2 from sub seabed CCS sites. To investigate the effects of acidification on abiotic conditions inside the sediment burrow micro-habitat (experiment 3) and regeneration capacities (experiment 4) we performed two additional experiments using the lower pH level of 7.0 which corresponded to a pCO_2 of 6400 µatm.

Abiotic parameters within burrows (Experiment 1)

O_2 and CO_2 profiles determined for burrows of *A. filiformis* demonstrate a progressive decrease of pO_2 and an increase of pCO_2 with depth (Fig. 5A-B). O_2 levels decrease down to 50.11 ± 7.3mmol l-1 (20% air saturation) and CO_2 levels increase to 0.13 ± 0.009 kPa (pH 7.64 ± 0.03) in depth of 3 cm (Figure 17A-B). No pH induced differences in O_2 profiles were recorded in sediments (Fig. 17C). During environmental acidification (pH 7), burrow water (BW) pH decreased to pH 6.98 ± 0.02 (pCO_2: 0.65 ± 0.05 *k*Pa) (Fig. 17A). Total alkalinity measured from BW (3 cm depth) was 2.17 ± 0.26 under control and 2.17 ± 0.48 under low pH conditions. Decreased seawater pH induced by hypercapnic conditions led to increases in BW pCO_2 in an additive fashion However, independent of the degree of sea water acidification (hypercapnic conditions) we observed a constant pCO_2 gradient of approximately 0.05 kPa between BW at 3 cm depth compared to the surrounding sea water.

Routine metabolic rates (RMR), ammonium excretion and O:N ratio (Experiment 2).

Routine metabolic rates were significantly influenced by decreased pH over the time course of 4 weeks (Fig. 18A), with a significant decrease at pH 7.0 levels down to 0.66 ± 0.06 µmol O_2 gFM^{-1} h^{-1} compared to 0.95 ± 0.06 to 1.07 ± 0.07 µmol O_2 gFM^{-1} h^{-1} under pH 8.1 and pH 7.3 respectively. Ammonium excretion rates significantly increased with increasing pCO_2 from 0.044 ± 0.007 µmol NH_4^+ gFM^{-1} h^{-1} under pH 8.1 conditions to 0.069 ± 0.009 $NH4^+$ gFM^{-1} h^{-1} at decreased pH (Fig. 18B). Accordingly, the O:N ratio decreased significantly with decreasing pH from 51.57 ± 8.59 at pH 8.1 down to 30.56 ± 5.46 at pH 7.0 (Fig. 18C). We could not observe any mortality during the entire experimental period.

Gene expression (Experiment 2)

In disc tissues of animals maintained for 4 weeks at pH 7.3 or pH 7.0 (Fig. 19, upper panel) the only significant change was observed for NHE_3 regulator which

was 0.36 ± 0.09 log^2-fold (22%) up regulated in response to pH 7.3. No significant differences were observed for other genes. In arm tissues (Fig. 19, lower panel) several significant changes were observed: among the ion regulatory genes, NBCe and AQP9 were 0.87 ± 0.35, 1.0 ± 0.46 and 1.72 ± 0.95 log^2-fold down regulated in pH 7.0 treatment. Among metabolic genes G6PDH transcript abundance was significantly affected in both treatment levels by 0.96 ± 0.37 (pH 7.3) and 1.61 ± 0.55 (pH 7.0) log^2-fold. LDH expression was significantly reduced by 0.52 ± 0.24 log^2-fold in pH 7.3. No significant changes were detected between pH for genes involved in amino acid catabolism including amino acid specific trans-aminases.

Biometrics and behavior (Experiment 2)

Along the experimental period no significant changes were detected in fresh mass (FM), dry mass (DM), ash-free dry mass (AFDM) and the ratio between ash dry mass (ADM) and DM for arms and bodies, respectively. However, a significant decrease of visible actively filter feeding arms were observed in decreased pH treated animals with only 43% of visible arms in pH 7.3 and 27% in pH 7.0 seawater (Fig. 20), whereas animals in pH 8.1 exposed up to 73% of their arms into the water column.

Extracellular acid-base status (Experiment 3)

In order to test in how far the brittlestar *A. filiformis* was able to control their extracellular pH homeostasis we used pH sensitive optodes to measure pHe in the coelomic cavity of control and CO_2 treated animals over a time course of 15 days (Fig. 21). Under pH 8.1 (0.05 *k*Pa pCO_2) conditions pHe was approximately 0.2 to 0.3 units below the environmental pH. When exposed to low pH conditions, the pHe dropped within 48 h to 7.64 ± 0.06 and 7.52 ± 0.05 at an ambient pH of 7.63 (0.15 *k*Pa pCO_2) and 7.3 (0.32 *k*Pa pCO_2), respectively (Fig. 21). Along the course of 10 days the pHe remained relatively stable at the respective pH level.

The calculation of HCO_3^- levels in the coelomic fluid indicated that already under pH 8.1 conditions *A. filiformis* had high extracellular HCO_3^- levels (6 - 7 mM) compared to the surrounding seawater (2 - 2.5 mM). When exposed to lowered sea water pH, animals significantly increased their extracellular fluid [HCO_3^-] to 8 -9 mM within 48 h (Fig. 21B). In the following days extracellular fluid [HCO_3^-] was maintained at elevated levels in decreased pH treated animals, compared to the control group.

Regeneration and RMR (Experiment 4)

Regeneration rates (RR in mm d^{-1}) were calculated as the coefficient of the significant linear regression between regenerate length (mm) and time (d). RR was significantly 3.5 times faster (ANCOVA; $F=73.03$; $p<0.0001$) in pH=8.1 (RR=0.083 ± 0.004 mm.d^{-1}; $F=369.36$, $p<0.0001$) compared to pH 7.0 (RR=0.024 ±0.002 mm.d^{-1}; $F=216.58$, $p<0.0001$) (Fig. 22). The differentiation index, (DI as a percentage), was calculated as the proportion in length of the regenerate that is completely differentiated (DI = (DL/RL) x 100) which serves as an indicator of functional recovery of the regenerate (for detailed description see Dupont and Thorndyke, 2006). This calculated DI was 59 ± 12 under control conditions after the regeneration period of days whereas no differentiation was observed in low pH treated animals. In both treatments the formation of a blastema had been observed, however regeneration seemed to be delayed or depressed in decreased pH treated animals (Fig. 22). The wound healing and regeneration process is accompanied by significant changes in routine metabolic rates (RMR) (Fig. 23). Under control pH conditions amputated animals increase metabolic rates immediately after amputation from 0.93 ± 0.06 to 1.04 ± 0.05 µmol O_2 gFM^{-1} h^{-1}. Up to 6 days these animals further increased their metabolic rate reaching up to 1.27 ± 0.07 µmol O_2 gFM^{-1} h^{-1} at 6 days post amputation (dpa). In the following days metabolic rates progressively declined back to starting levels at the time point of 18 days post amputation (Fig. 22A). In contrast, metabolic rates

of animals regenerating in pH 7.0 seemed to initially increase metabolic rates as observed for pH 8.0 animals. However, instead of increased metabolic rates until 6 dpa, respiration rate declined after 2 dpa and stayed at background levels of approximately 0.9 to 1.0µmol O2 gFM^{-1} h^{-1} (Fig. 22B).

3.3.3 Discussion

Abiotic conditions within burrows

While surrounding seawater has a pH of 8.1, *A. filiformis* experiences a pH lower than 7.7 and strong hypoxic conditions within their sediment burrows. Oxygenation of *A. filiformis* burrows and the oxygenation of the surrounding sediment reported in the literature corroborates with our findings, demonstrating that O_2 concentrations decrease with depth in burrows (Vopel et al., 2003). This is a consequence of anoxic conditions within the sediment itself and the metabolism of the animal that demands continuous uptake of O_2. The fact that acidified conditions negatively affected the sweeping activity of arms in the water column could suggest that active ventilation of the burrows by the animal may be affected as well. These findings indicate the importance to consider not only the environmental and local variations in abiotic parameters (McElhany and Busch, 2013), but also those seen by many organisms in their micro-habitats. In this context the present work demonstrates that already now *A. filiformis* is confronted with pH levels predicted for the coming 100 years in open ocean surface waters. pH 7.7 is the extreme of present pH natural variability experienced today and pH down to 7.3 may be extreme of near-future natural variability. Moreover, potential leakage of pure CO_2 from CCS sites can easily lead to a local acidification of burrow water < pH 7.3 and can be expected to significantly affect physiological processes of burrowing organisms like *A. filiformis* which are discussed in the following.

Physiology and behaviour

Our pHe measurements suggest that *A. filiformis* is a weak acid-base regulator similar to other echinoderms which cannot fully compensate an extracellular acidosis during environmental hypercapnia. However, in accordance to other echinoderms (summarized in Stumpp et al., 2012), *A. filiformis* was able to accumulate up to 1.5 – 2.5 mM HCO_3^- in body fluids in response to an extracellular acidosis. In contrast, organisms that can fully restore pHe during acidosis (e.g. fish and crustaceans) can actively accumulate HCO_3^- in the 10-20 mM range at hypercapnia levels of 0.5 -1 *k*Pa pCO_2 (Larsen et al., 1997, Pane and Barry, 2007). An uncompensated acidosis has been demonstrated to induce metabolic depression (PÖrtner et al., 2000, PÖrtner et al., 2004). Metabolic depression is a major strategy in most invertebrate phyla and all vertebrate classes to survive environmental stress (Dezwaan and Wijsman, 1976, Guppy, 2004, Ramnanan and Storey, 2006). A number of recent studies indicated that mild hypercapnia does not affect or even stimulates metabolism whereas more severe hypercapnic conditions can lead to depressed metabolic rates (Thomsen and Melzner, 2010, Dorey et al., 2013). In accordance to these observations our work demonstrated that moderate seawater acidification (pH 7.3) does not elicit metabolic depression, but in turn has the tendency to increase metabolic rates during a four week acclimation period. A similar moderate acidification induced stimulation of metabolic rates has been previously observed in several marine invertebrates, including brittlestars (*A. filiformis* and *Ophiocten sericeum*) (Wood et al., 2008, Wood et al., 2011), the bivalve *Mytilus edulis* (Thomsen and Melzner, 2010), pluteus larvae of sea urchins (Stumpp et al., 2011b) and the Arctic pteropod *Limacina helicina* (Comeau et al., 2010). These studies suggested increased energetic demands to fuel compensatory mechanisms to maintain calcification rates and acid base homeostasis. However, this study also demonstrated that prolonged exposure to stronger acidification (pH 7.0) resulted in a 31% reduction of routine metabolic rates in *A. filiformis*. Similar observations were made in other marine invertebrates like the worm *Sipunculus nudus*, where hypercapnia induced reductions in pHe (and not in pHi) were

demonstrated to elicit metabolic depression as indicated by reduced oxygen consumption rates in isolated muscle tissues (Reipschälger and Pörtner, 1996). Furthermore, a recent work conducted on sea urchin larvae demonstrated that moderate acidification increase metabolic rates whereas pH levels < 6.5 significantly reduced respiration rates (Dorey et al., 2013). In the present work the depression of metabolic rates in the low pH treatment is further underlined by a general down regulation pattern in arm tissues of genes involved in energy consuming (e.g. acid-base genes) as well as energy providing (metabolic genes) processes. Although not tested on the protein level these results suggest that genes coding for metabolic and acid-base regulatory processes are expressed at lower rates under acidified conditions. Such a modulation of physiological processes (e.g. reduction of protein synthesis and down regulation of energy providing processes) has been described to be a general feature of organisms undergoing metabolic depression (Guppy and Withers, 1999). Interestingly, expression profiles of body tissues did not demonstrate such a clear down-regulation pattern as observed for arm tissues. This differential, tissue-specific response could have 2 possible explanations: i) body tissues maintain routine functionality despite metabolic depression in arm tissues or ii) arm tissues are the major site of energy metabolism, respiration and ion-regulation whereas body tissues mainly serve reproduction and nutrient absorption. The latter is supported by behavioral observations demonstrating that during exposure to acidified conditions *A. filiformis* retract their arms into burrows. This behavioral response could be interpreted as an energy saving mechanism to reduce additional energetic costs for suspension feeding by sweeping their arms in the water column. Reduced activity may also translate into decreased bioturbation of sediments, which has been hypothesized to affect nutrient flux between water and sediment (Wood et al., 2009). Moreover, retraction of arms during hypercapnic exposure may lead to reduced feeding rates as well as ventilation of burrows, which could have a negative feedback on the animal's energy budget and hypercapnic conditions inside the sediment burrows. Thus, determinations

of feeding rates in combination with energetic expenses during environmental hypercapnia represent an important task for future research. Besides increased metabolic rates at moderate and decreased metabolic rates at low pH levels the present work demonstrates that *A. filiformis* increases NH_4^+ excretion rates at both, moderate and high hypercapnia levels. Higher NH_4^+ excretion rates in response to decreased seawater pH significantly decreased the O:N ratio from control to low pH treatments. Measured metabolic and NH_4^+ excretion rates as well as O:N ratios are comparable to published values for *A. filiformis* and other brittlestars from temperate latitudes (Davoult et al., 1991, Christensen and Colacino, 2000, Talbot and Lawrence, 2002, Vopel et al., 2003) as well as other echinoderms such as the sea urchins *Psammechinus miliaris* (Ottero-Villanueva et al., 2004) and *Sterechinus neumayeri* (Hill and Lawrence, 2006). Moreover, the O:N ratio in *P. miliaris* maintained on an algal diet was 37 while urchins fed with mussel or salmon tissue decreased the ratio to 29 and 12, respectively (Ottero-Villanueva et al., 2004). This indicates that the diet composition directly translates into altered O:N ratios. Based on histological studies, Wood *et al*. (2008) suggested that unfed *A. filiformis* increases protein metabolism by the breakdown of muscle tissues to fuel increased energetic demands in response to seawater acidification. It is likely that enhanced catabolism and cell turnover/dedifferentiation of muscle tissues following traumatic amputation in brittlestars and crinoids is related to cell differentiation to produce stem cells to regenerate lost tissues rather that fueling calcification processes (Candia-Carnevali and Bonaroso, 2001, Biressi et al., 2010). However, despite increased NH_4^+ excretion rates the present study could not demonstrate any changes in ratios between ADM and DM between pH treatments that would have indicated an increased utilization of muscle tissue or other amino acid compounds as energy source. However, higher NH_4^+ excretion rates can be directly linked to acid-base compensatory processes (Shih et al., 2008, Wu et al., 2010). It has been hypothesized that secretion of NH_4^+ derived from protein catabolism serves as an additional acid extrusion mechanism in the mussel *Mytilus edulis* (Thomsen

and Melzner, 2010), the sea urchin *Strongylocentrotus droebachiensis* (Stumpp et al., 2012) and the worm *Sipunculus nudus* (Langenbuch and PÖrtner, 2002). In this context the importance of Rhesus proteins (RhP) needs to be mentioned, which were recently discovered to be associated with acid-base regulatory abilities in various marine taxa (Weihrauch et al., 2009, Nawata et al., 2010, Hu et al., 2013). Future studies are needed to broaden our understanding regarding the pathways of NH_4^+ based acid-base regulatory mechanisms in marine invertebrates.

Regeneration potential in acidified environments

The present work demonstrates increased demands of aerobic metabolism during the course of regeneration of autotomized arm tissues. The initial phase of regeneration, is characterized by cell proliferation (Thorndyke et al., 2001, Dupont and Thorndyke, 2006), seems to require significant energetic costs that derive from aerobic metabolism. Environmental acidification leads to significant energetic costs and a reduction of the metabolic scope of regeneration. This reduction in metabolic scope for regeneration processes is associated with a significant decrease in regeneration rates in *A. filiformis* exposed to decreased pH. This contrasts with Wood et al. (2008) who reported increased regeneration rates in the same species exposed to decreased seawater pH. These different findings are probably due to methodological differences: in the study by Wood *et al*. (2008); amputations were not standardized whereas in the present study arms were cut at 1 cm from the disc in all animals which minimized differences in regeneration rates (Dupont and Thorndyke, 2006). However, the results of the present work are in general accordance to observations made in brittlestars exposed to osmotic challenges. For example, the brittlestar *Ophiophragmus filograneus* has been demonstrated to reduce regeneration rates down to 25% compared to control animals when exposed to hyposaline conditions (Talbot and Lawrence, 2002). Interestingly, under this hypoosmotic stress reduced regeneration rates of *O. filograneus* were accompanied with reduced metabolic

rates and increased NH_4^+ excretion, in a similar manner as observed in the present study. It can be hypothesized that ionic disturbances in general, including acid-base and osmotic challenges, may trigger very similar physiological responses at certain threshold levels.

3.3.4 Conclusion

Our work highlights the importance to consider micro-habitats when considering the impacts of elevated pCO_2 from CCS leakage. The infaunal brittlestar *A. filiformis* experiences naturally low O_2 and pH conditions within sediment burrows. A decrease in seawater pH is additive to the naturally low pH within the burrows. More studies on abiotic parameters in sediment burrows are needed in order to fully understand the effects of acidification on these microhabitats, and in how far organisms are able to control and cope with this microenvironment. Our results also indicate that *A. filiformis* cannot tolerate prolonged exposure to pH 7.0 as indicated by the onset of metabolic depression. This effect on aerobic metabolism reduces the metabolic scope for regeneration that in turn, has severe repercussion on regenerative capacities of autotomized arm tissues. Our results suggest that adult *A. fliformis* are relatively robust to CO_2 induced seawater acidification (down to pH 7.3). However, stronger acidification down to a pH of 7.0 as it can be locally expected for potential leakage from CCS sites can significantly affect the fitness of this ecologically important species.

3.4 Response of early life-stages *[Chan et al. 2012; Dorey et al. 2013; Stumpp et al. 2012]*

The above three publications have been presented as appendices to this report. They detail the impacts of elevated pCO_2 on larval stages of sea urchins *Strongylocentrotus droebachiensis* and *Strongylocentrotus purpuratus.* Elevated pCO_2 was shown to have impacts on larval digestion, larval (mortality, symmetry, growth, morphometry and respiration and ontogeny.

In general, larvae were found to be more susceptible than adults to elevated pCO_2/reduced pH but that different sub-lethal effects are produced at different critical pH thresholds. Larvae exhibit the ability to up-regulate their metabolism and to compensate in the pH range 8.1- ~7.5. Below ~ pH 7.5 impacts to development and growth precede more significant impacts to symmetry and survival at extreme pH. It is clear from these publications that, whilst adults of some species may appear robust to acute or even chronic exposure to elevated pCO_2 that might be associated with a CCS reservoir failure, depending on the time of year, significant impacts might be seen in larval and juvenile stages within the water column. Impacts to these sensitive stages will potentially affect the recruitment and persistence of populations of key ecosystem engineers adjacent to even diffuse leaking reservoirs.

4.0 General discussion

As a result of investigations within the *E*CO_2 project and in collaboration with other CCS projects, it is possible to make informed predictions on the susceptibility and tolerance of marine organisms faced with chronic acidification derived from a CCS leakage scenario. One of the most notable findings is the variation in the degree of tolerance observed by different organisms and at various life stages. However, the variability in organism CO_2 tolerance requires more understanding into how this will impact food webs and local ecosystems. In addition, more research is required to understand the combined effects of a CCS leakage scenario with other environmental stressors associated with a changing marine climate.

A useful indicator of CO_2 stress is the behavioural observations recorded in infaunal organisms moving to the top of sediment beds and appearing moribund (Shade et al 2013). Key findings from the work investigating the borrowing conditions of echinoderms during CCS leakage scenarios indicate a progressive hypoxia compounded by increasing hypercapnia and declining pH. This effectively reduces metabolite scope and leads to ecosystem impacts resulting from reduced bioturbation activity, in turn reducing energy budgets and increasing ammonia excretion. Complementing the work completed within *E*CO_2, additional research has helped to formulate a better understanding of how organisms may interact during a CCS leakage scenario. Experiments on biological impacts of direct injection of CO_2 on the seabed meiofauna demonstrated a decline in sediment community oxygen consumption, at 20,000 uatm pCO_2, suggesting a weakening meiobenthos activity with time as acidification intensifies (Ishida et al., 2013). A decline in bacteria and archaea communities was also noted as an initial response to high CO_2, however both microbial groups increased abundance after 2 weeks, an indication that in the

absence of other trophic groups there was reduced predation on bacteria, allowing them to proliferate (Ishida et al., 2013).

There are pronounced differences in bivalve tolerance between the three studied species investigated by Shade et al., (2013). These differences indicate that more experiments are necessary to understand the response of communities dominated by different bivalve species to leakage of CO_2-rich water. While we found significant, yet moderate, impacts of seawater acidification on microbial and meiofauna communities at the highest treatment level, it is clear that other macrofauna species (e.g. *Cerastoderma edule Nereis* spp., *Corophium* spp.), which exert a strong influence on habitat microbiota and meiofauna (Nascimento et al. 2012), might also suffer high mortality during long-term exposure to acidified water.

Cerastoderma edule was found to be very sensitive to most CO_2 levels tested. While significantly elevated mortality occurred only at high levels of acidification (24,000 µatm when averaged over all size classes), dissolution of the shell already occurred at lower pCO_2. This indicates that longer term leakage events with average pCO_2 >1,500 µatm could be detrimental to species' fitness, as shell corrosion ultimately leads to invasion of microorganisms and allocation of resources towards shell maintenance and immune processes, leaving less resources available for growth and reproduction. Such detrimental energy budget re-allocation has also been observed in other marine invertebrates during long-term acclimation to high seawater pCO_2 (e.g. Stumpp et al. 2012). Detailed determination of energy budgets of all three tested bivalve species are necessary to better understand sub-lethal, fitness relevant impacts of long-term exposure to acidified seawater. Smaller *C. edule* were very susceptible to acidification, with higher mortality than larger size classes evident at pCO_2 >6,000 µatm. This suggests that it will be important to investigate responses of

freshly settled *C. edule* to sediments with calcium carbonate undersaturation. Green et al. (2004) demonstrated exponentially decreasing rates of dissolution-induced mortality in the infaunal clam *M. mercenaria* with decreasing size, indicating that freshly settled juveniles might potentially constitute an ontogenetic bottleneck in terms of tolerance of acidic seawater.

Outer shell corrosion has been observed in a number of gastropod and bivalve molluscs (e.g. Hall-Spencer et al. 2008, Ries et al. 2009) and has often been linked to presence of a thick and intact periostracum (Ries et al. 2009). *C. edule* is characterized by a very thin periostracum (ca. 2 µm) and a shell that is exclusively composed of aragonite, the more soluble calcium carbonate polymorph. In addition, *C. edule*, through its frequent movement within the sandy sediment (Flach 1996), erodes its periostracum with time. Mytilid mussels, which are protected by a periostracum that is >20 µm thick (Harper 1997), can live in seawater that is strongly undersaturated with respect to calcium carbonate (Ω_{arag}<0.2) if their periostracum is intact. However, corrosion occurs when the periostracum is mechanically damaged (Thomsen et al. 2010), leading to complete dissolution of the shell in extreme cases in deep-sea hydrothermal vent mussels that live in strongly acidic waters with pH <6 (Tunnicliffe et al. 2009). Internal dissolution of shells has also been reported, both in response to internal, hemolymph acidification during tidal emersion (Akberali 1980, Akberali et al. 1983) and during prolonged exposure to acidified seawater (Melzner et al. 2011). Internal dissolution is likely to occur, as bivalve body fluids (hemolymph and extrapallial fluid) are typically more acidic and more strongly undersaturated with calcium carbonate than the surrounding seawater (see Thomsen et al. 2010, Melzner et al. 2013 for an extended discussion). While bivalves are able to repair holes and fractures in their shells (e.g. Mount et al. 2004), it is apparent from our results that *C. edule* in the highly acidified treatments were not able to allocate sufficient resources into shell repair mechanisms, or that the rate of dissolution simply outpaced 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 protein from the extrapallial fluid, the fluid that is in contact with the inner side of the shell (Kadar 2008). In summary, progressing shell corrosion, even at the lower treatment levels (i.e. 1,500 µatm), constitutes a significant problem, as shell integrity is essential for fitness of bivalve species. In addition, evidence of spine dissolution in *P. lividus* was also noted above 1000 µatm by Morgan and Hauton (unpublished) leading to a reduction in spine length and increasing the vulnerability of *P. lividis* to predation.

The physiological capacity of echinoderms to tolerate elevated pCO_2 is limited due to the because of a reliance on diffusive respiratory gas exchange. Therefore any disturbances in acid base status are difficult to buffer due to lack of a respiratory pigment or substantial protein concentration in the coelomic fluid. This is compounded by weak ionic regulatory ability, combined with a vulnerable calcareous test. The physiological limitations of these organisms compromise their ability to adapt to changing conditions if unable to compensate via acid base buffering capacity, which we have herein shown to be limited. Present findings demonstrate very little ability to compensate chronic acidification in the urchin *P. lividus*, an economically important species used in aquaculture. The pCO_2 tension they could be potentially exposed to in the event of a CCS leak would pose a serious threat to their survival; however, the duration of exposure appears to be critical. Furthermore, weak acid base compensation was also shown in *A. filiformis*. Acidosis, arising from uncompensated acid base disturbance, has been demonstrated to induce metabolic depression (PÖrtner et al., 2000, PÖrtner et al., 2004). In the absence of a buffering capacity, metabolic depression can help alleviate the hypercapnic stress by reducing O_2 demand, as observed in most invertebrate phyla and all vertebrate classes in order to survive environmental stress (Dezwaan and Wijsman, 1976, Guppy, 2004, Ramnanan and Storey, 2006). Numerous studies have found that mild

hypercpnia does not affect, or even stimulates, metabolism - whereas more severe hypercapnic conditions can lead to depressed metabolic rates (Thomsen and Melzner, 2010, Dorey et al., 2013). However, this is species specific and in the present investigation *A. filiformis* did not elicit metabolic depression (> pH 7.3), in fact an increase in metabolic rates during a four week acclimation period was observed. These findings compliment other marine invertebrates, including brittlestars (*A. filiformis* and *Ophiocten sericeum*) (Wood et al., 2008, Wood et al., 2011), the bivalve *Mytilus edulis* (Thomsen and Melzner, 2010), pluteus larvae of sea urchins (Stumpp et al., 2011b) and the Arctic pteropod *Limacina helicina* (Comeau et al., 2010).

While intermediate levels of acidification (i.e. <4,000 µatm) have been shown to often lead to elevated metabolic rates (e.g. Thomsen & Melzner 2010, Beniash et al. 2010) and increased oxidative stress (Tomanek et al. 2011) in a number of bivalve species, studies using treatment levels of >4,000 µatm have generally found a metabolic reduction (Thomsen & Melzner 2010, Michaelidis et al. 2005). Metabolic suppression is a strategy to survive short-term abiotic stress and is beneficial during periods of e.g. tidal emersion to conserve energy stores. Internal, metabolism-induced hemolymph acidification during valve closure may be the signal for metabolic reductions during such situations (Guppy & Withers 1999). When employed as a strategy in the long run, metabolic suppression will lead to consumption of endogenous energy stores and reduced fitness. In support of that view, CO_2-exposed *C. edule* suffered reductions in shell-free dry mass when compared to all other experimental groups. Clearly, it seems important to investigate aerobic metabolism and energy allocation in this species to better understand the observed responses. 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 (e.g. Stumpp et al. 2011, 2012 Dorey et al. 2013). While the mineralogy is an important factor in defining susceptibility of bivalves (Ries et al. 2009), there is a high variation in vulnerability between species with the same shell mineralogy (Gazeau et al. 2013). Crystal size and the proportion of organic matrix within the shell play an additional factor in resistance against environmental stress such as acidified seawater (Harper 2000). Our results certainly confirm our original notion that the species most sensitive to hypoxia / anoxia (Dries & Theede 1974) are also most vulnerable to seawater acidification.

More pronounced changes in benthic microbial community structure with elevated pCO_2 were found in experimental biofilms from the Great Barrier Reef (Witt et al. 2011) and along CO_2 clines in the Mediterranean (Lidbury et al. 2012). Lidbury et al. (2012) observed significant increases in biofilm production with moderate pCO_2 (600-1,600 µatm) that went along with significant shifts in microbial community composition. While changes in benthic biofilm composition might be strongly determined by increased CO_2 availability for CO_2 limited autotrophic organisms, Kerfahi et al. (2014) demonstrated shifts in sediment microbial communities (top 2 cm of the sediment) along CO_2 clines (600 – 1,600 µatm) in the Mediterranean based on 16S rRNA sequence information. These authors found abundances of most dominant genera to be unaffected by CO_2, while only 5% of the genera differed in abundance along the CO_2 cline. Clearly, 16S rRNA sequence based information is necessary to further resolve the fine changes in microbial community structure that we observed in the present study with high seawater pCO_2.

Other studies have also found significant changes in meiofauna composition in comparable experimental approaches: Widdicombe et al. (2009) found a significant reduction in nematode community structure and diversity after 20 weeks. However, significant changes were only found at pH values <6.0,

suggesting that nematodes are more resistant to high seawater pCO_2 than macrofauna assemblages (Widdicombe et al. 2009). Dashfield et al. (2008) also did not find strong changes in infauna nematode abundance and diversity after seven weeks acclimation to pH 7.5.

Within ECO_2 we have also demonstrated that acidification in the overlying sea water leads to an additive acidification in the burrow water (Hu et al., 2014*)*. Investigating the borrowing habitats of echinoderms, extreme hypoxia was noted in the burrows of *A. filiformis* which increased with the depth of the burrow. Two-dimensional pH distributions in marine sediments have demonstrated rapid changes in pH by ± 2 units within 24 millimeters ranging from pH 6 to pH 8 (Zhu et al., 2006). Such acidified conditions in marine sediments can be explained by the aerobic decomposition of organic matter producing CO_2 and the re-oxidation of anaerobic metabolites such as NH_4^+, HS-, and Fe_2^+ (Zhu et al., 2006). The fact that burrows contained live animals suggest that despite the potential ability of *A. filiformis* to ventilate their burrows with their arms (see Vopel et al., 2003), pCO_2 levels within burrows were significantly affected by CO_2 induced seawater acidification. Ventilation to control abiotic conditions inside of sediment burrows has been reported for other marine taxa including polychaetes (Marinelli, 1994), crustaceans (Stamhuis and Videler, 1998) and some burrowing fish species (Atkinson et al., 1987). Elevated pCO_2 impacted *A. filformis'* ability to ventilate its burrows, which was compounded by hypoxia and resulted in a reduction in whole organism energy budgets.

A. filiformis retracted into their burrows during exposure to chronic acidification and was identified as an energy saving mechanism, albeit reducing their suspension feeding capacity. A reduction in movement observed on retraction into its burrows also has implications for the burrow ecosystem, reducing bioturbation and affecting nutrient flux between water and sediment (Wood et

al., 2009). *A. filiformis* increases NH_4^+ excretion rates at both, moderate and high hypercapnia levels, suggesting a higher NH_4^+ excretion rate in response to decreased seawater pH, which significantly decreased the O:N ratio from control to low pH treatments, in conjunction to increased metabolic rates at moderate and decreased metabolic rates at low pH levels. The present finding were in agreement to published values of *A. filiformis* and other brittlestars from temperate latitudes (Davoult et al., 1991, Christensen and Colacino, 2000, Talbot and Lawrence, 2002, Vopel et al., 2003) as well as other echinoderms such as the sea urchins *Psammechinus miliaris* (Ottero-Villanueva et al., 2004) and *Sterechinus neumayeri* (Hill and Lawrence, 2006). In addition, higher NH_4^+ excretion rates can be directly linked to acid-base compensatory processes (Shih et al., 2008, Wu et al., 2010). It is hypothesized that the secretion of NH_4^+ derived from protein catabolism serves as an additional acid extrusion mechanism in the mussel *Mytilus edulis* (Thomsen and Melzner, 2010), the sea urchin *Strongylocentrotus droebachiensis* (Stumpp et al., 2012) and the worm *Sipunculus nudus* (Langenbuch and PÖrtner, 2002). Furthermore, the importance of Rhesus proteins (RhP) needs to be mentioned, which were recently discovered to be associated with acid-base regulatory abilities in various marine taxa (Weihrauch et al., 2009, Nawata et al., 2010, Hu et al., 2013). Future studies are required to broaden our understanding regarding the pathways of NH_4^+ based acid-base regulatory mechanisms in marine invertebrates. Arm regeneration during environmental acidification will lead to significant energetic costs.. However, a reduction in metabolic scope for regeneration processes is associated with a significant decrease in regeneration rates in *A. filiformis* exposed to decreased pH. Previous investigations have contrasted the present findings and reported increased regeneration rates in the same species exposed to decreased seawater pH (Wood et al. 2008); however, this could be due to methodological differences.

The present findings demonstrate critical changes in micro-habitats during a

CCS leakage scenario and the importance of understanding local changes in habitat biochemistry. Burrowing organisms are already exposed to hypoxia and hypercapnic environments, highlighting them as vulnerable to further increases in acidification that would compound hypoxia/hypercapnia. Further investigations monitoring the abiotic variables within sediment burrows are required to enable more accurate predictions on the impacts of localized acidification arising from a CCS leakage scenario. *A*. *filiformis* cannot tolerate prolonged exposure to pH 7.0 as indicated by the onset of metabolic depression, which reduces metabolic scope for regeneration that in turn, has severe repercussion on regenerative capacities of automised arm tissues. Adult *A. fliformis* can tolerate CO_2-induced sea water acidification (down to pH 7.3). However, it is intolerant of stronger acidification down to a pH of 7.0. The expected duration and depth of acidification is therefore critical in determining the tolerance of ecologically important species.

5.0 Overall conclusions

We highlight the varying degrees of species susceptibility to CO_2 acidification from a CCS leakage scenario. This will be critical to understand whole ecosystem susceptibility to CCS leakage and as a result there is an imperative requirement for more large-scale community response investigations.

Most organisms investigated can tolerate a large degree of acidification (i.e pH 7.3) before significant mortality is observed (pH >7.0). However, this tolerance also needs to be considered alongside the compounding effects of present day anthropogenic stressors such as ocean acidification and warming events. In addition, the sensitivity of compounding environmental stressors acting in unison are evident from sediment micro burrow investigations, illustrating that hypoxia-sensitive species are also susceptible to hypercapnia.

Furthermore, whilst the duration and depth of acidification has been investigated on separate scales; more work is required to define critical thresholds of community responses combining both scales. Duration of localised CO_2 acidification arising from a leakage scenario is dependent on tides and currents (UK QICS Project *www.bgs.ac.uk/qics/home.html*) suggesting organisms will only be exposed to localised chronic acidification for short time periods (<1 day), elevating the likelihood of survival of exposed organisms. As such, the duration of exposure to CO_2 may be more significant than the depth of acidification experienced by benthic organisms.

The remote monitoring (high resolution video or still images) of the behavioural responses of macrofauna has considerable promise as a low cost approach to real time monitoring of CCS leakage events.

6.0 Recommendations

- Develop a catalogue of species-specific critical pH or pCO_2 tensions that will severely inhibit organism 1) growth, 2) reproduction, 3) recovery;
- Vulnerable ecosystems, i.e sediment micro burrows, already experience hypoxia and hypercapnia. Further elevations in pCO_2 will compound environmental stress in these environments affecting important sedimentary ecosystem processes;
- Behavioural responses such as massive accumulation of bivalves on the seafloor could be used as a cheap and efficient monitoring tool for future monitoring of sub-seabed CCS storage sites, e.g. by towing camera systems across large sea floor areas;
- The impact of unequal susceptibility pCO_2 on local food chains requires further investigation as this may lead to fishery issues by indirectly affecting commercially-targeted species.

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9.0 Tables and Figures

Table 1. *Cerastoderma edule* mortality, statistics; (a) results of Kruskal-Wallis analysis. The upper half shows results for cumulative mortality (significances marked in bold letters); the lower half shows statistical results for shell corrosion of dead cockles (significances marked in bold letters); (b, c) results of the Kruskal-Wallis analysis for mortality of different size classes tested against each other. Tables show statistical results for (b) treatment 2 (2,900 µatm) and treatment 3 (6,600 µatm) and (c) treatment 4 (12,800 µatm) and 5 (24,400 µatm). Significances are marked in bold letters.

a		Cummulative mortality	X²=28.8132	p-value= 0.0000	df=5	Critical difference=17.8541	
		900 µatm	1 500 µatm	2 900 µatm	6 600 µatm	12 800 µatm	24 400 µatm
% dissolved	900 µatm		4.3333	0.1667	11.5833	14.2500	**22.3333**
X²=26.7528	1 500 µatm	0.000		4.5000	15.9167	**18.5833**	**26.6667**
p-value=0.0000	2 900 µatm	5.8333	5.8333		11.4167	14.0833	**22.1667**
df=5	6 600 µatm	13.5000	13.5000	7.6667		2.6667	10.7500
	12 800 µatm	**18.3333**	**18.3333**	12.5000	4.8333		8.0833
	24 400 µatm	**22.3333**	**22.3333**	16.5000	8.8333	4.0000	

b		2 900 µatm	X²=10.0954	p-value= 0.0389	df=4	Critical difference=14.2672
		2 - 2.5 cm	1.5 - 2 cm	1 - 1.5 cm	0.5 - 1 cm	0 - 0.5 cm
6 600 µatm	2 - 2.5 cm		2.5000	2.1667	7.8333	11.6667
X²=11.2657	1.5 – 2 cm	2.3333		0.3333	5.3333	9.1667
p-value=0.0237	1 – 1.5 cm	4.9167	2.5833		5.6667	9.5000
df=4	0.5 – 1 cm	4.500	6.8333	9.4167		3.8333
	0 – 0.5 cm	9.4167	11.7500	**14.3333**	4.9167	

c		12 800 µatm	X²=22.858	p-value= 0.0001	df=4	Critical difference=14.2672
		2 - 2.5 cm	1.5 - 2 cm	1 - 1.5 cm	0.5 - 1 cm	0 - 0.5 cm
24 400 µatm	2 - 2.5 cm		0.0000	3.3333	8.6667	**18.0000**
X²=19.1817	1.5 – 2 cm	3.4167		3.3333	8.6667	**18.0000**
p-value=0.0007	1 – 1.5 cm	4.5000	1.0833		5.3333	**14.6667**
df=4	0.5 – 1 cm	13.1667	9.7500	8.6667		9.3333
	0 – 0.5 cm	**19.1667**	**15.7500**	**14.6667**	6.0000	

Table 2. Analysis for bacterial diversity, Bray Curtis similarity, 10,000 permutations, R<0.5 indicates no good separation of groups, significances are indicated by bold numbers; (a) analysis of all samples, R=0.2302; (b) analysis of all samples taken after 6 weeks, R=0.3133; (c) analysis of all samples taken after 12 weeks, R=0.2486; (d) response: Hellinger-transformed ARISA data.

a	900 µatm	1 500 µatm	2 900 µatm	6 600 µatm	12 800 µatm	24 400 µatm
900 µatm	0	0.4665	1	0.066	0	0
1 500 µatm	0.4665	0	1	0.8475	0	0
2 900 µatm	1	1	0	1	0.084	0
6 600 µatm	0.066	0.8475	1	0	1	0.0165
12 800 µatm	**0**	**0**	0.084	1	0	0.0045
24 400 µatm	**0**	**0**	**0**	**0.0165**	**0.0045**	0

b	900	1 500	2 900	6 600	12 800	24 400
900 µatm	0	0.264	1	0.9345	0.249	0.03
1 500 µatm	0.264	0	0.177	0.0645	0.033	0.0285
2 900 µatm	1	0.177	0	0.675	0.447	0.057
6 600 µatm	0.9345	0.0645	0.675	0	1	0.822
12 800 µatm	0.249	**0.033**	0.447	1	0	0.0645
24 400 µatm	**0.03**	**0.0285**	0.057	0.822	0.0645	0

c	900	1 500	2 900	6 600	12 800	24 400
900 µatm	0	1	1	0.093	0.0615	0.033
1 500 µatm	1	0	1	1	0.1425	0.018
2 900 µatm	1	1	0	1	1	0.2055
6 600 µatm	0.093	1	1	0	1	0.1845
12 800 µatm	0.0615	0.1425	1	1	0	0.681
24 400 µatm	**0.033**	**0.018**	0.2055	0.1845	0.681	0

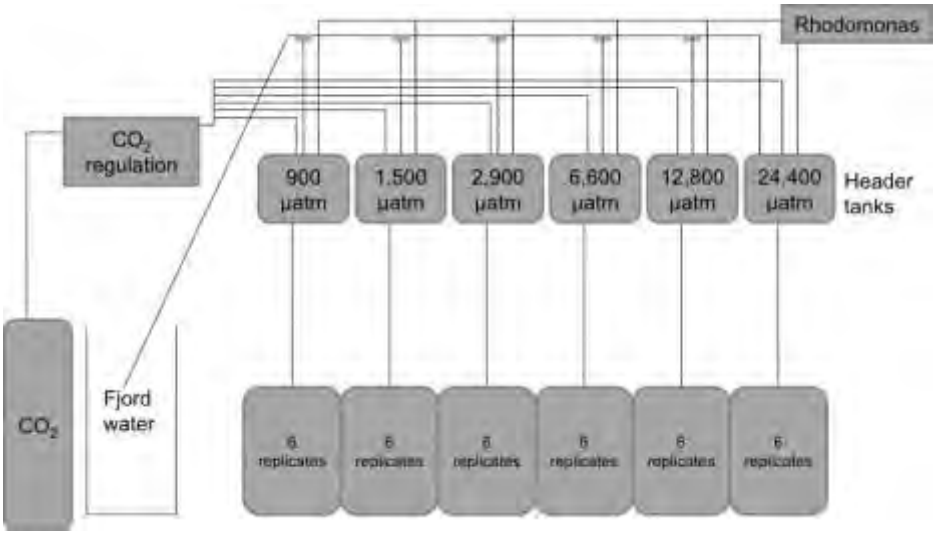
d	df	%variance explained	F	P
Time + Treatment	2	11.7	5.5858	0.001
Time	1	5.0	4.8737	0.001
Treatment	1	6.9	6.3053	0.11
Time*Treatment	1	1.3	1.0342	0.38

Table 3. Seawater physiochemical conditions of the four different hypercapnia experiments.

	pH (NBS)	Temperature (°C)	Salinity	Alkalinity (mmol)	pCO_2 (µatm)	TCO_2 (mmol)
	measured				calculated	
Experiment 1						
	7.93 ± 0.02	8.70 ± 0.27	31.20 ± 0.10	2.18 ± 0.27	695.78 ± 39.81	2.09 ± 0.27
	7.03 ± 0.03	8.60 ± 0.22	31.16 ± 0.09	2.18 ± 0.32	6347.76 ± 556.30	2.44 ± 0.35
Experiment 2						
	8.06 ± 0.05	10.23 ± 0.90	31.19 ± 0.07	2.22 ± 0.02	526.34 ± 19.01	2.10 ± 0.02
	7.29 ± 0.04	10.17 ± 0.95	31.22 ± 0.06	2.23 ± 0.04	3395.83 ± 236.59	2.36 ± 0.05
	7.00 ± 0.04	10.25 ± 0.86	31.17 ± 0.06	2.20 ± 0.03	6643.84 ± 185.20	2.49 ± 0.02
Experiment 3						
	8.04 ± 0.08	8.47 ± 0.14	31.57 ± 0.17	2.25 ± 0.04	492.46 78.79	2.13 0.05
	7.63 ± 0.07	8.42 ± 0.16	31.46 ± 0.15	2.27 ± 0.02	1473.40 241.60	2.28 0.02
	7.30 ± 0.03	8.42 ± 0.18	31.64 ± 0.08	2.29 ± 0.02	3213.10 218.46	2.40 0.02
Experiment 4						
	7.99 ± 0.08	11.10 ± 0.14	31.55 ± 0.35	2.20 ± 0.04	614.60 ± 126.43	2.09 ± 0.01
	7.01 ± 0.01	11.05 ± 0.07	31.65 ± 0.49	2.19 ± 0.01	6399.70 ± 252.10	2.46 ± 0.03

Table 4 Primer sequences used for qRT-PCR.

Gene	Abbreviation	Function	Primer Sequence 5'-3' (forward and reverse)	Amplicon length
Ion regulation				
Na/H-exchanger 3 isoform	NHE3/fr	Regulation of NHE3	F GAAAAAGGAAAACACGGACAATTTATAC R CCTGGTTTCACTCCCGCTAA	77
V-type H-ATPase subunit V0	VHAV0	Active proton secretion	F AACAATTCCACTATGGCGTCTTCT R GCTACGCATTCTGCTATCCATACTAT	66
Na/HCO3 cotransporter	NBCe	Secondary active ion transport	F CCCAAGTTGTCGGCTGTAGAG R GCATTGGAATAACCAGTAATAATTTGTC	104
Na/K-ATPase	NKA	Electrochemical gradient	F CATGCCACACTGCTTTCTTTG R GTGAGTTCTTCCTGGTCTTGCA	81
Aquaporin 3	AQP3	Channel protein	F GATGAACGGCTCCATACCATCT R TTGGCACACTTCTACTGATGCTTT	86
Metabolism				
Lactate dehydrogenase	LDH	Anaerobic metabolism	F ATGTGTTCAGCCAGGAGTAACAAG R GCACCAGGATCTATAGGAAAGCAT	84
Glucose-6-phosphate dehydrogenase	G6PDH	Glycolysis	F GGTTCCACCGCCAAAAATATC R TAAAGCCTTAAACGAACGTAAAGCA	79
Succinate dehydrogenase	SuDH	Respiratory chain/Krebs cycle	F GAACAGTTTGCTACGTGCGAAT R CAACATGGCAGGCATGTTTT	75
Amino acid catabolism				
Glutamate dehydrogenase	GlutDH	Protein metabolism	F GGTGCTACGGAGAAGGATATAGTGA R TGCTGTTCTCATAATTTGCCTAGCT	84
Alanine transaminase	AlaT	Protein metabolism	F TGAAGAGAGTGGTTGGAGTCTACAGA R CTACCATAGCAACTGGTTTACAGTGTTT	89
Aspartate transaminase	AspT	Protein metabolism	F CATGGGTCTCTATGGTGAACGA R GTGACTCACATCTCTTGGCTTCAT	80
Reference gene				
Ubiquitin-conjugated enzyme	UCE	Protein degradation	F TTTCACAACTAAGATCTATCATCCAAACA R TGGTGACCACTGTGACCTCAAG	82



a.

b.

Figure 1. (a) Schematic illustration of the experimental set up; six header tanks with six different CO_2 levels generated through pH controlled CO_2 addition (IKS Aquastar); *Rhodomonas sp.* supplied to six header tanks; six replicates for each treatment; (b) Image of the experimental set- up in a temperature – controlled climate chamber at GEOMAR, with header tanks (left) supplying the experimental units (bottom) with CO_2 enriched water and food algae.

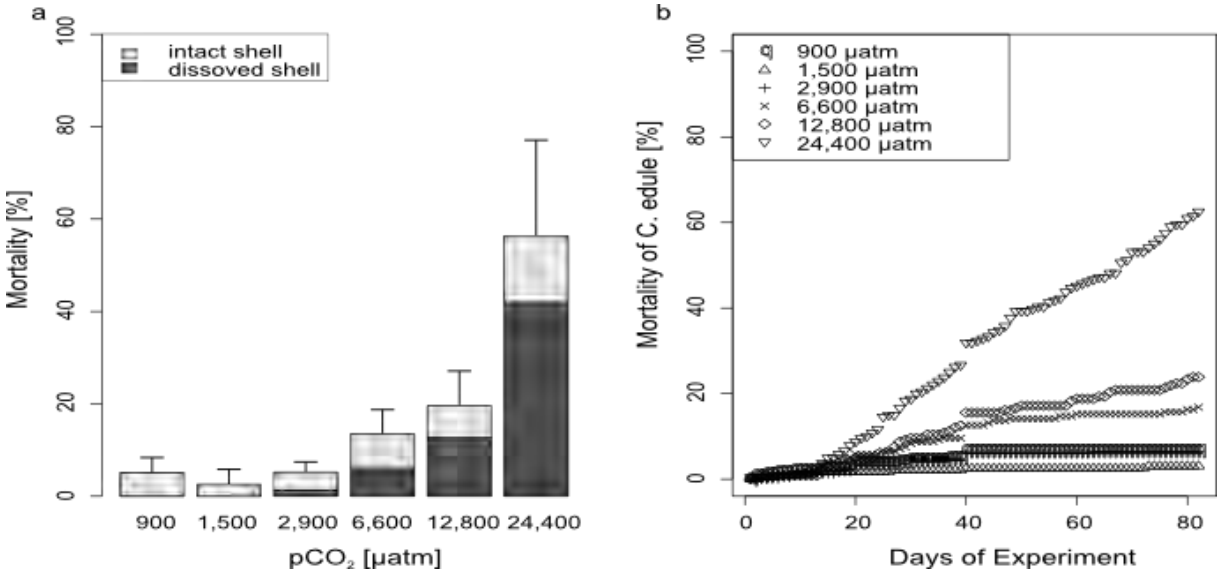


Figure 2. (a) Bars showing *Cerastoderma edule* mortality over the entire experimental duration (mean ±SD); color coding showing fraction of corroded vs. intact shell (white=intact, grey=corroded). (b) Cumulative mortality plotted over the duration of the experiment. The first sampling after 6 weeks leads to the break in the mortality curves; 50% mortality in treatment 5 was reached at day 68.

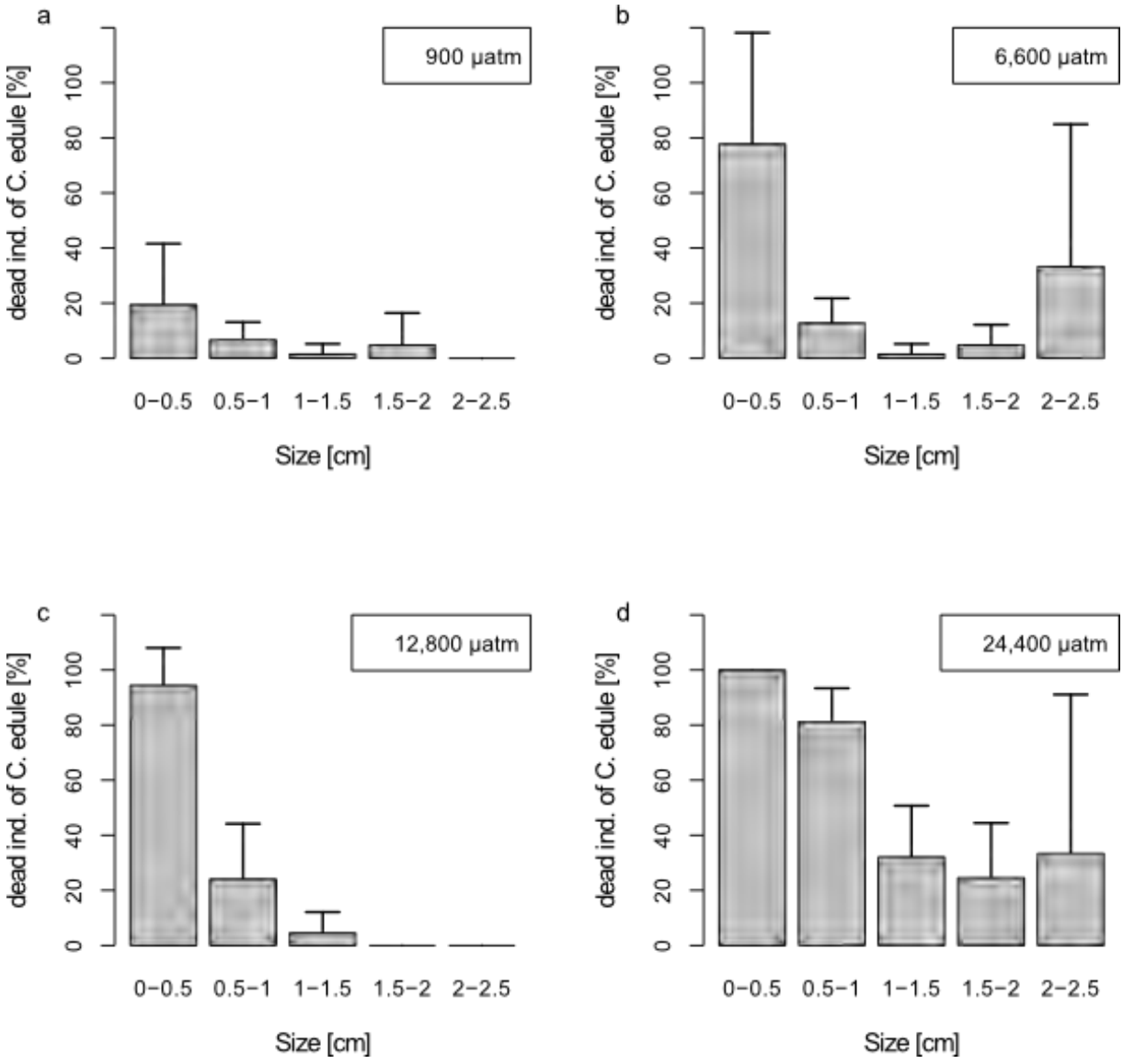


Figure 3. Mortality of different *C. edule* size classes during the experiment for the control (a), treatment 3 (6,600 µatm) (b), 4 (12,800 µatm) (c) and 5 (24,400 µatm) (d).

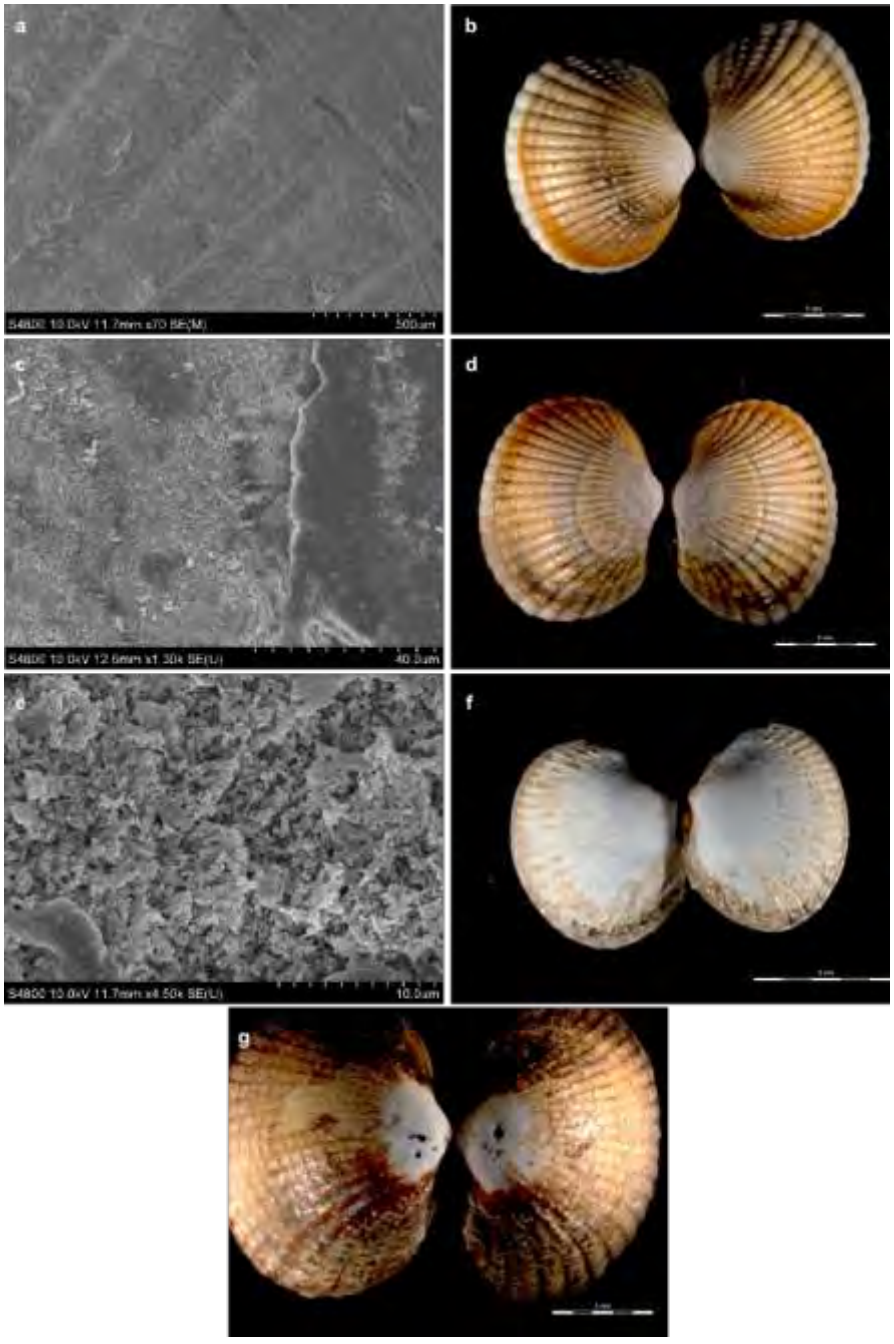


Figure 4. Shell corrosion in *Cerastoderma edule*. (a, b) control (900 µatm), no shell corrosion visible on the outside of the shell. (a) SEM image, scale bar 500 µm; (b) Stereo microscopic image, scale bar 5 mm; (c, d) Treatment 1 (1,500 µatm), shell corrosion on the outside of the shell. (c) SEM image; Scale bar 40 µm, (d) stereo microscopic image, scale bar 5 mm; (e, f) Treatment 3 (6,600 µatm), shell dissolution on the outside of the shell. (e) SEM image, scale bar 10 µm, (f) stereo - microscopic image scale, bar 5 mm; (g) Treatment 5 (24,400 µatm), strong dissolution signs, holes. Stereo microscopic image, scale bar 5 mm.

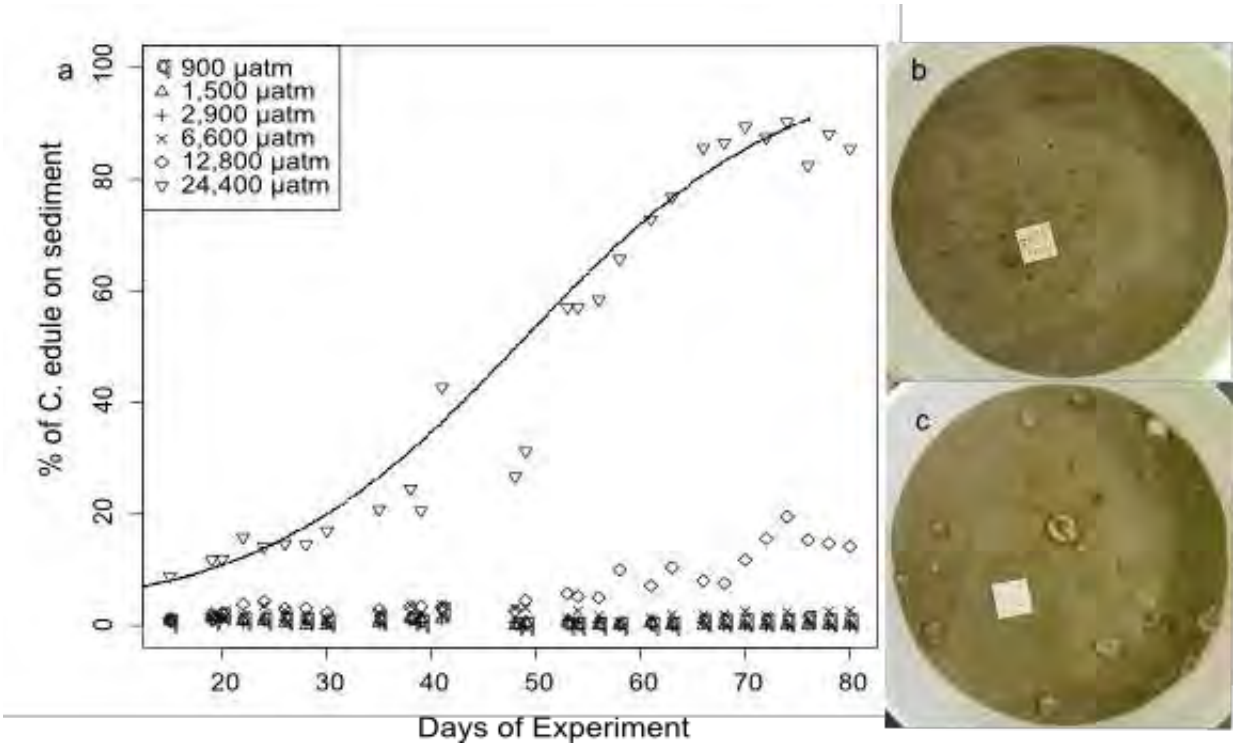


Figure 5. *Cerastoderma edule* behavior. (a) Average abundance of non-buried *C. edule* over the complete experimental phase in % of total, curve fitted for treatment 5 including 95% confidence interval; (b) image of control experimental unit, sediment surface with siphos opened and visible, no *C. edule* on the sediment surface; (c) Treatment 5, accumulation of *C. edule* on the sediment surface towards the end of the experiment.

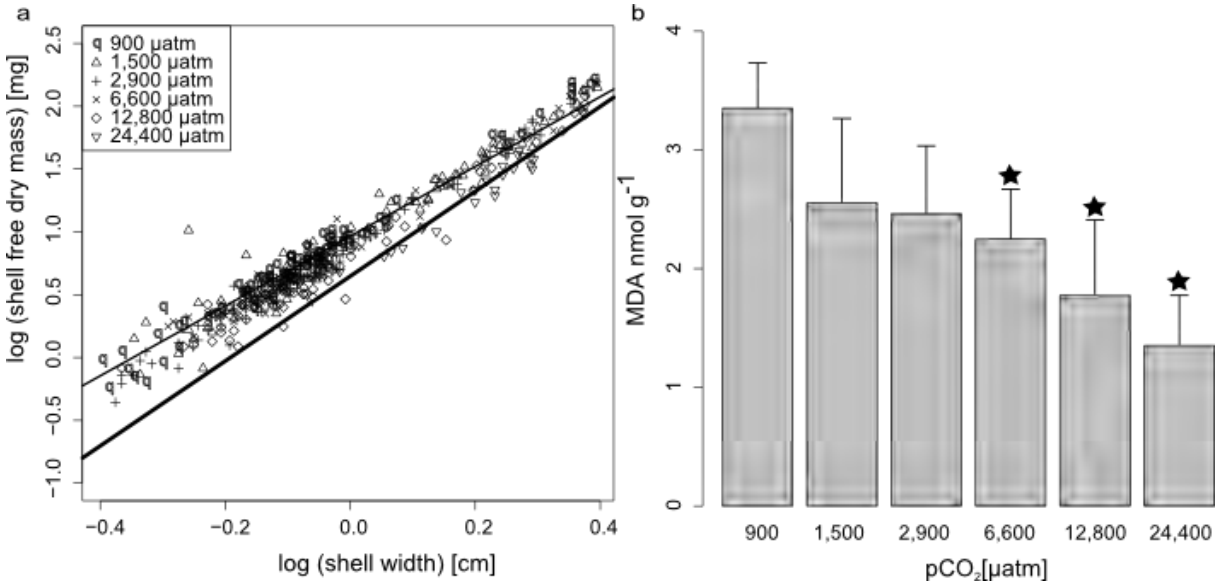


Figure 6. *Cerastoderma edule* condition and MDA accumulation. (a) log shell free dry mass plotted versus log shell mass. Regression line plotted for the control (thinner line) and treatment 5 (thicker line). (b) average of whole body MDA content [nmol g^{-1}] for the different treatments. Means and standard deviation, stars indicate significant differences with respect to the control.

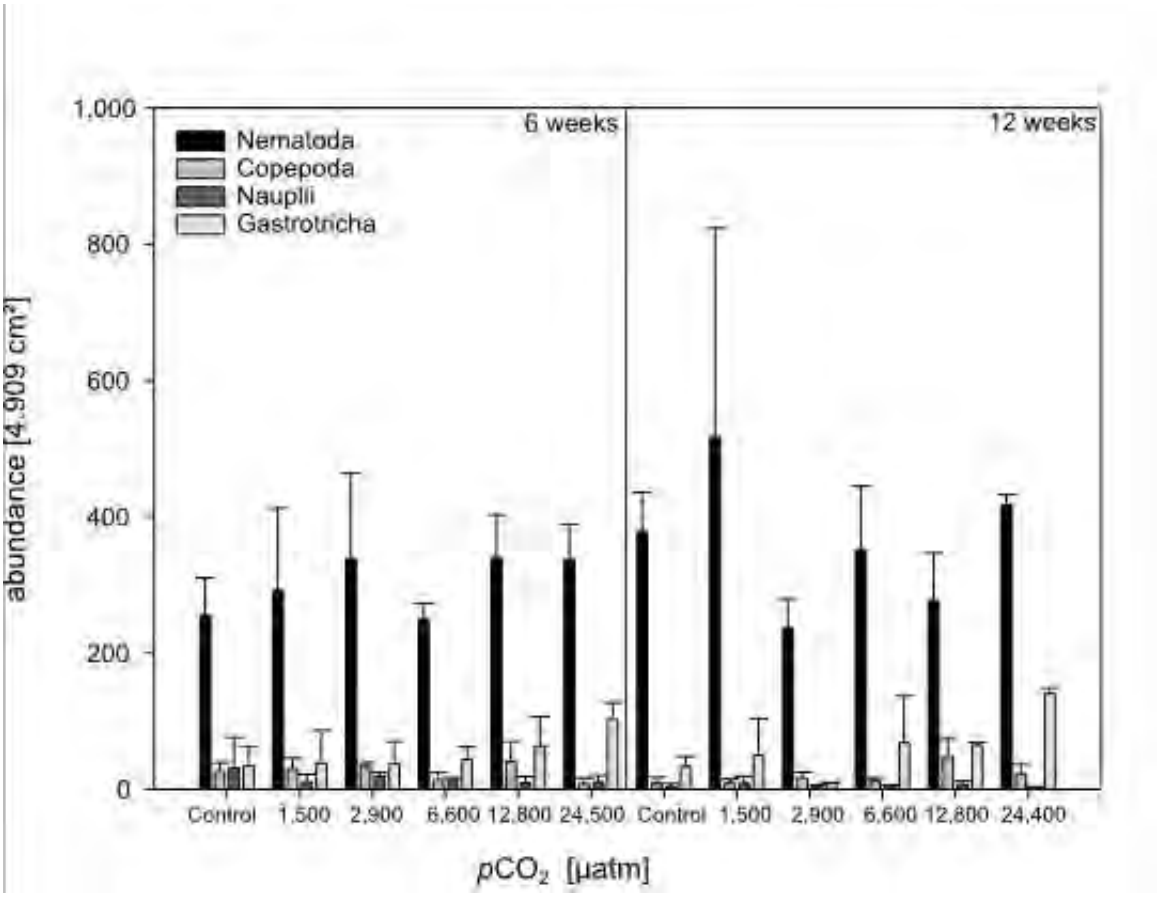
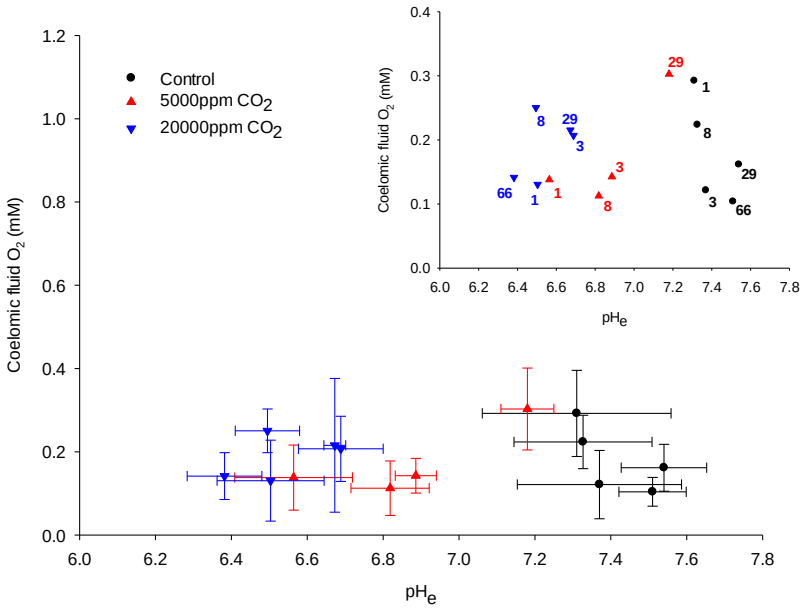
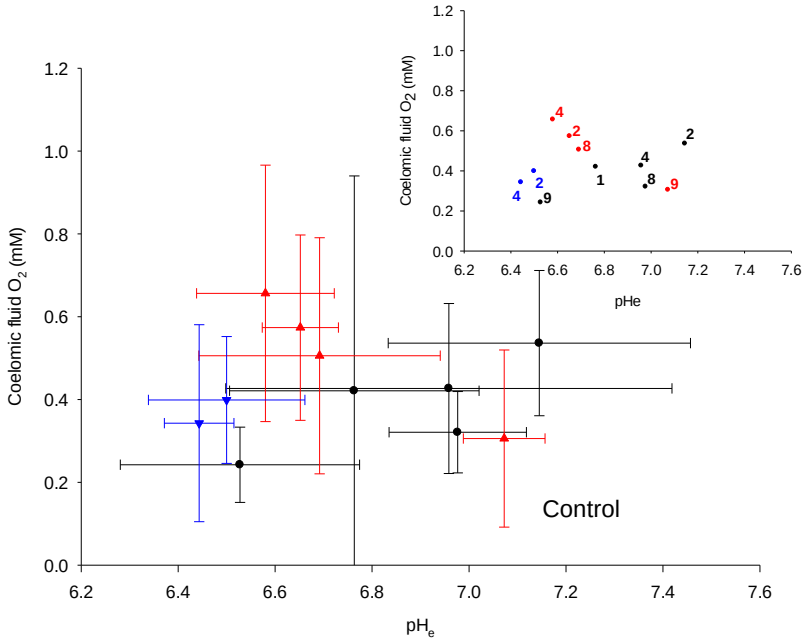


Figure 7. Meiofauna abundance, main groups. Samples were taken after 6 weeks (left) and 12 weeks (right). Means and standard deviation.



a.



b.

Figure. 8. Coelomic fluid O_2 content in relation to extracellular pH (pHe) of *Paracentrotus lividus* to elevated pCO_2 exposures. Data points are means ± SD for each time point. (a.) 2 month exposure to elevated pCO2, (b.) 7 day exposure to elevated pCO_2. Inset shows means with date of each measurement.

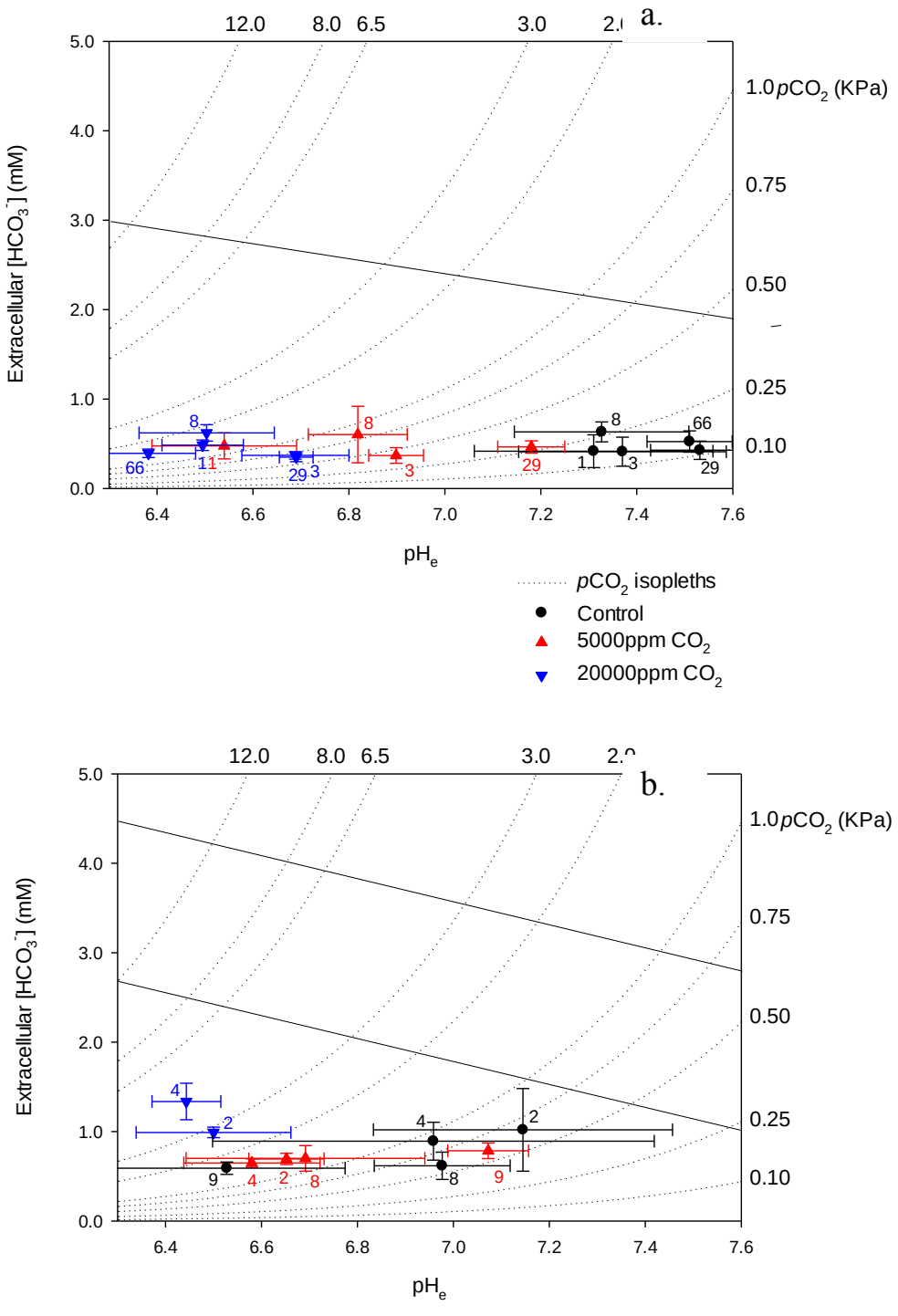


Figure. 9. Davenport diagrams showing the acid base response of the coelomic fluid of *P. lividus* to elevated pCO_2 exposures. Data points are means ±SE for each time point. (a.) 2 month exposure to elevated pCO2, (b.) 7 day exposure to elevated pCO_2.

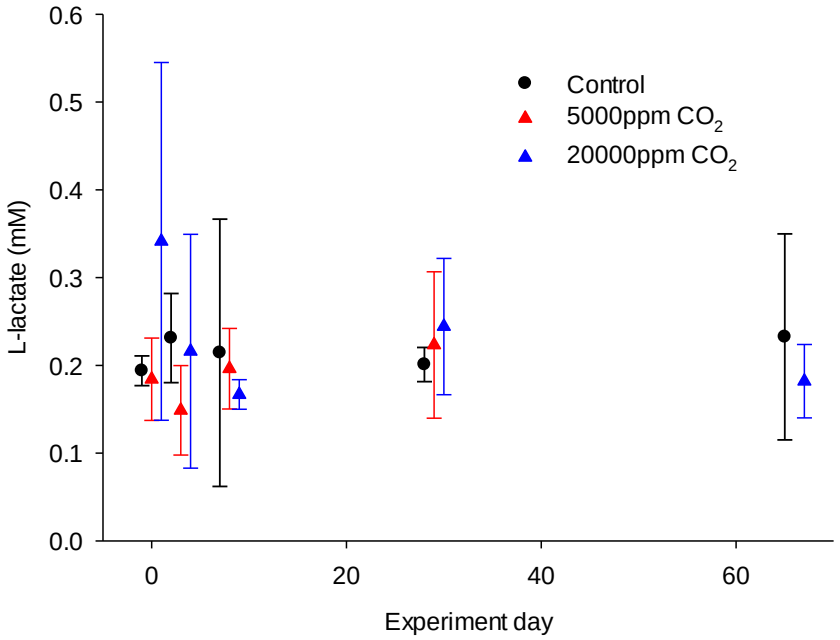


Figure 10. Concentration of L-lactate in coelomic fluid of *Paracentrotus lividus* exposed to elevated pCO_2 for a period of approximately two months (mean ± SD).

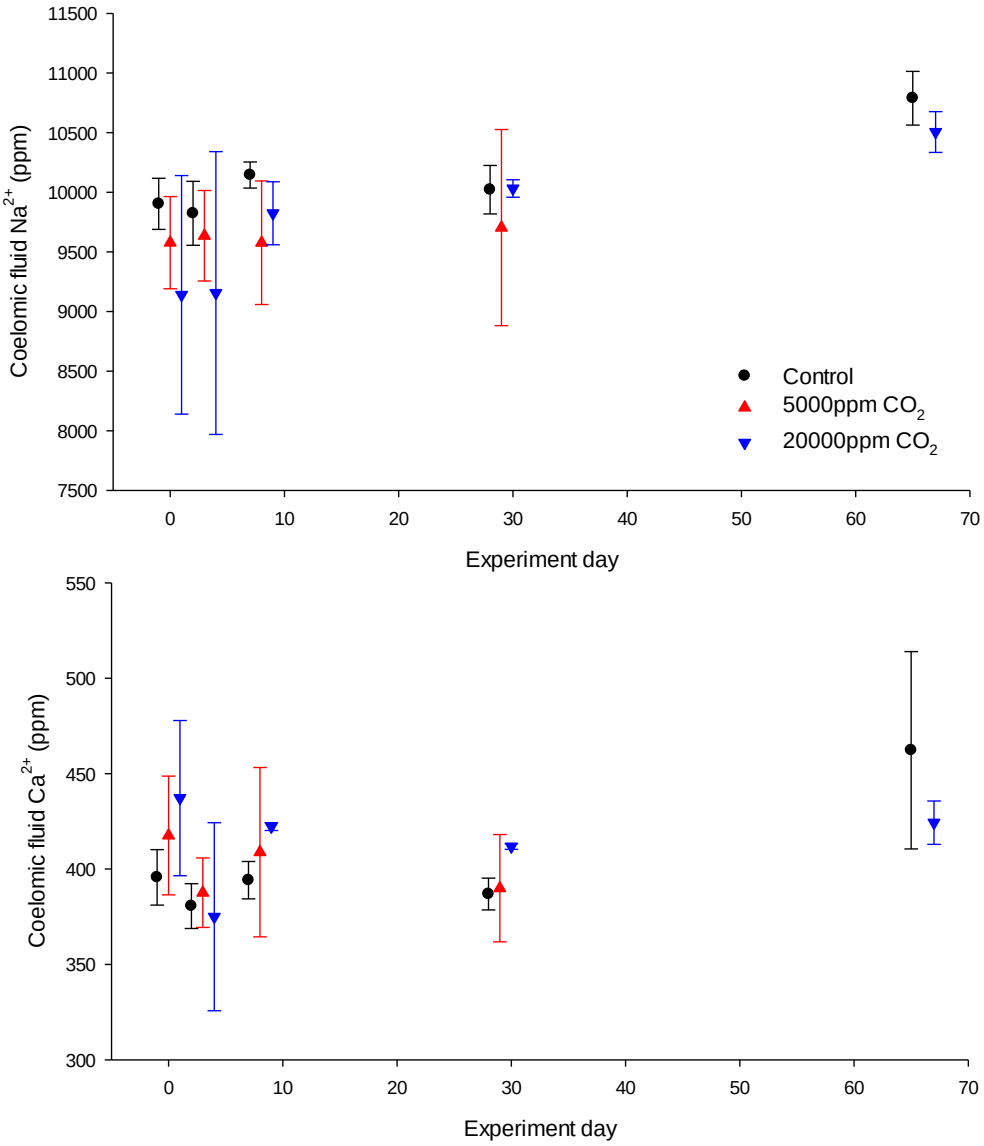


Figure. 11. Changes in coelomic fluid calcium (Ca^{2+}) and sodium (Na^{+}) of *Paracentrotus lividus* with time during the two month exposure to elevated pCO2 (nominally 20000ppm), showing the mean ± SD.

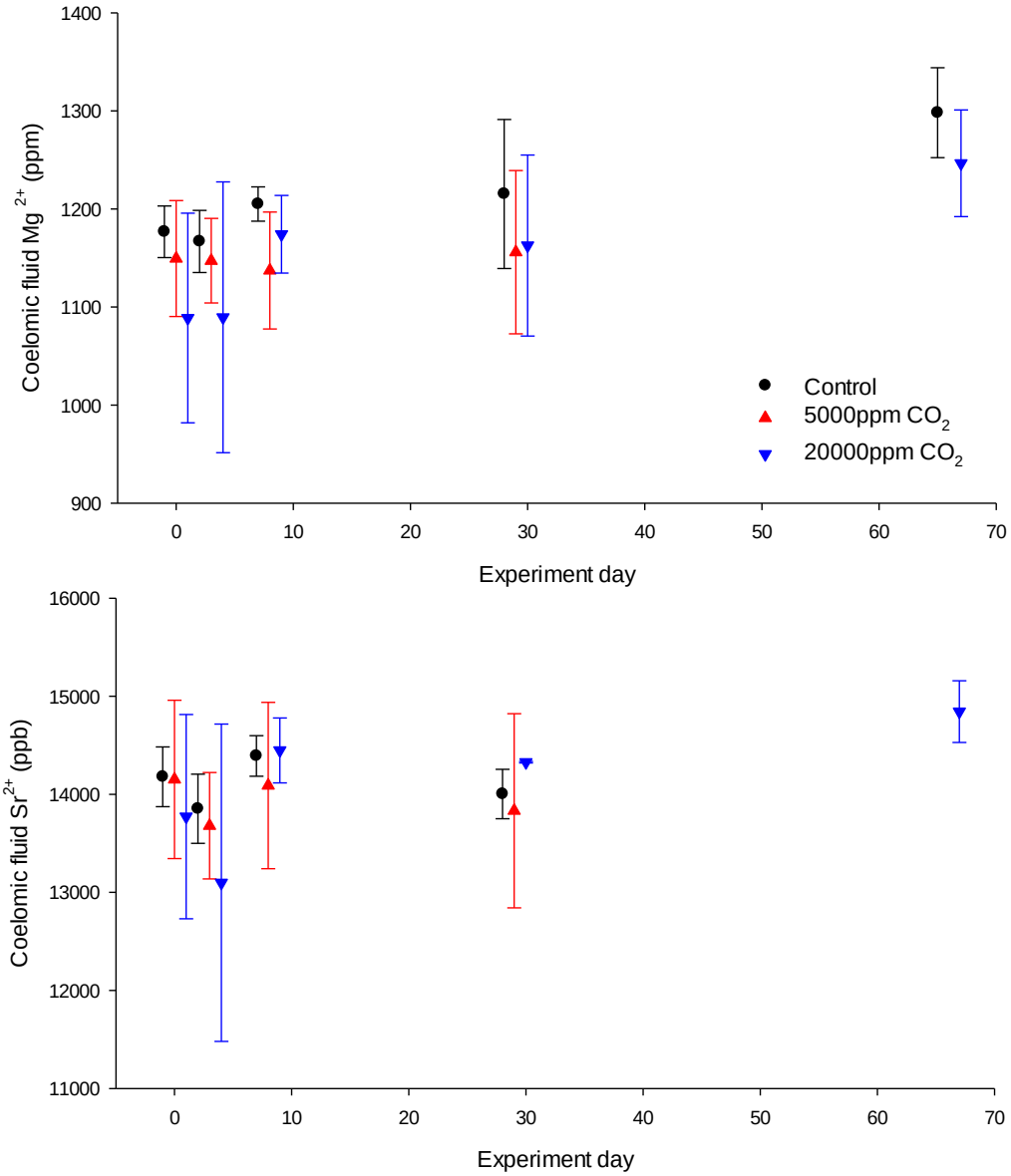


Figure. 12. Changes in coelomic fluid calcium (Mg^{2+}) and sodium (Sr^{+}) of *Paracentrotus lividus* with time during the two month exposure to elevated pCO2 (nominally 20000ppm), showing the mean ± SD.

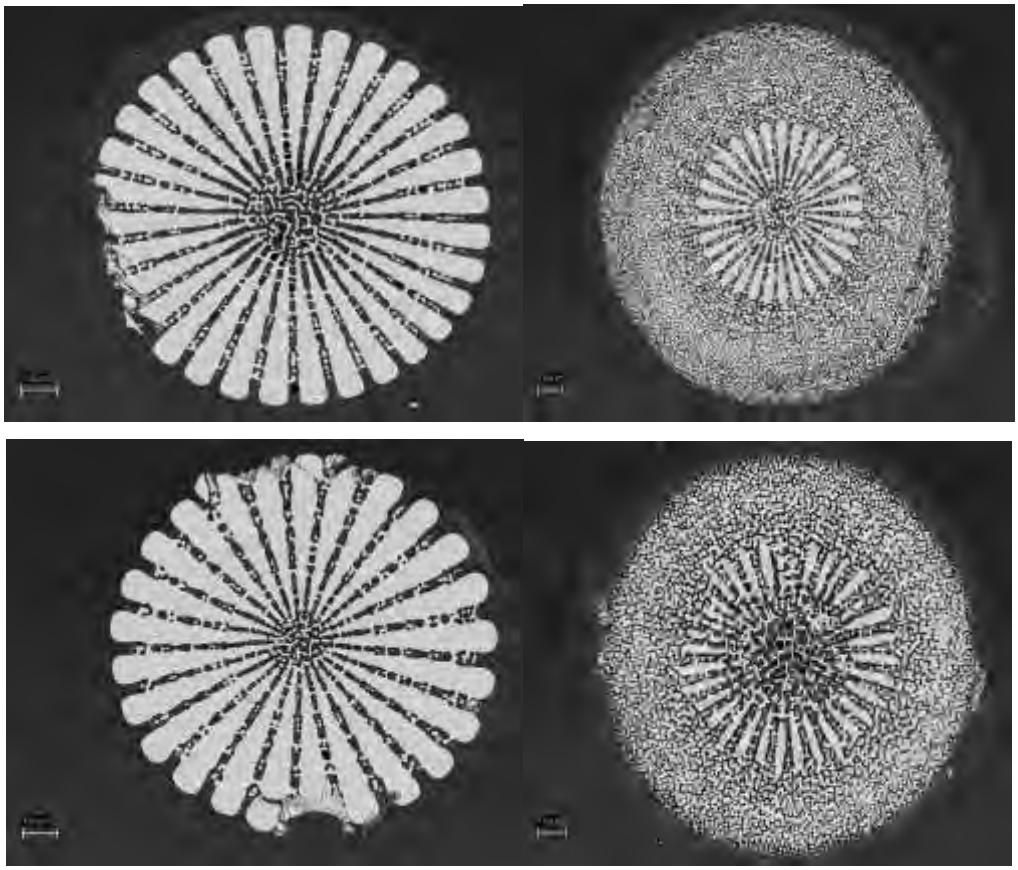


Figure 13. Cross sectional SEM images of *Paracentrotus lividus* spines, illustrating spine dissolution in acidified spine samples from urchins exposure to 20,000 ppm for 2 months (right hand side) compared to urchins maintained in control aquarium for the same period (left hand side). Scale bar = 100 μm.

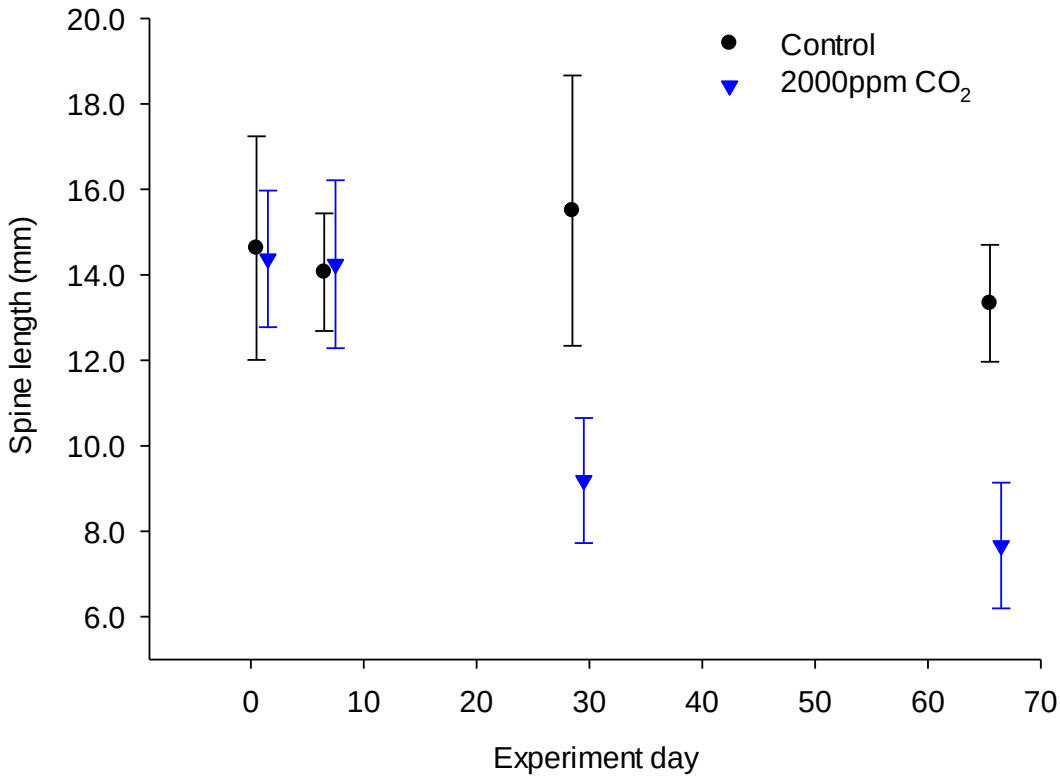


Figure 14. Changes in mean (± SD) spine length of *Paracentrotus lividus* incubated in the control and nominally 20000ppm CO_2 for 67 days, showing a significant decrease in spine length in the treatment group.

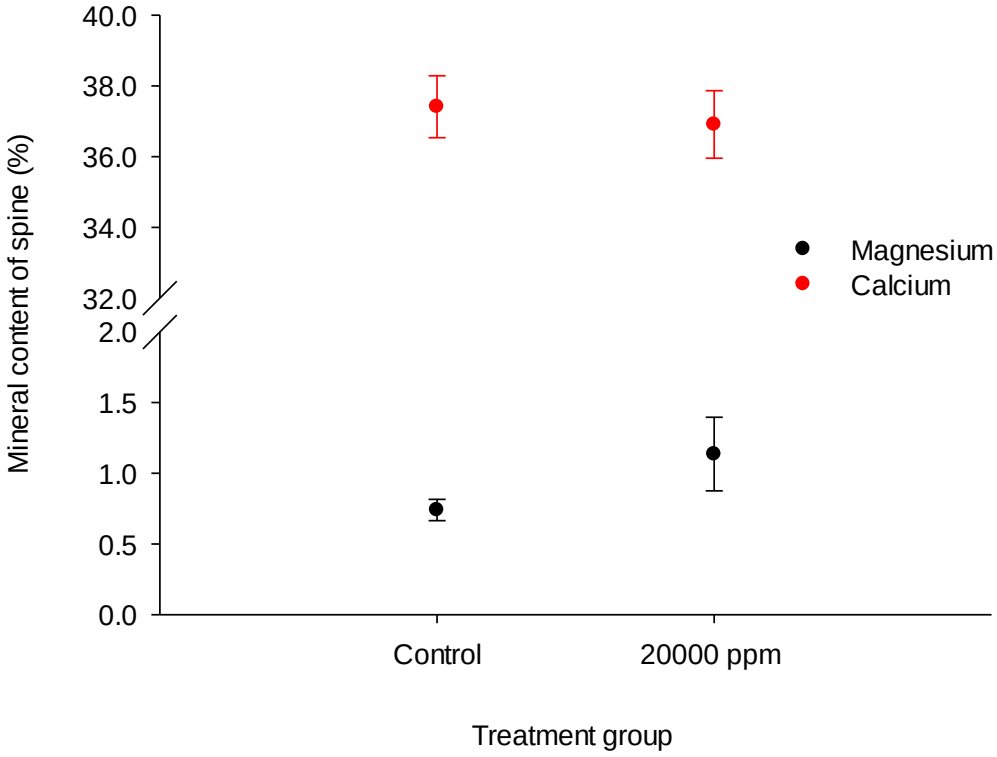


Figure 15. Changes in proportions of calcium and magnesium in the spines of *Paracentrotus lividus* incubated in the control and nominally 20000ppm CO_2 for 67 days, showing a slight but significant increase in the % of magnesium in the control group.

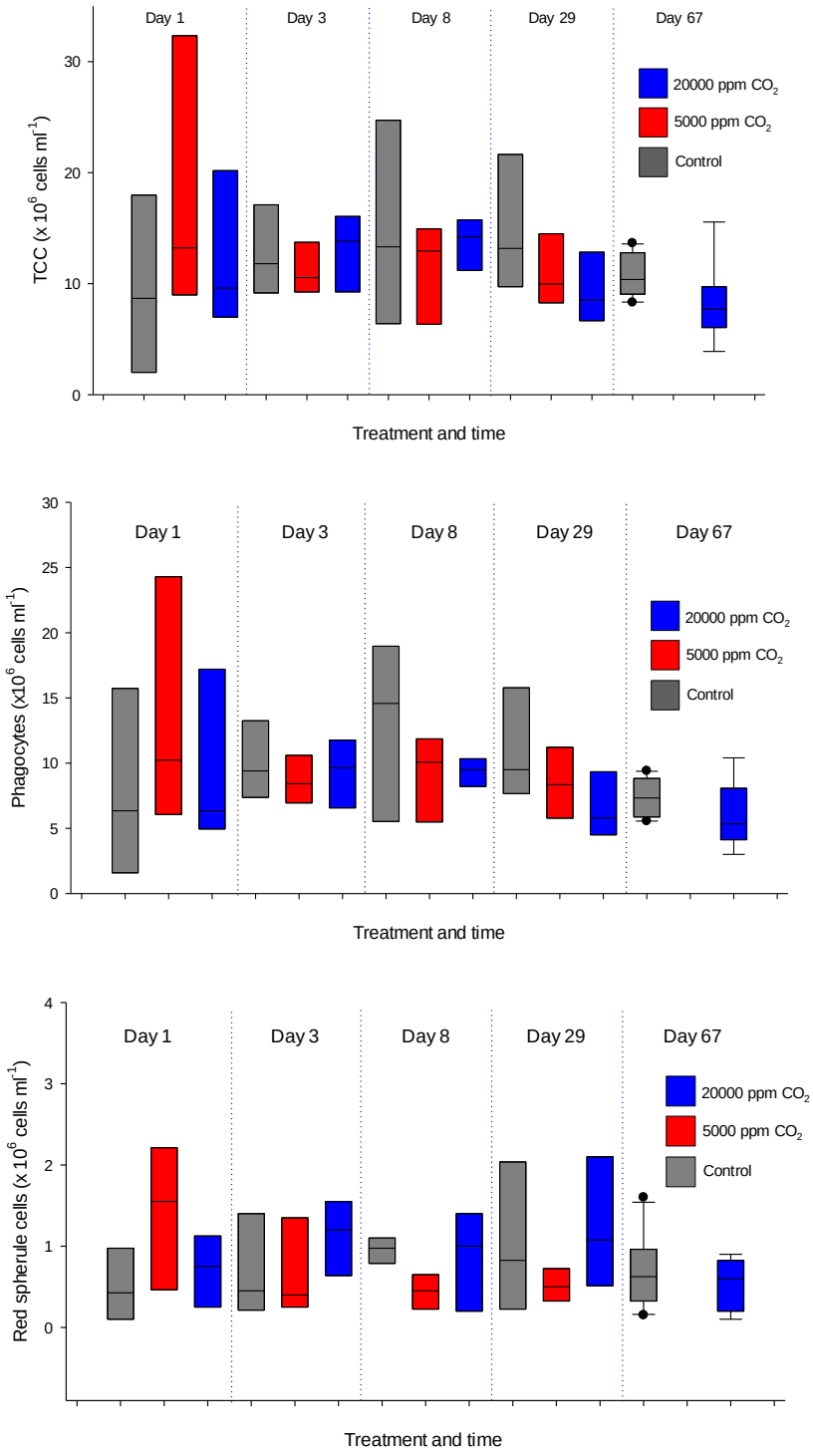


Figure 16. Box and whisker plots of differential coelomocyte counts in Paracentrotus lividus exposed to 5000 and 20000 ppm CO_2 showing no consistent or significant changes in total coelomocytes (TCC, top), phagocytes (middle) and red spherule cells (bottom) with time or treatment.

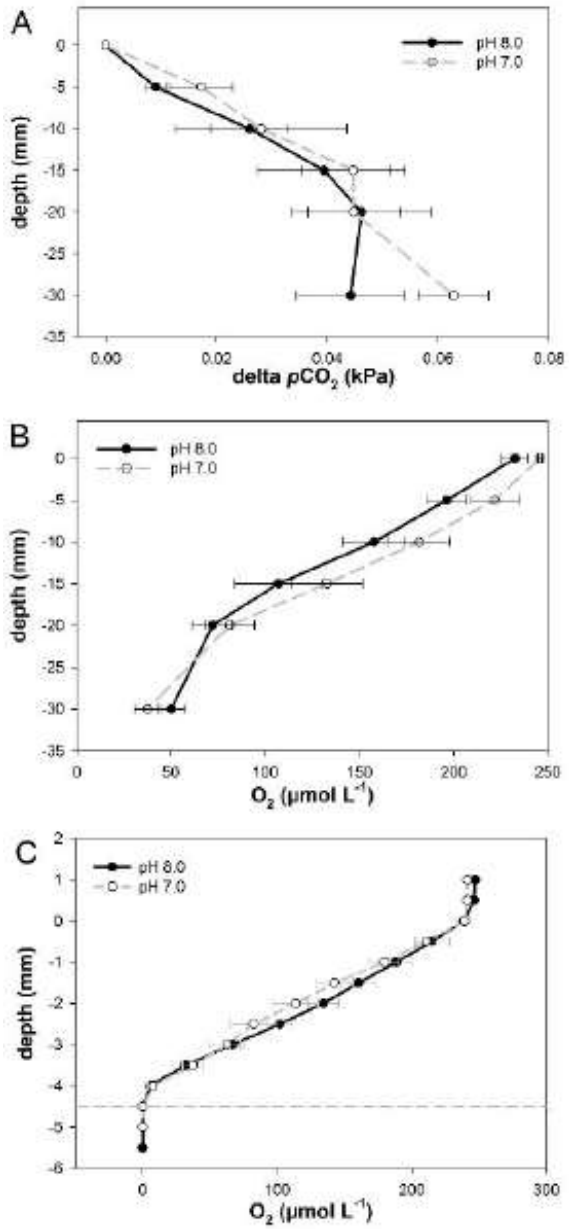


Figure 17. Abiotic parameters in *Amphiura filiformis* burrows. pCO_2 profiles (delta pCO2 relative to pCO2 at 0 mm depth) in burrows of *Amphiura filiformis* (A). CO_2 concentrations were calculated from total alkalinity and pH measured in different depth and pH treatments. Values are given as means ± SE (n=4). Oxygen profiles in burrows of *Amphiura filiformis* (B) and in the sediment surface (C). Oxygen concentrations were measured in different depth and CO_2 treatments. Values are given as means ± SE (n=4).

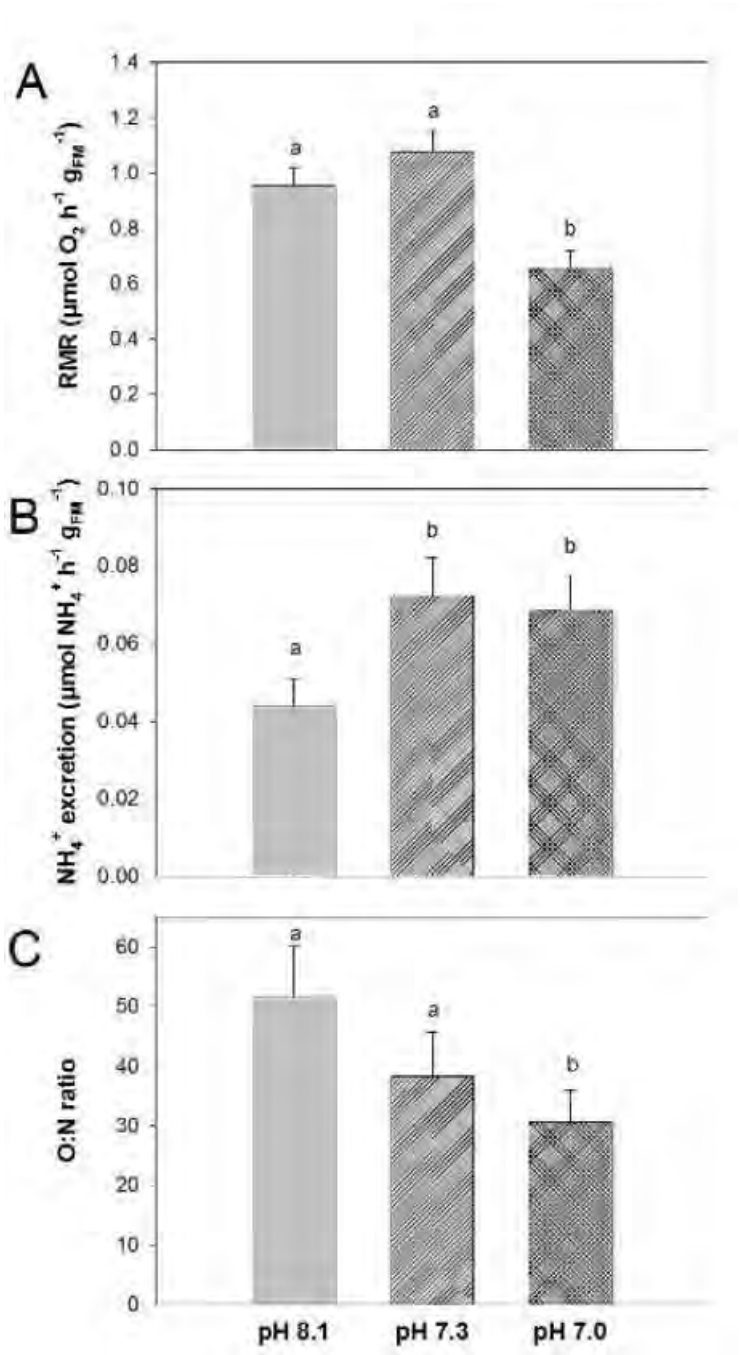


Figure 18. Effects of seawater acidification on metabolism and $NH4^+$ excretion. Routine metabolic rates (RMR) and $NH4^+$ excretion rates in *Amphiura filiformis* exposed to three pH levels over a period of 4 weeks. (A) routine metabolic rate, (B) NH_4^+ excretion rate and (C) O:N ratio. Values are given as means ± SE. Significant differences between treatments are presented by different letters (One-Way-ANOVA, $p<0.05$, $n=4$).

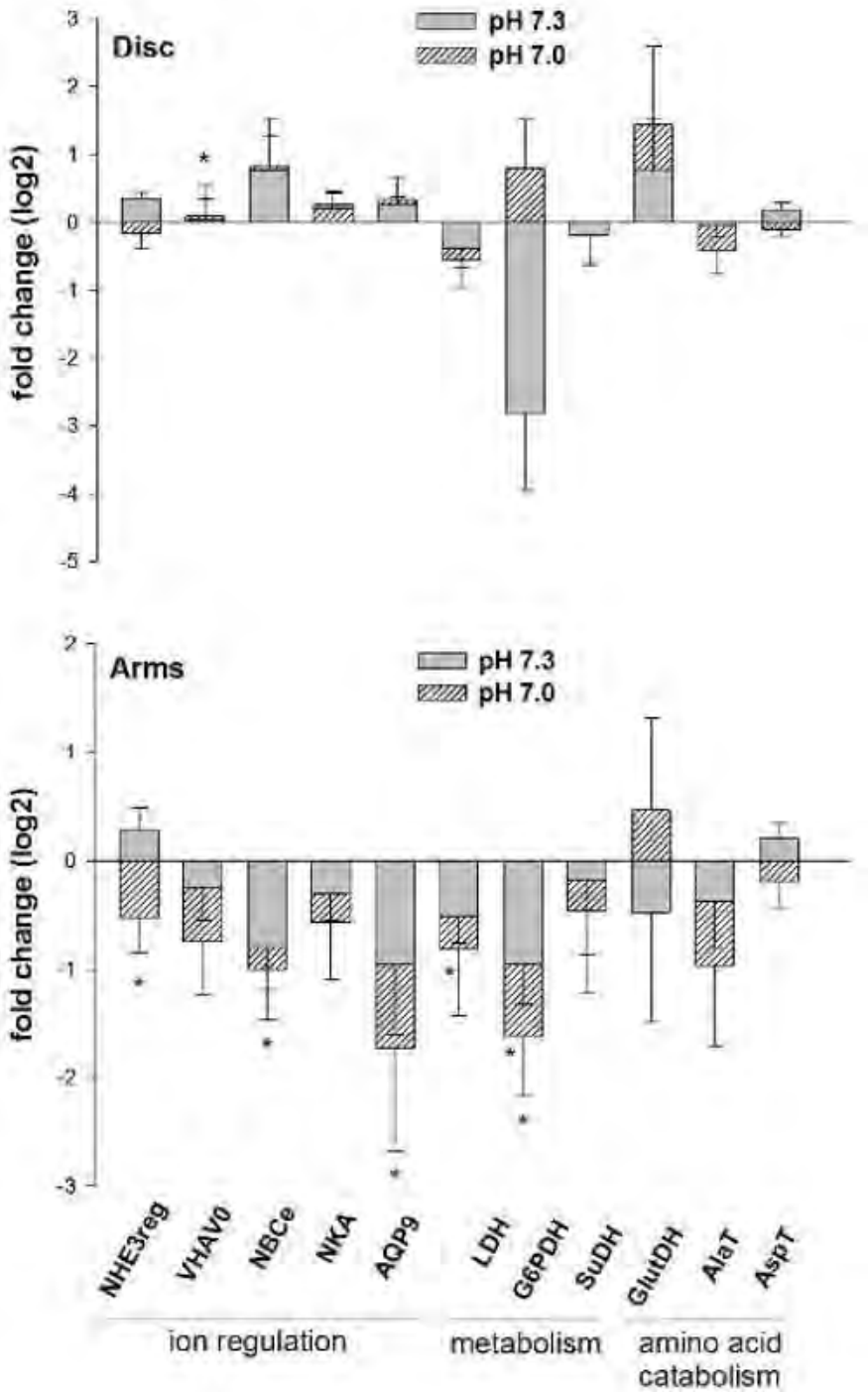


Figure 19. Gene expression profiles in disc and arm tissues. Expression profiles from selected genes determined for disc and arm tissues of *Amphiura filiformis* after exposure to three different pH levels (pH 8.1, 7.3 and 7.0) for 4 weeks. Expression of the gene candidates were normalized to UCE. Asterisks denote a significant change (One- Way-ANOVA, $p<0.05$) in gene expression ($n = 4$).

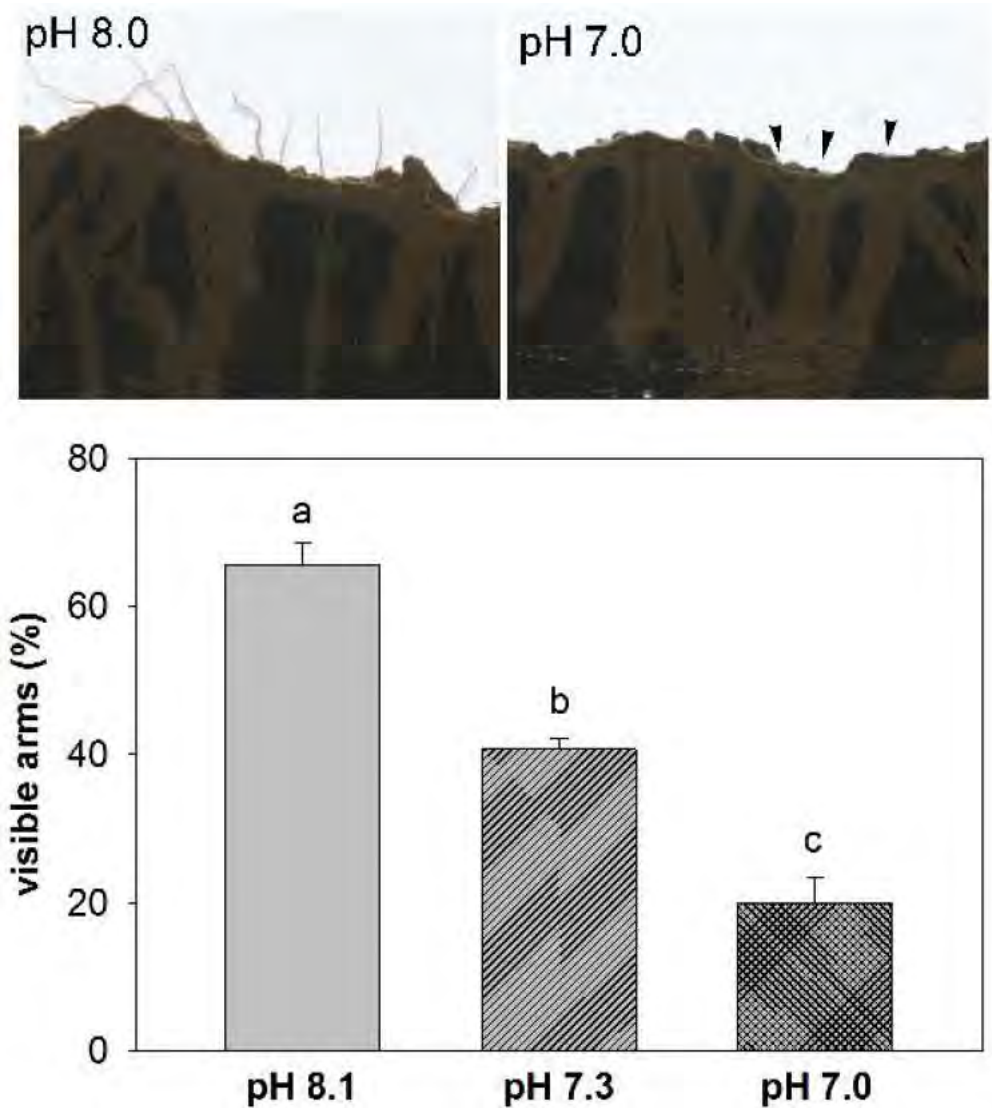


Figure 20. Amount of arms visible during the incubation time of 4 weeks in the different pH treatments. Photographs taken in "thin" aquaria of pH 8.1 and pH 7.0 treated animals after two weeks incubation time. Arrows indicate position of visible arms lying on the sediment surface in the low pH treatment. Values are given as means ± SE. Significant differences between treatments are presented by different letters (One- Way-ANOVA, $F = 65.202$, $p < 0.001$, followed by *post-hoc* Holm-Sidak test, $n = 4$).

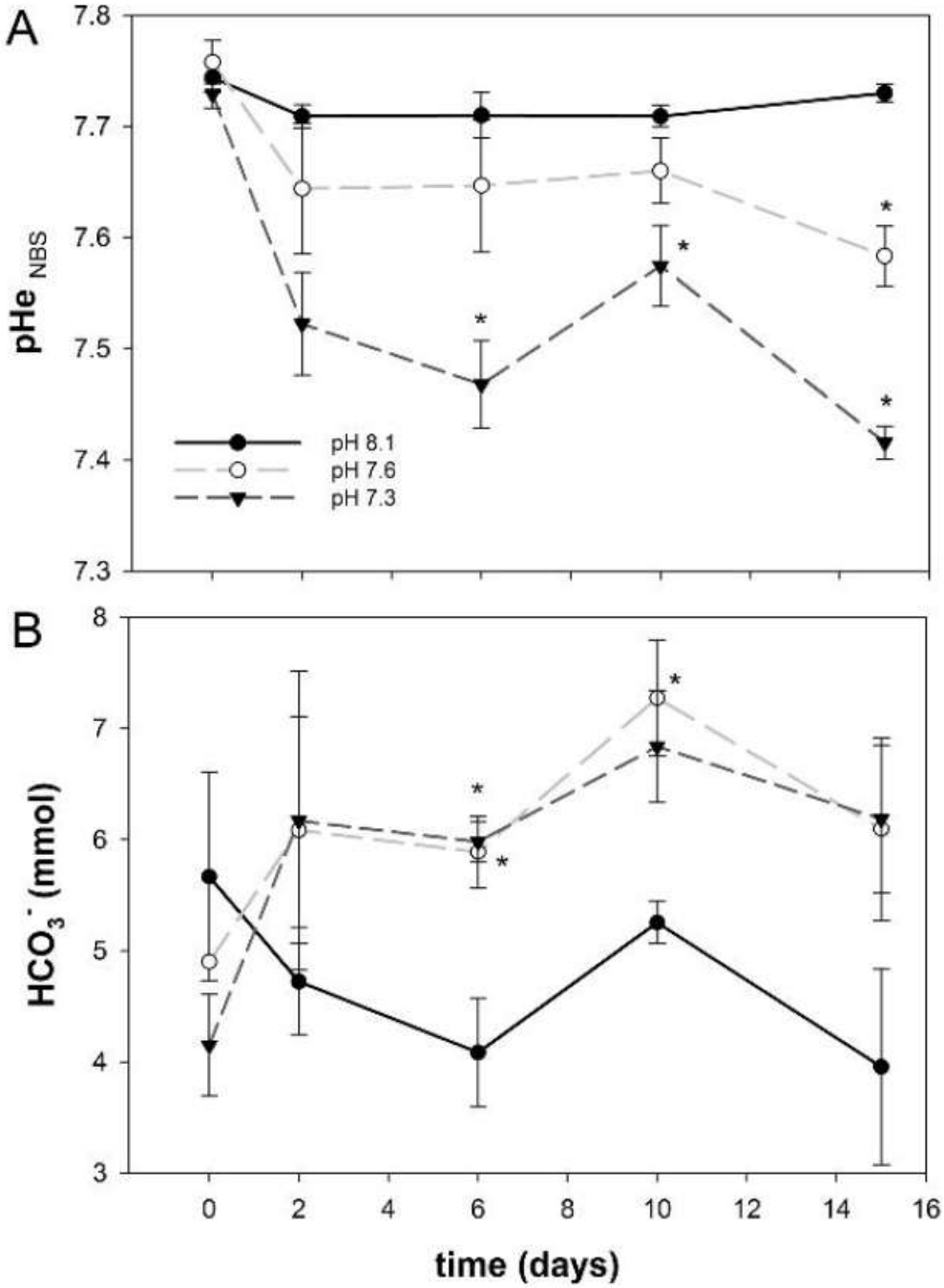


Figure 21. Extracellular acid-base status during hypercapnia treatment in the coelomic cavity of *Amphiura filiformis*. Animals were exposed to three different pH levels (pH 8.1, 7.6, 7.3) for the duration of 15 days. Extracellular pH (A) and body fluid HCO3- levels (B) were determined before the start of the experiment and at 4 time points along the 1 incubation period. Asterisks denote significant differences between treatment groups (One-Way-ANOVA, $p<0.05$). Values are given as means ± SE (n = 6).

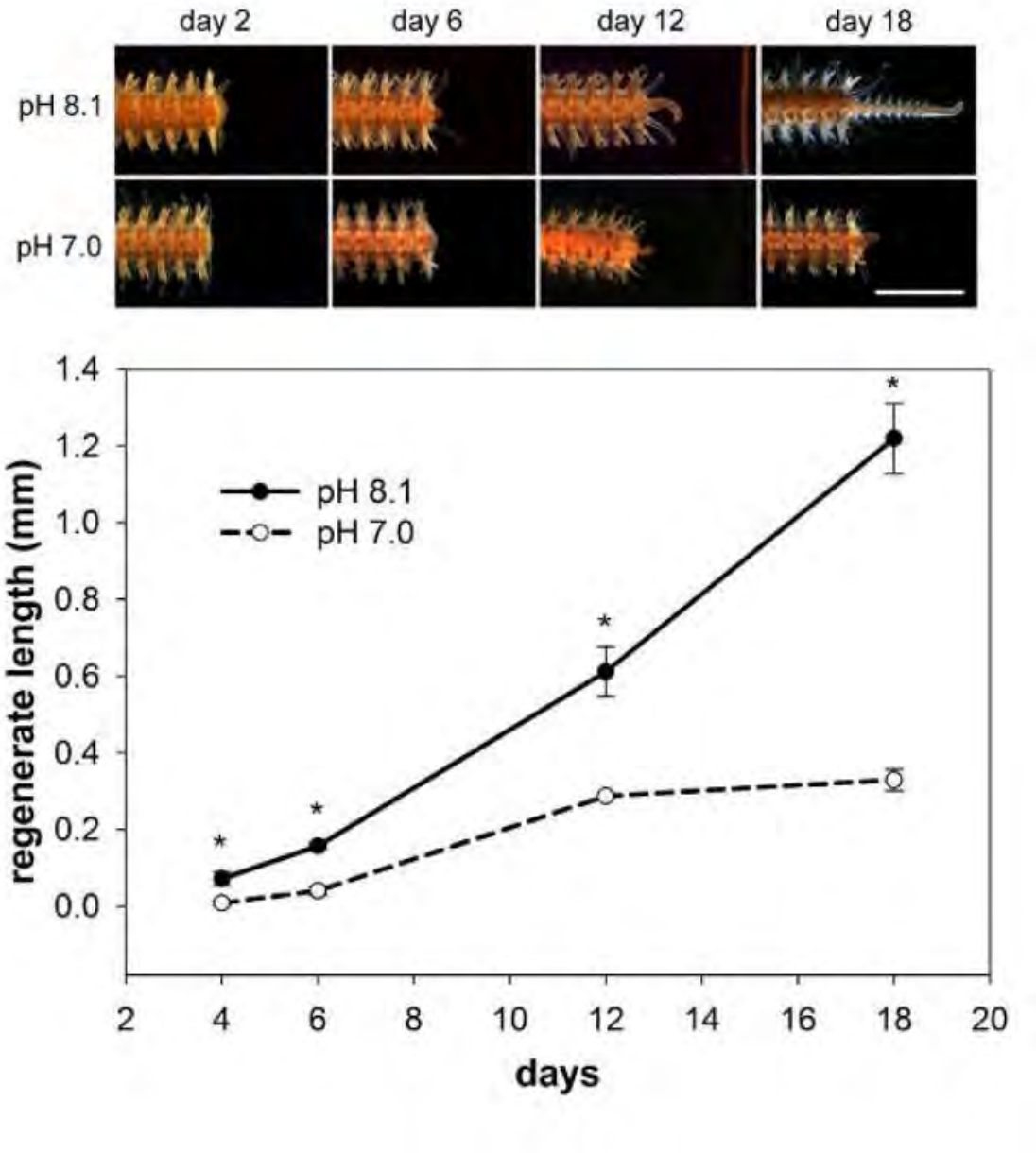


Figure 22. Effects of seawater acidification on regeneration. Regeneration during pH 8.1 and pH 7.0 treatments along the experimental period of 18 days. Images showing sequential regeneration and regenerate length for animals exposed to pH 8.1 and pH 7.0 conditions. Values are given as means ± SE (n=4-6). Asterisks denote a significant difference between treatments ($p<0.05$).

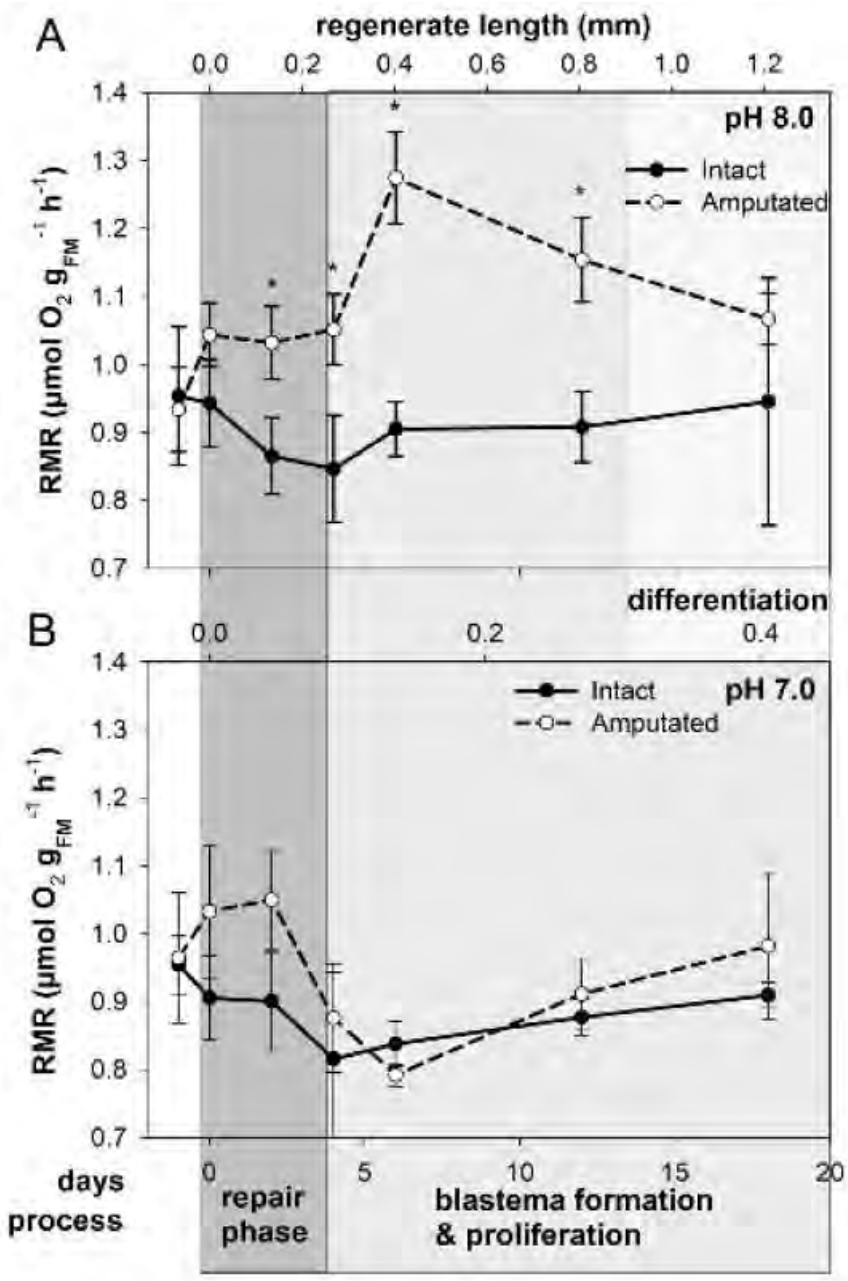


Figure 23. Metabolic rates during regeneration under acidified conditions. Comparison of routine metabolic rates (RMR) along a regeneration period of 18 days between *Amphiura filiformis* exposed to pH 8.1 (A) and pH 7.0 (B) conditions. Non amputated animals (solid line) were used as control group. RMR as a function of regenerate length is given by the x-axis on the top. Different grey shades indicate phases of the healing and regeneration process. Asterisks denote significant differences (student´s t-test,$p<0.05$) between treatments. Values are given as means ± SE (n = 6).

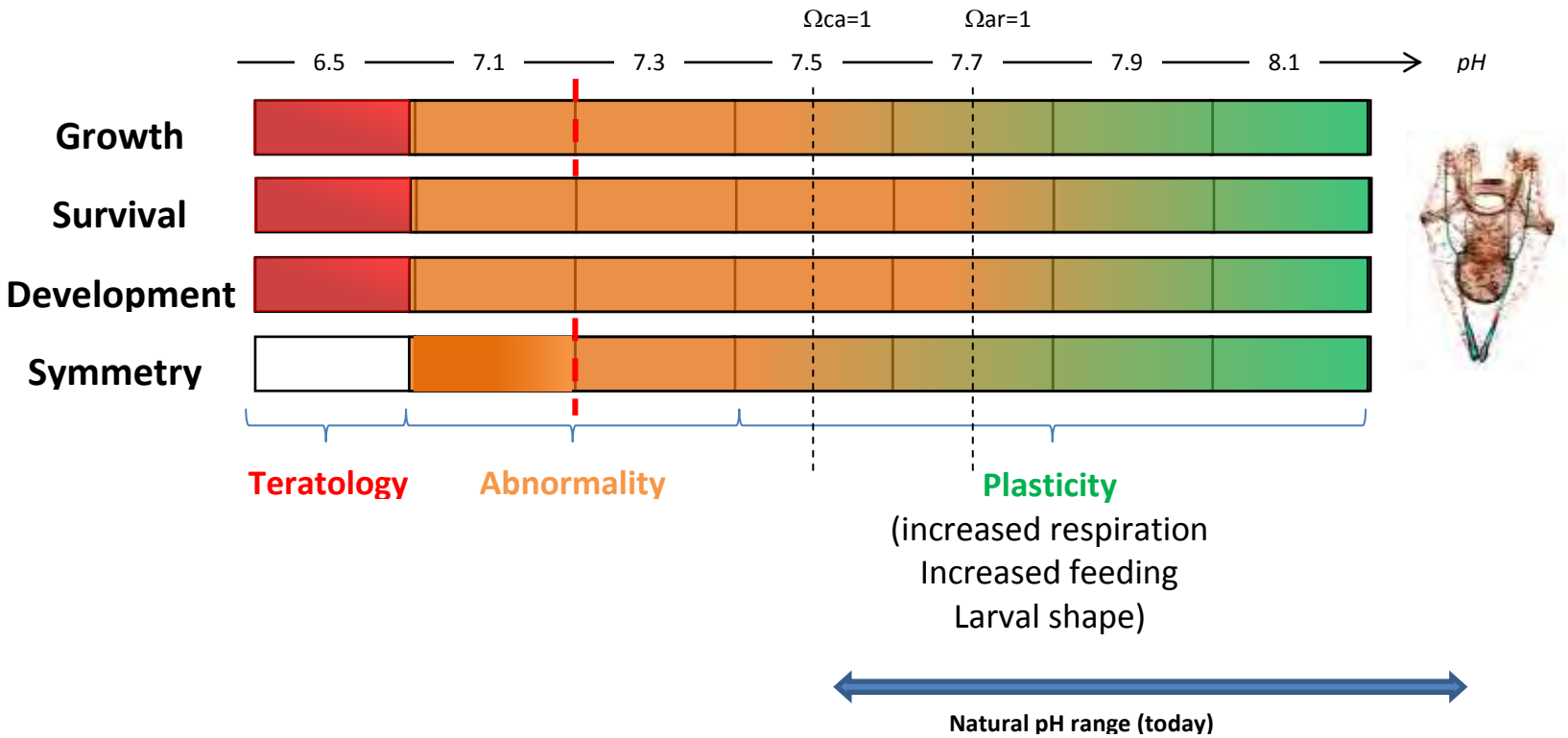


Figure 24. Synthesis of the work of Chan et al. 2012; Dorey et al. 2013; Stumpp et al. 2012 summarising the imapcts of elevated pCO_2 on larval biology using the urchins *Strongylocentrotus droebachiensis* and *S. purpuratus* as models. In general, larvae are found to be more susceptible than adults to elevated pCO_2/reduced pH but that different sub-lethal effects are produced at different pH tipping points.