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The toxicological interaction between ocean acidity and metals in coastal meiobenthic copepods

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ABSTRACT

Increased atmospheric CO_2 concentrations are causing greater dissolution of CO_2 into seawater, and are ultimately responsible for today's ongoing ocean acidification. We manipulated seawater acidity by addition of HCl and by increasing CO_2 concentration and observed that two coastal harpacticoid copepods, *Amphiascoides atopus* and *Schizopera knabeni* were both more sensitive to increased acidity when generated by CO_2. The present study indicates that copepods living in environments more prone to hypercapnia, such as mudflats where *S. knabeni* lives, may be less sensitive to future acidification. Ocean acidification is also expected to alter the toxicity of waterborne metals by influencing their speciation in seawater. CO_2 enrichment did not affect the free-ion concentration of Cd but did increase the free-ion concentration of Cu. Antagonistic toxicities were observed between CO_2 with Cd, Cu and Cu free-ion in *A. atopus*. This interaction could be due to a competition for H^+ and metals for binding sites.

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1. Introduction

Over the last century, the atmospheric concentration of CO_2 has risen at a rate 100 times faster than any change observed during the past 650,000 years (Siegenthaler et al., 2005). There is broad consensus that this ongoing change is a direct result of human activity, principally by fossil fuel burning, cement production and changing land use (Hansen et al., 2007). Atmospheric levels of CO_2 have consequently increased from pre-industrial levels of 280 ppm to a concentration of approximately 380 ppm (Feely et al., 2004). Almost 50% of all anthropogenic CO_2 emitted to the atmosphere has diffused passively into the ocean, significantly decreasing the rate of global warming (Sabine et al., 2004). Concentrations of atmospheric CO_2 are rising at a rate of 3.3% per year and will likely continue to rise (Canadell et al., 2007).

Political, social and environmental pressures to reduce atmospheric CO_2 have led governments to seek new options for CO_2 mitigation. A potential approach is the injection of CO_2 in underground porous reservoir rocks. Sub-seabed sequestration of CO_2 is considered to be a practical tool (Gibbins et al., 2006), and is already in use in the Norwegian sector of the North Sea (Holloway, 2005). Subsurface leakage is possible over time and has the potential to considerably increase local CO_2 concentration (Hawkins, 2004).

The net effect of CO_2 enrichment to seawater is to increase the concentration of carbonic acid (H_2CO_3), bicarbonate ion (HCO_3^-) and hydrogen ion (H^+), and to decrease the concentration of carbonate (CO_3^{2-}). The production of H^+ lowers the pH and causes the phenomenon called "ocean acidification". Carbonate ions can react with excess H^+ to form HCO_3^-, and it was thought that the carbonate buffering system would allow oceans to retain a stable pH despite rising emissions. However, as the partial pressure of CO_2 increases, the buffering capacity of seawater decreases and the ocean has been reduced by 0.1 pH unit since start of the industrial revolution, representing a 30% increase in the concentration of H^+ ions (Caldeira and Wickett, 2003; Key et al., 2004). Recently, an increase in the area exposed to corrosive seawaters during seasonal upwelling was observed along the North American Pacific continental shelf (Feely et al., 2008). Hydrological models predict that, based on proposed future emissions of CO_2, the average oceanic pH will decline by 0.3–0.5 U by the year 2100 and by 0.7 U within the next 300 years (Caldeira and Wickett, 2003). Leakage from CO_2 seabed storage would create locally faster and stronger acidification than that induced by atmospheric CO_2 (Hawkins, 2004).

Reduced availability of carbonate ions, induced by increased CO_2 concentrations, decreases calcification rates of organisms producing $CaCO_3$ shells or skeletons (Gattuso et al., 1998; Kleypas et al., 2006; Riebesell et al., 2000). In addition to calcification, a number of other physiological processes, including growth, development, metabolism, ionoregulation and acid–base balance, can be affected directly by increases in CO_2 (hypercapnia) or resulting acidosis (Fabry et al., 2008; Pörtner, 2008; Pörtner et al., 2004; Widdicombe and Spicer, 2008).

It appears inevitable that marine biota will be faced with ocean acidification for decades or centuries to come. Further, effects of

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acidification may exacerbate the potential effects of other anthropogenic stressors such as heavy-metal enrichment, especially in coastal sediments. Specifically, ocean acidification will change the organic and inorganic speciation of metals and will modify interactions of metals with marine organisms (Millero et al., 2009).

In the general context of ocean acidification, as well as in the context of CO_2 leakage from seabed storage, data are urgently needed to quantify the potential effects of CO_2 on organisms from shallow coastal areas (Raven et al., 2005). Meiofauna are the most abundant metazoans living in soft sediment, and this environment constitutes the majority of seabed habitats. The first aim of the present study was to compare toxic impacts of acidity induced by HCl and CO_2 on two species of harpacticoid copepods: *Schizopera knabeni* and *Amphiascoides atopus* in aqueous exposures. A wide range of acidity conditions was tested (including conditions that caused 100% mortality) to examine the entire H^+ concentration–response curve. A second aim of the present study was to evaluate the interactive toxicity of CO_2 with cadmium (Cd) and copper (Cu) for the copepod *A. atopus*. Those heavy metals are produced by human activity and both are widespread in the marine environment. Cd and Cu were chosen for study because they have different behaviors in CO_2 acidified water, which may create differences in toxicity.

2. Materials and methods

2.1. Test organism and laboratory culture

A culture of *S. knabeni* was initiated in 1993 using animals from a salt marsh near Port Fourchon, Louisiana (Lotufo, 1997), and a culture of *A. atopus* was established in 1992 (Lotufo and Fleeger, 1995). Both cultures were kept at 25 °C in 1 L sediment-free Erlenmeyer flasks containing 600 mL of 30 psu artificial seawater (ASW), which was renewed every 14 days. Copepods were fed weekly with T-*Isochrisis* paste (Brine Shrimp Direct, Odgen, UT, USA). Continuous reproduction in both species was apparent. Copepod were harvested by rinsing culture medium through a 125 μm aperture screen and retained copepods were sorted under a stereo dissection microscope via Pasteur pipette.

2.2. Ninety-six hours toxicity tests

All materials used to conduct bioassays were acid-cleaned prior to use. Fifteen nonovigerous adult female copepods (*A. atopus* or *S. knabeni*) were placed in 600 mL ASW plastic flasks, covered with a lid to avoid exchange with CO_2 from the air, and three replicates were used for each treatment. ASW was previously oxygenated by bubbling air for 24 h. Incubations at each condition were performed for 96 h at 25 °C and 16:8 light:dark photoperiod in an environmental chamber.

Acidity was manipulated by adding mineral acid (HCl) to ASW in flasks. Range-finding tests were used to determine acidities that resulted in 100% mortality to both copepod species. *A. atopus* and *S. knabeni* were exposed to 5 (ranging from 5.62 to 6.22) and 8 (ranging from 4.75 to 5.67) different pH conditions, respectively ($N = 3 \times 15$ animals for each pH level). The pH (NBS scale) was measured, using a pH meter (Oakton) at the beginning and at the end of the incubation. pH varied by an average of 0.059 ± 0.015 U over the course of the exposures, representing an average H^+ concentration variation of 0.250 ± 0.145 μmol L^{-1}. Carbon dioxide effects were studied by bubbling pure CO_2 in ASW. This CO_2-rich water (pH 4.5) was mixed with air-bubbled ASW in order to achieve desired pH levels. CO_2 concentrations were tested at increasing levels until 100% mortality was achieved. *A. atopus* and *S. knabeni* were exposed to 10 (ranging from 5.47 to 6.67) and 9 (ranging from 5.29 to 6.44) different CO_2-induced pH conditions, respectively ($N = 3 \times 15$ specimens). Mean variations of pH and concentration of H^+ measured between the end and the beginning of incubation were 0.089 ± 0.053 and 0.132 ± 0.093 μmol L^{-1}, respectively. Total alkalinity in the ASW used under the conditions of our experiments was determined potentiometrically using a Gran function (Dickson et al., 2007). The pCO_2 was calculated from pH and total alkalinity using the R package seacarb (Proyle, 2003).

In the present study, the pH values measured at the end of the incubations were used for the calculation of LC_{50}s.

Only *A. atopus* was studied for metal exposure. A small volume (20 μL) of acidified solution with the calculated amount of Cd and Cu to achieve a target concentration was spiked into each 600 mL incubation flask. At pH of 8.20, 6.30, 6.23 and 6.07, respectively, 9, 6, 5 and 5 different Cd concentrations were tested ($N = 3 \times 15$ specimens). At pH of 8.20, 6.36, 6.23, 6.15 and 6.08, respectively, 6, 3, 3, 4 and 3 different Cu concentrations were tested ($N = 3 \times 15$ specimens).

After 96 h, water was sampled for pH and metal concentrations. Contents of each microcosm were rinsed through a 125 μm mesh sieve and copepods retained were enumerated as live or dead. Missing copepods were presumed dead and percent mortality was calculated. For *A. atopus* and *S. knabeni*, mean fractions of missing copepods represented 7.27 ± 14.22% and 6.42 ± 14.18% of the total animals, respectively.

2.3. Metal measurements

Total Cd concentrations were determined by flame r atomic absorption spectroscopy (Varian AA240FS), and Cu concentrations were analyzed using ICP-OEC (Inductively Coupled Plasma, Optical Emission Spectrometer). All modeling for Cd and Cu was performed using the geochemical equilibrium modeling software program MINEQL+, version 4.6. Modeling simulations were run using components from the existing internal MINEQL+ database including, Cl^-, Na^+, SO_4^{2-}, Mg^{2+}, Ca^{2+}, K^+, HCO^{3-}, Br^-, BO_3, Sr and F^-, at the nominal concentrations typical of 30 psu ASW (Instant Ocean). Two successive iterations were performed; the first at a fixed pH of 4.5 with an open atmosphere in order to obtain the dissolved inorganic carbon (DIC) concentration, and the second using this DIC in a closed system, and using the components at the concentrations as described above, plus the different measured total Cd and Cu concentrations added to the components. These conditions mimicked the experimental protocols used in this study, which included bubbling CO_2 into ASW to a pH of 4.5 (under open atmosphere conditions), then mixing this water with air-bubbled ASW to achieve a desired pH (as described above) before sealing the container to container with copepods inside.

Concentrations of H^+, metals and free-ion metal concentrations that caused 10% (LC_{10}) and 50% (LC_{50}) lethality were estimated, as well as their 95% confidence intervals using log-probit analysis (SPSS Version 17.0.1, Chicago, IL).

The sum toxic unit approach (Sprague, 1970) was used to assess the joint effects of binary mixtures of (i) CO_2/Cd, (ii) CO_2/Cu and (iii) CO_2/Cu free-ion. Toxic units (TU) are a means of expressing the toxicity of a mixture of compounds as a portion of its threshold-effect concentration (Sprague, 1970). The toxicity of a mixture of compounds is expressed as a sum of the ratios of the exposure concentration and threshold-effect concentrations (LC_{50} expressed as concentration that causes 50% mortality [LR_{50}]) of the individual compounds in the mixture, where sum toxic units = (concentration x/LR_{50} x) + (concentration y/LR_{50} y). If compounds in a mixture are acting additively, the sum TU required to cause a 50% mortality of the exposed organisms will equal 1. A TU including 1 within the 95% confident interval was considered additive, a TU that did not include 1 in its high end 95% confidence interval (<1) was

zconsidered greater than additive (synergistic), while a TU that did not include 1 in its low end 95% confidence interval was considered less than additive (antagonistic).

2.4. Statistical analysis

Differences between (i) acidity tolerances of *A. atopus* versus *S. knabeni* and (ii) acidity induced by HCl versus CO_2 were tested with analysis of covariance (ANCOVA) (Green et al., 1996) using SAS software (SAS-Institute, 1985). The observed survival proportions were transformed via angular transformation (Anscombe, 1948).

3. Results

3.1. Acid toxicity

Over the 96-h exposure period, mortality of *A. atopus* and *S. knabeni* ranged, respectively, from 0% to 2.2% and from 0% to 4.4% in control experiments.

Amphiascoides atopus had an LC_{10} of 5.99 (5.86–6.19) pH units and the LC_{50} was 5.75 (5.63–5.84) pH units when acidity was manipulated with HCl. When water was acidified with CO_2, LC_{10} was 6.41 (6.29–6.82) pH units and LC_{50} was 6.11 (6.04–6.17) pH units, equivalent to LC_{10} of 23.6 (9.1–31.1) and LC_{50} of 47.2 (41.0–55.4) matm CO_2 (Fig. 1).

Schizopera knabeni, had an LC_{10} of 5.51 (5.38–5.90) pH units and the LC_{50} was 5.00 (4.90–5.06) pH units when acidity was manipulated with HCl. When water was acidified with CO_2, LC_{10} was 6.06 (5.92–6.39) pH units and LC_{50} was 5.54 (5.49–5.59) pH units, equivalent to LC_{10} of 52.9 (24.7–73.1) and LC_{50} of 175.1 (156.6–197.1) matm CO_2 (Fig. 2).

ANCOVA of the angular-transformed survival proportions showed that both species were significantly more sensitive to acidification from CO_2 relative to acidification from HCl ($p < 0.001$). Furthermore, regardless of the method used to acidify the ASW, *A. atopus* was significantly more sensitive to acidity than *S. knabeni* ($p < 0.001$).

3.2. Metal toxicity

The 96-h LC_{50} of *A. atopus* for Cd was 1.38 mg L^{-1} (1.26–1.52) at pH 8.20. When acidity was changed by adding CO_2, Cd LC_{50} was 1.41 mg L^{-1} (1.22–1.60) at pH 6.30, 0.98 mg L^{-1} (0.72–1.18) at pH 6.23 and 0.05 mg L^{-1} (0–0.307) at pH 6.07 (Fig. 3). The toxic unit required to create 50% mortality was 1.47 mg L^{-1} (1.28–1.62) (Fig. 4). As TU did not include 1 in its low end 95% confidence interval, CO_2 and Cd can be considered to have antagonistic toxicity. MINEQL+ modeling revealed that the Cd free-ion concentration was not affected by CO_2 under the conditions of the experiment.

The 96-h LC_{50} of *A. atopus* for Cu was 0.65 mg L^{-1} (0.40–0.99) at pH 8.20. When acidity was changed by adding CO_2 this Cu LC_{50} was

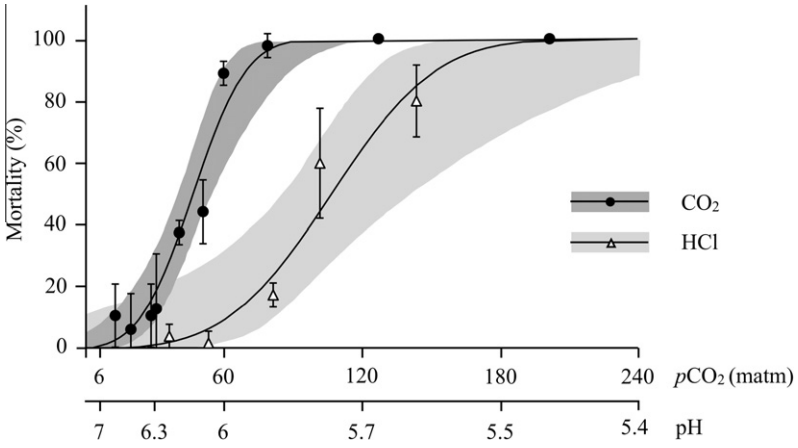

Fig. 1. Mortality (%) of *A. atopus* (±SD, $N = 3 \times 15$ specimens) versus pCO_2 (matm) and versus pH (using either CO_2 or HCl). Dose response and ±95% confidence intervals (shaded areas) using log-probit analysis.

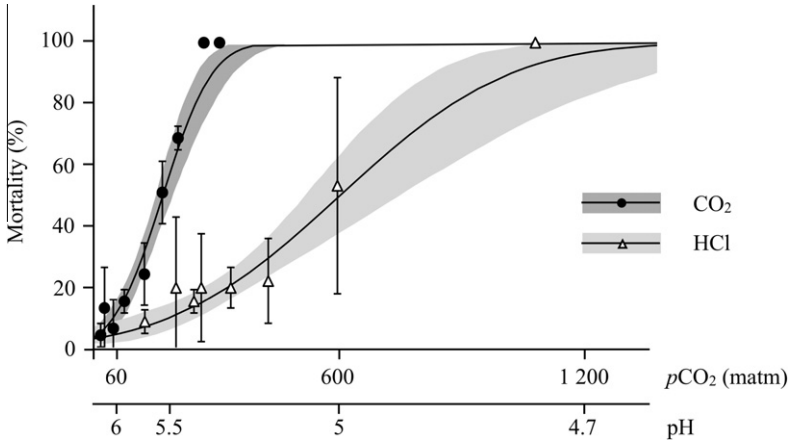

Fig. 2. Mortality (%) of *S. knabeni* (±SD, $N = 3 \times 15$ specimens) versus pCO_2 (matm) and versus pH (using either CO_2 or HCl). Dose response and ±95% confidence intervals (shaded areas) using log-probit analysis.

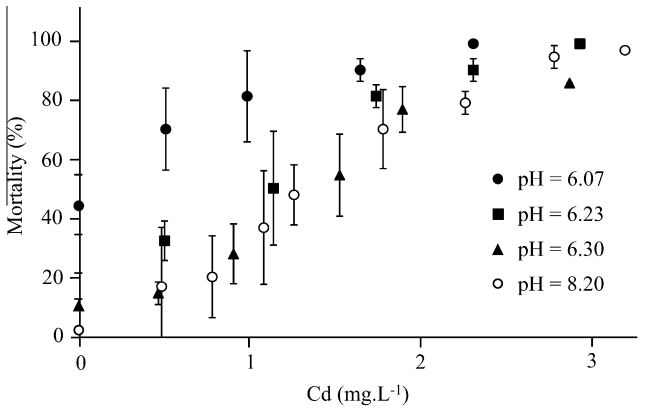

Fig. 3. Mortality (%) of *A. atopus* (±SD, $N = 3 \times 15$ specimens) versus total Cd concentrations at four different pH values induced by CO_2.

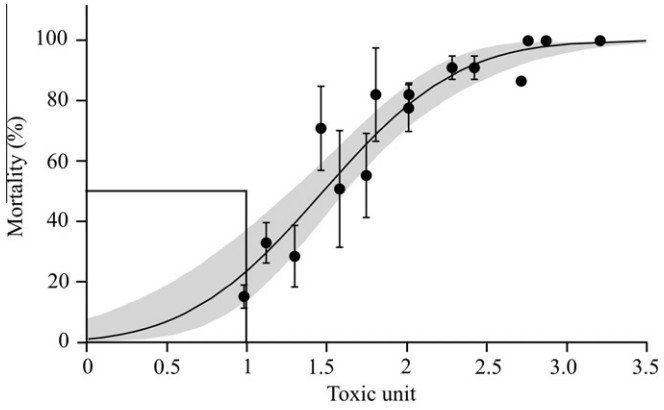

Fig. 4. Effect of Cd–CO_2 mixture on *A. atopus* mortality (±SD, $N = 3 \times 15$ specimens). Dose–response curve represents the best fit to toxic-unit data (shaded area; ±95% confidence intervals). Crosshair represents the theoretical point at which the center of the dose–response curve should pass if both components were to act in a dose-additive manner.

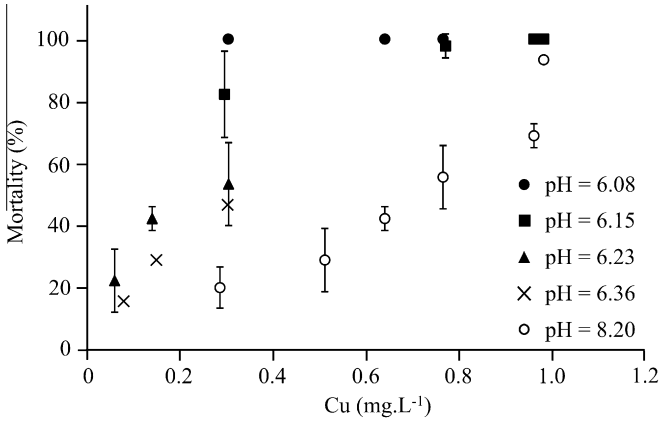

Fig. 5. Mortality (%) of *A. atopus* (±SD, $N = 3 \times 15$ specimens) versus Cu concentrations at five different pH levels induced by CO_2.

0.32 mg L^{-1} (0.25–0.55) at pH 6.37 and 0.26 mg L^{-1} (0.19–0.48) at pH 6.23 (Fig. 5). The toxic unit required to create 50% mortality was 1.17 (1.01–1.25). TU was slightly >1 in its low end 95% confidence interval (Fig. 6), which indicated that the toxicity of CO_2 and Cu were antagonistic. MINEQL+ modeling revealed that the free-ion

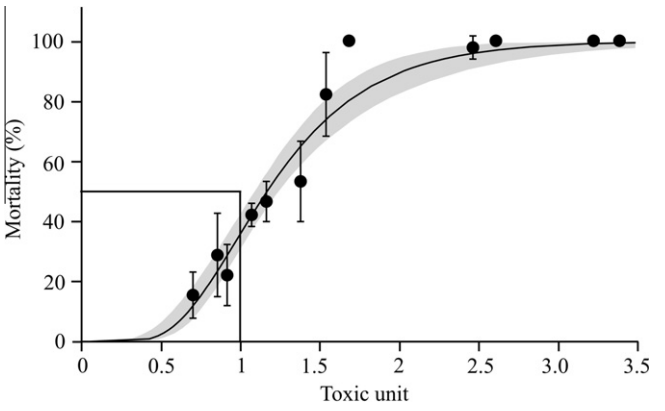

Fig. 6. Effect of Cu–CO_2 mixture on *A. atopus* mortality (±SD, $N = 3 \times 15$ specimens). Dose–response curve represents fit to toxic-unit data (shaded area; ±95% confidence intervals). Crosshair represents the theoretical point at which the center of the dose–response curve should pass if both components act in dose-additive manner.

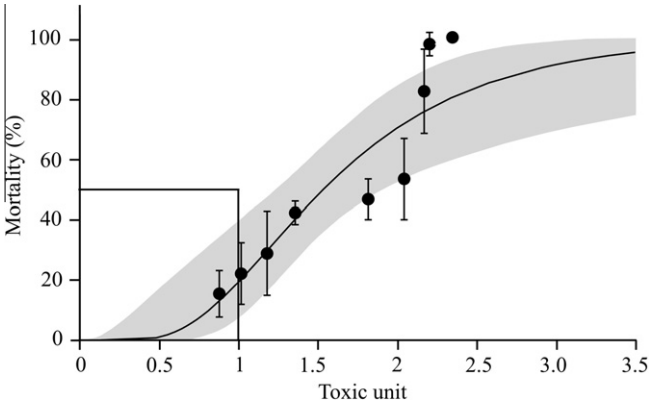

Fig. 7. Effect of free Cu^{2+} ion–CO_2 mixture on *A. atopus* mortality (±SD, $N = 3 \times 15$ specimens). Dose–response curve represents fit to toxic-unit data (shaded area; ±95% confidence intervals). Crosshair represents the theoretical point at which the center of the dose–response curve should pass if both components act in dose-additive manner.

concentration of Cu^{2+} was affected by CO_2. The 96 h LC_{50} free-ion Cu^{2+} concentration was 2.73 μM (2.08–3.32) at pH 8.2. Free-ion concentrations of Cu^{2+} were evaluated for each incubation condition and TU was calculated according to the individual toxicities of CO_2 and free-ion Cu. The TU that created 50% mortality was 1.53 (1.24–1.92) (Fig. 7). Consequently, when calculated with free-ion concentrations, toxicological interactions between CO_2 and Cu appeared to be more strongly antagonist than when calculated with total Cu concentrations (TU = 1.17 (1.01–1.25)). Interactions between CO_2 and Cd as well as CO_2 and free-ion Cu displayed a similar range of antagonist effects with TU LC_{50}s of 1.47 (1.28–1.62) and 1.53 (1.24–1.918), respectively.

4. Discussion

4.1. Methodological consideration

To our knowledge, this is the first study of the influence of CO_2 on shallow marine harpacticoid copepods. However, the present results should be interpreted with caution as several methodological aspects may underestimate the toxicity of elevated CO_2 concentrations in seawater. Sublethal effects may produce CO_2 sensitivity affecting many physiological parameters. Mechanisms

that mitigate acidity by acid–base balance come at a metabolic cost and, while not life threatening, could decrease growth and reproductive capacity (Langenbuch and Pörtner, 2004). For instance, egg production in the pelagic copepod *Acartia steueri* was found to decrease at pH of 6.8 (Kurihara et al., 2004). The fact that only the response of adults was studied, may also lead to an underestimation of CO_2 toxicity. It is widely accepted that early life history stages may be more sensitive to high pCO_2 (Pörtner and Farrell, 2008), especially in invertebrates (Dupont and Thorndyke, 2009a; Kurihara, 2008; Mayor et al., 2007). Early developmental stages of copepods are especially sensitive to contaminants (Green et al., 1996; Lotufo, 1997).

However, other aspects of the present study may lead to an overestimate of the impact of ocean acidification. Transfers of animals to each condition were abrupt and consequently not representative of the gradual changes that animals would experience in natural environments. Specifically, our acute exposures did not allow for either physiological acclimation or genetic modification in response to altered environmental conditions. A multigenerational experiment on the pelagic copepod *Acartia tonsa* showed that only one generation is needed to cope with impacts of ocean acidification on life cycle dynamics (Dupont and Thorndyke, 2009b).

Such potential biases must be kept in mind when interpreting results dealing with the toxicity of acidity. However, the impact of those biases would not apply to a (i) comparison of toxicity between CO_2 and HCl, (ii) comparison of CO_2 tolerance between species or (iii) comparison of metal and CO_2 interactive toxicity.

4.2. CO_2 versus HCl toxicity

The present study suggests that acidification using CO_2 produced an LC_{10} of pH 6.41 for *A. atopus* (Fig. 1) and pH 6.06 for *S. knabeni* (Fig. 2). A model of the Southern North Sea suggests an average decrease of 0.1 pH unit over the next 50 years (Blackford and Gilbert, 2007), while a global model predicts ocean pH values as low 7.5 within the next 300 years (Caldeira and Wickett, 2003). Both predictions are, however, well above observed toxicities. Our results suggest that adult benthic copepods from coastal environment may be relatively insensitive to CO_2 and may not be highly affected by an increase of ocean acidity. However, leakage from CO_2 seabed storage can create a faster and stronger local acidification (Hawkins, 2004) than the one induced by atmospheric CO_2. The present study suggests that some harpacticoid species may be sensitive to those strong changes expected for seepage scenario.

The 96-h LC_{50} observed for *A. atopus* and *S. knabeni* were, respectively, pH 5.75 (Fig. 1) and 5.00 (Fig. 2) when acidity was manipulated with HCl. In a similar type of experiment, the sensitivity of pelagic copepods to acidity was shown to be species-specific: among 10 oceanic zooplankton species studied, only two had lower sensitivity to acidification than we observed: *Eucalanus bungii bungii* and *Themisto japonica* LC_{50}'s of pH 5.16 and 5.00, respectively (Yamada and Ikeda, 1999). Thus, adult benthic copepods may be slightly more tolerant to acidity than pelagic copepods.

Unmixed sediments are characterized by strong geochemical gradients. pH changes of 1 U with depth (Fisher and Matisoff, 1981) and 1.5 U over short time scales (Gnaiger et al., 1978) have been measured. In such variable environments, it is unlikely that benthic animals would be affected by small changes of pH. However, some meiofauna appear to be specialists, living at preferred depth locations within the sediment (Fleeger and Gee, 1986; Steyaert et al., 2003). Depth specialists could be adapted to highly specific conditions from that sediment stratum and consequently some species may be intolerant to small changes in pH.

CO_2 sensitivity of benthic fauna has been studied in the deep sea in order to evaluate the potential impact of large-scale sequestration of CO_2. Deep-sea harpacticoid copepods suffered 70–100% mortality when exposed to a reduction in pH of 0.75 U (Thistle et al., 2005). Copepods may not be able to burrow deeper to avoid CO_2 (Thistle et al., 2006), but they may be able to swim into the water column in order to attempt escape from the advancing front of carbon dioxide-rich seawater CO_2 (Thistle et al., 2007). Other studies have illustrated sensitivity of deep-sea nematodes, foraminifera and euglenoid flagellates (Barry et al., 2004; Fleeger et al., 2006, 2010; Ishida et al., 2005; Ricketts et al., 2009) at pH decreases of only 0.1–0.2 U (Barry et al., 2005). The deep sea is characterized by highly stable physico-chemical parameters. In contrast, shallow-dwelling aquatic animals have evolved acid–base regulatory capabilities to combat seasonal, or even daily fluctuations in water pH. Deep-sea animals are unlikely to possess well-developed acid–base regulatory capacity (Pane and Barry, 2007). Consequently, the high CO_2 sensitivity of deep-sea copepods may not be observed among species from shallow coastal environments.

To our knowledge, this is the first study to examine the impact of changing seawater pH on harpacticoid copepods from a shallow-water environment. Among other members of shallow meiofauna, nematodes have a higher tolerance to CO_2 than do macrofauna (Dashfield et al., 2008; Widdicombe et al., 2009) and this apparently short-term tolerance could be due to their impermeable proteinaceous cuticle. Three species of nematodes from subtidal coastal environments were highly resistant to high concentrations of CO_2 and lethal effects were only observed after increasing acidity to pH 5.5–6 (Takeuchi et al., 1997). At pH 7.5, a subtidal nematode community was unaffected by high CO_2 after several days of exposure (Dashfield et al., 2008), but a pH of 7.3 produced a change in nematode community composition (Widdicombe et al., 2009). Nematodes and copepods share the same habitat and have consequently evolved in similar environmental conditions. The present study suggests that, as with nematodes, shallow-water harpacticoid copepods would be largely unaffected by future ocean acidification.

Carbonate chemistry of seawater can be manipulated in various ways, and the most commonly used is by bubbling seawater with gases at different pCO_2 values. In the present study, different pCO_2 seawaters were prepared by mixing different ratios of water bubbled with pure CO_2 and water bubbled with air. This technique increases dissolved inorganic carbon at constant total alkalinity, and provides realistic simulations of past and predicted changes of ocean chemistry (Gattuso and Lavigne, 2009). Adding a strong acid (HCl) to a system does not alter the concentration of dissolved inorganic carbon but does modify total alkalinity. This method of acidification is thus more artificial because alkalinity is not expected to change over this century. Consequently at similar pH values, water acidified with CO_2 will contain more dissolved CO_2, HCO_3^- and CO_3^{2-} than water acidified with a strong acid. CO_2 diffuses easily into intracellular compartments and rapidly reacts with water to form H^+ and HCO_3^-. This intracellular acidosis can affect many physiological processes and may explain why the strongest toxic effects were observed when CO_2 was used to manipulate acidity in the present study. Stronger toxicity of early developmental stages of sea bream was also observed when acidity was manipulated by CO_2 (Kikkawa et al., 2004). These results emphasize that the addition of strong acid does not fully mimic the changes in carbonate chemistry expected with ocean acidification and would likely lead to an underestimation of the toxic effect.

4.3. Schizopera knabeni and A. atopus sensitivity

Even though ocean acidification is expected to reduce biodiversity (Widdicombe and Spicer, 2008), some species may benefit from these new environmental conditions (Dupont and Thorndyke, 2009a). In several experiments, increased pCO_2 has modified the meiofaunal community composition of deep-sea (Bernhard et al.,

2009; Thistle et al., 2006) and shallow (Widdicombe et al., 2009) sediments. Those experiments indicate that not all species within a community have the same sensitivity to CO_2. Differences were also observed between species of deep-sea copepod in their ability to escape CO_2 (Thistle et al., 2007). Such natural differences could explain the observed lower sensitivity of *S. knabeni* relative to *A. atopus*.

Different physiological mechanisms allow organisms to counteract acidification via: (i) passive buffering of intra- and extracellular fluids, (ii) transport of CO_2 in the blood of species that have respiratory pigments and (iii) metabolic suppression during periods of elevated CO_2. Species adapted to hypercapnic environments have evolved such mechanisms to counteract CO_2 more efficiently than other species (Seibel and Walsh, 2001, 2003).

The two copepod species studied are associated with different environments. The natural habitat and the distribution of *A. atopus* are unknown, but species in this genus are exclusively found on beaches comprised of large particles (large sand grains to small cobble) (Lotufo and Fleeger, 1995). The porosity of such sediment being high, the porewater pH is similar to the pH of overlying water with little change over space and time. Cultures of *S. knabeni* were initiated with specimens collected from estuarine sediments in Louisiana (Lotufo, 1997). The pH of sediment porewater in this environment is approximately 7.55 (Ho and Lane, 1973). This type of muddy sediment is poorly permeable and pH decreases strongly with depth at the millimeter scale (e.g., Fisher and Matisoff, 1981). Moreover, in muddy sediments, the majority of meiofauna lives in the uppermost surficial layer. Bioturbation increases water penetration to depth (Aller and Aller, 1992), creating millimeter lateral heterogeneity in pH (Zhu et al., 2006). In addition to differences in spatial and temporal pH heterogeneity, muddy sediments have generally lower oxygen availability and higher CO_2 concentration than sandy sediment and cobble. Mud-dwelling meiofauna may consequently be better adapted to hypercapnic environments than the fauna of sand and cobble. Our observations are consistent with this hypothesis. Widdicombe et al. (2009) similarly observed that a nematode community from a mud environment was less sensitive to CO_2^- rich water than was a nematode community coming from sandy sediment.

4.4. Metal toxicity

Anthropogenic activities have led to significant metal pollution in many parts of the world, especially in inshore sediments (Nixon, 1995). Physical adsorption and chemical binding contribute to heavy metal concentrations that are several orders of magnitude higher in sediment than in overlying water. As a result, sediments generally represent sinks for pollutants. Sediment dwellers like copepods can potentially be exposed by taking up dissolved porewater metals across their thin exoskeletons and soft tissues and/or by ingesting sediment and associated metal.

Compared to other heavy metals, Cd is relatively soluble, tends to bioaccumulate and is consequently considered to be a significant environmental threat (Goldberg, 1984). Cu can enter the aquatic environment primarily from mining activities (Lewis, 1995) but also from antifouling paints. Cu is an essential micronutrient for all organisms. However at high concentrations, it becomes toxic for plants and animals. In the present study, the Cu 96-h LC_{50} of *A. atopus* was 650 $\mu g\,L^{-1}$ at a pH of 8.2, which is in the range of LC_{50}s found for the intertidal copepod genus *Tigriopus* (Barka et al., 2001; Kwok and Leung, 2005; O'Brien et al., 1988).

4.5. Metal/CO_2 toxicity

In the future ocean, marine life will have to face acidification simultaneously with other anthropogenic stressors such as metal contamination. Prediction of the toxicology of multiple stressors is challenging when the combined effects of toxicants cannot be predicted from the individual effect of each. A review of studies of mixtures of heavy metals reveals that synergisms and antagonisms are more common than response-additive toxicity (Norwood et al., 2003). Such interactive toxicology was observed in copepods (Fleeger et al., 2007; Hagopian-Schlekat et al., 2001). However, the combined effect of CO_2 and metal toxicity for marine organisms have rarely been studied (Millero et al., 2009).

A consequence of ocean acidification is a decreased concentration of OH^- and CO_3^{2-}. These anions form strong complexes in ocean water with divalent and trivalent metals (Millero et al., 2009). This reduction is expected to change the speciation of numerous metal ions in seawater (Byrne, 2002). Metals such as Cd^{2+} form strong complexes with chloride. However, because Cl^- is pH insensitive, speciation of Cd will be minimally affected by ocean acidification. Indeed, modeling conducted in the present study revealed that the free-ion concentration of Cd was not affected by acidity. Other metals such as Cu^{2+} form strong complexes with carbonate. Such metals will be more strongly affected by acidification and the concentration of their free ionic form will increase. In the present study, free-ion modeling with MINEQL+ revealed that in contrast to Cd, the free-ion form of Cu was increased with increasing acidity. Free-ion forms of metals are generally more toxic than complex forms (Allen et al., 1980). Comparison between LC_{50} calculated using total Cu or free-ion Cu allows an evaluation of the influence of CO_2 on speciation. The approach using total Cu measured permits an evaluation of toxicity of combined (i) Cu, (ii) CO_2 and (iii) increased concentration of free-ion Cu according to CO_2 conditions. With the Cu approach, only Cu and CO_2 effects are evaluated. The strongest antagonism observed with the free-ion approach confirmed that CO_2 increased the free-ion form of Cu, thus making it more toxic.

Acidification of the ocean also leads to an increased concentration of H^+. As a result, competition for binding sites increases between H^+ and metals. Surface sites become less available to adsorb metals in the presence of increasing H^+, potentially making acidification and metal toxicity antagonistic. In the present study, an antagonism was observed between CO_2 and Cd and of CO_2 and the free-ion of Cu, suggesting a competition for binding sites with H^+ for both metals. CO_2 concentration decreased toxicity of both metals by the same order of magnitude, suggesting that the binding sites of both metals may be similar. However, other processes could lead to the observed antagonist effect. Indeed, one mechanism to counteract acidosis is to depress metabolism (Guppy and Withers, 1999), thus reducing the rate of metal transport in the animals. Cd mimics Ca, and a reduced calcification rate can, for instance, reduce the rate of Cd uptake. The extent to which such processes operate in harpacticoid copepods is not well understood. To our knowledge, only one other study dealing with CO_2 and metal toxicity has been performed in the marine environment. In this case, antagonism was also observed between CO_2 and Cd: when CO_2 concentration was increased, the uptake of Cd decreased in the hatchling tissue of cuttlefish *Sepia officinalis* (Lacoue-Labarthe et al., 2009). Studies of freshwater organisms are more common and reveal competition for binding sites between H^+ and metals in aquatic biota (e.g., Hare and Tessier, 1996; Yu and Wang, 2002). Similarly, uptake of metal by soil-dwelling animals was reduced when pH was changed by adding lime (e.g., Oste et al., 2001).

4.6. Extrapolation to natural environment

The present study was conducted in water and allows evaluation of pharmacological interactions between metals and CO_2. However, exposure in sediments can produce other kinds of

interactions that are difficult to predict. The effects of mixtures in sediments are especially complex for deposit-feeding animals that are exposed to both dissolved contaminants and contaminated food/sediment. For example, the narcotic effect of PAH has been shown to slow the uptake of Cd-contaminated sediment in a bulk deposit-feeding oligochaete by reducing his feeding rate (Millward et al., 2001). CO_2 also has a narcotic effect but its toxic interactions with metals in sediment remains uncertain, as copepods are not considered as bulk deposit feeders (Green et al., 1993). Other interactions may differ between aquatic and sediment environments. High amounts of metal can potentially be bound to sediment and those complex forms of metals are not necessarily bioavailable and toxic for sediment dwelling animals such as harpacticoid copepods. Acidification releases bound metals from sediments (Ardelan et al., 2009) rendering them more toxic in a free form. The amount of free metal released from sediment may be more important than amount available from pelagic environments. Such mechanisms are largely unknown and therefore studies are needed to better evaluate such potential indirect effects of ocean acidification.

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