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RESEARCH ARTICLE

Lethal and sub-lethal effects of elevated CO_2 concentrations on marine benthic invertebrates and fish

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Abstract Concern about leakage of carbon dioxide (CO_2) from deep-sea storage in geological reservoirs is increasing because of its possible adverse effects on marine organisms locally or at nearby coastal areas both in sediment and water column. In the present study, we examined how elevated CO_2 affects various intertidal epibenthic (benthic copepod), intertidal endobenthic (Manila clam and Venus clam), sub-tidal benthic (brittle starfish), and free-living (marine medaka) organisms in areas expected to be impacted by leakage. Acute lethal and sub-lethal effects were detected in the adult stage of all test organisms exposed to varying concentrations of CO_2, due to the associated decline in pH (8.3 to 5.2) during 96-h exposure. However, intertidal organisms (such as benthic copepods and clams) showed remarkable resistance to elevated CO_2, with the Venus clam being the most tolerant ($LpH_{50} = 5.45$). Sub-tidal species (such as brittle starfish [$LpH_{50} = 6.16$] and marine medaka [$LpH_{50} = 5.91$]) were more sensitive to elevated CO_2 compared to intertidal species, possibly because they have fewer defensive capabilities. Of note, the exposure duration might regulate the degree of acute sub-lethal effects, as evidenced by the Venus clam, which showed a time-dependent effect to elevated CO_2. Finally, copper was chosen as a model toxic element to find out the synergistic or antagonistic effects between ocean acidification and metal pollution. Combination of CO_2 and Cu exposure enhances the adverse effects to organisms, generally supporting a synergistic effect scenario. Overall, the significant variation in the degree to which CO_2 adversely affected organisms (viz., working range and strength) was clearly observed, supporting the general concept of species-dependent effects of elevated CO_2.

Keywords Carbon dioxide · CO_2 capture and storage (CCS) · pH · Intertidal organism · Sub-tidal organism

Introduction

Efforts to mitigate the emission of anthropogenic carbon dioxide (CO_2) are becoming increasingly important towards attempt to manage climate change (Pachauri et al. 2014). Carbon dioxide capture and storage (CCS) represents one such promising CO_2 mitigation technology. In this process, CO_2 is captured from the atmosphere, then compressed, and transported to sub-seabed storage sites for (semi)-permanent long-term isolation. However, there is a risk of injected CO_2 escaping (or leaking) from maritime CCS storage sites and dispersing and/or dissolving into water column or seabed sediment (Carroll et al. 2014; De Vries et al. 2013). Consequently, CO_2 leakage may have possible adverse effects on both water column and sediment-dwelling organisms.

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The primary environmental impact of CO_2 leakage is a decline in waterborne pH (Blackford et al. 2008). Yet, this change might be slow or negligible in the open ocean, due to carbonate buffering. However, local acidification as low as pH 5 might occur under certain condition (i.e., when seawater circulation is limited), which may lead to possible acute adverse effects on aquatic animals (Auerbach et al. 1997; Caulfield et al. 1997; Payán et al. 2012). Indeed, several studies have reported acute effects of elevated CO_2 on various marine organisms, including copepods (McConville et al. 2013), bivalves (Gazeau et al. 2010), and fish (Lee et al. 2003). Acute adverse effects are primarily associated with ionic imbalances caused by a rapid increase of hydrogen ions in the organism.

In fact, body chemistry regulates whether toxic effects are acute or chronic in different species (Pörtner et al. 2004). Because the toxic effects of chemicals are species-specific, various marine organisms are expected to exhibit a broad range of threshold effect concentrations of CO_2. In parallel, the toxic endpoints identifying such adverse effects may vary across molecular, cellular, individual, and population levels, depending on the sensitivity of test species. Copepods are one of the more CO_2-sensitive organisms and represent good test animals because they are small, with low locomotive capacity to avoid CO_2 plumes (Auerbach et al. 1997; Halsband and Kurihara 2013). Clams and starfish are representative calcifying organisms and are the more sensitive animals to ocean acidification; yet, reports about the adverse effects of elevated CO_2 on these organisms remain limited (Gazeau et al. 2010; Guinotte and Fabry 2008; Ries et al. 2009). Fish are also good test animals because their early developmental stages are the more sensitive to elevated waterborne CO_2, despite being generally considered as more CO_2-tolerant than marine invertebrates (Forsgren et al. 2013; Kikkawa et al. 2003). Compared to those of other classic toxic chemicals, studies about the lethal and sub-lethal effects of elevated CO_2 conditions remain limited on these groups of organisms.

In this study, we investigate how elevated CO_2 concentrations adversely affect various marine organisms, including copepods, clams, starfish, and fish as a part of Korean CCS Environmental risk and impact assessment research (Kim et al. 2016) (Table 1 and Fig. 1). The test organisms were selected from typical habitats or areas likely to be exposed to the scenario of CO_2 in situ, specifically, intertidal zone (benthic copepod, Manila clam, and Venus clam), sub-tidal zone (brittle starfish), and open water column (marine medaka). First, we aimed to examine acute lethal and sub-lethal effects of elevated CO_2 on species belonging to the five marine groups. Second, effect of exposure duration on a daily basis (viz., 0, 24, 48, 72, and 96 h) was tested for selected organisms (two clam species) as part of an acute test. Third, chronic lethal effects were also tested for starfish by a longer period of exposure (viz., 10 days), to determine long-term toxicological effects under a continuing exposure scenario of elevated CO_2. Fourth, combined effect of CO_2 and copper exposure, as a co-occurring potential toxic element, was investigated using the Manila clam, to consider the effect of possible interactions between micro-pollutants and CO_2 in the natural environment. A toxicological database on the pH level that affects various marine organisms was compiled and integrated into our results, as part of the study. Our results are expected to provide insights into species-specific and endpoint-specific biological effects of elevated CO_2 on various marine organisms.

Materials and methods

Collection and handling of test organisms

Benthic copepods (*Tigriopus* sp.) were collected from tidal pools of rocky intertidal zone, located in Manlipo, on the west coast of Korea (Fig. S1 of Supplemental materials (S)). Collected organisms were transported to the laboratory and cultured in filtered in situ seawater (GF/F, Whatman, Kent, UK; 30 psu) in a temperature-controlled room, maintained at 20 °C. Benthic copepods were fed a combination of *Isochrysis galbana*, *Tetraselmis suecica*, and *Chaetoceros gracilis* obtained from Korea Marine Microalgae Culture Center. Seawater exchange and larval collection were conducted once a week. For the CO_2 exposure experiments, benthic copepods were individually selected (200-μm mesh sieved) and acclimated in 1 L autoclaved seawater for 24 h without food.

Manila clams (*Ruditapes philippinarum*) and Venus clams (*Macridiscus aequilatera*) were collected from the tidal flats of Seonjae Island on west coast of Korea and the coastal area of Jumunjin on the east coast of Korea, respectively (Fig. S1). Collected clams were transported to the laboratory and sorted for individuals of 25–35 and 39–59 mm in length for Manila and Venus clams, respectively. Individuals were acclimated in filtered seawater for 2 days, which was maintained at 15 °C. On the first day of acclimation, seawater was frequently renewed to remove gut contents. Clams were not fed, and dead individuals were immediately removed to maintain water quality.

Brittle starfish (*Ophiura* sp.) was collected by using a sediment grab sampler (1-mm mesh sieved) in the coastal area (30–50 m seabed depth) of Samcheok on the east coast of Korea (Fig. S1). Collected organisms were transported to the laboratory and placed in water tanks containing filtered seawater. Individuals were cultured at 10 °C and 35 psu, similar to in situ conditions. Finally, the stock of marine medaka (*Oryzias melastigma*) used in our experiment was obtained from NeoEnbiz Co. (Korea) and has been reared in Laboratory of Marine Benthic Ecology at Seoul National University (Korea) for over 10 generations. Continuously cultivated marine medaka was used for the fish exposure tests. The organisms were cultured in 25-L glass tank at 26 °C with

Table 1 Details of test organisms, exposure conditions of CO_2, and endpoints in the present study

	Intertidal organisms			Sub-tidal organisms	
	Benthic copepods	Manila clams	Venus clams	Brittle starfish	Marine medaka
Test organism					
Species	*Tigriopus* sp.	*Ruditapes philippinarum*	*Macridiscus aequilatera*	*Ophiura* sp.	*Oryzias melastigma*
Life stage	Adult (>200 μm)	Adult (25–35 mm)	Adult (39–59 mm)	Adult (>35 mm)	Adult (>40 mm)
CO_2 exposure					
Exposure route	Indirect exposure (seawater)	Direct exposure (sediment-seawater)	Direct exposure (sediment-seawater)	Direct exposure (sediment-seawater)	Direct exposure (seawater)
Control	Air	Air	Air	Air	Air
% $CO_{2\ gas}$	1, 5, 10, 20, and 30	5, 10, 20, and 50	1, 5, 10, 20, and 30	1, 5, 10, and 15	1, 10, 20, and 30
pH range	5.5–8.0	5.2–8.1	5.5–7.8	6.2–7.9	5.6–8.3
Exposure system	Fig. 1a	Fig. 1b	Fig. 1b	Fig. 1b	Fig. 1b′
Condition details					
Type	Static	Static-renewal (80 % every 2 days)	Static-renewal (80 % every 2 days)	Static-renewal (80 % every 2 days)	Static-renewal (80 % every 2 days)
Duration (h)	96	96	96	96 and 240	96
Frequency of monitoring (h)	24	24	12	24	24
Temperature (°C)	23	20	15	10	25
Salinity (psu)	30	30	30	35	35
Water volume (L)	0.025	2.5	2.5	2.5	2.5
Number of organism	10	10	10	10	10
Number of replicates	5	3	3	3	3
Endpoints					
Lethal effect	Mortality	Mortality	Mortality	Mortality	Mortality
Sub-lethal effects	Swimming inhibition	Siphon releasing Shell closed failure Response delay	Siphon releasing Shell closed failure Response delay		
Data presented in	Fig. 2	Fig. 2 and Table S3	Fig. 2 and Table S4	Fig. 3	Fig. 3

a light/dark photoperiod of 14/10 h and 30 psu. The fish were fed brine shrimp and dry flake once a day until satiation.

Design of CO_2 exposure systems

A strict quality assurance and quality control (QA/QC) was performed to develop CO_2 exposure systems, particularly targeting the maintenance of waterborne pH during exposure with minimum physical disturbance to test organisms (Fig. 1). Two systems were used: (1) an air tight box system (indirect exposure) and (2) the glass chamber system (direct exposure), depending on the purpose of the experiment (Table 1). Under both systems, the targeted pH in the water column was successfully maintained with minor variation (< ±0.1) during the CO_2 exposure. Dissolved oxygen (DO) was controlled as >6.0 mg L^{-1} and >80 % (ASTM 2007, 2008) using O_2-balanced air, ensuring standard water quality throughout the exposure period.

The air tight box system was primarily designed for small-sized test organisms (for benthic copepods). This system was an indirect exposure system, avoiding the direct bubbling of CO_2 gas in small beakers (Kita et al. 2013) (Fig. 1a). The CO_2 concentration in the air tight box system was maintained by a continuous supply of air (control) or CO_2 gas mixture (1 and 5 % of CO_2-balanced with air; 10, 20, and 30 % of CO_2-balanced with 20 % O_2). Two large beakers (500 mL) filled with seawater were placed inside the air tight box. One beaker was bubbled by the direct injection of air or CO_2-mixed gases. The other beaker (without gas bubbling) was used to monitor pH and DO in the system daily. The preliminary test confirmed that the pH in the test beaker could be stabilized by continuously dissolving gas, reaching targeted CO_2 concentrations within 48 h.

The glass chamber system was used for macro-organisms. This system represents a direct exposure system, because large-sized animals did not seem to be affected by the

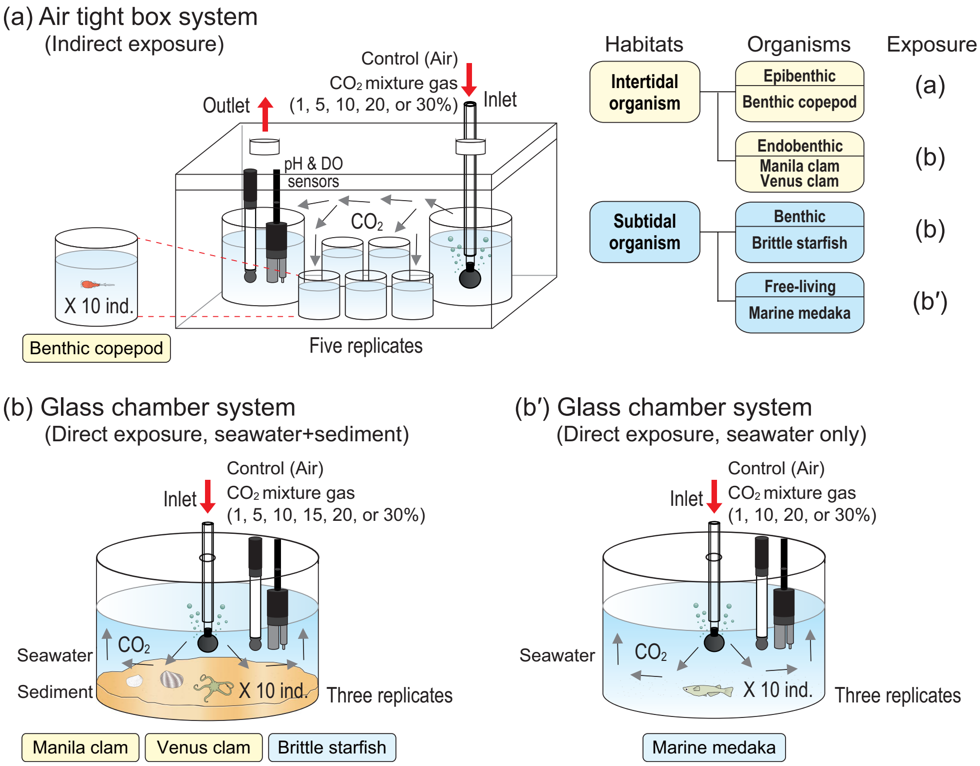

Fig. 1 Schematics of CO_2 exposure systems. (*a*) Air tight box system (*indirect exposure*) for the benthic copepod experiment. (*b*) Glass chamber system (*direct exposure*, *seawater-sediment*) for the Manila clam, Venus clam, and brittle starfish experiments. (*b′*) Glass chamber system (*direct exposure*, *seawater only*) for marine medaka. Experimental conditions are detailed in Table 1

vigorous gas flow (Fig. 1b, b′). In this system, the CO_2 gas mixture (1 and 5 % of CO_2-balanced with air; 10, 15, 20, 30, and 50 % of CO_2 balanced with 20 % of O_2) was directly injected to each test chamber containing 2.5 L filtered seawater. The preliminary test confirmed that CO_2 and DO in the glass chamber system (viz., direct exposure) were saturated faster (<3 h) than those in the air tight box system (viz., indirect exposure). Exposure experiments were initiated with decreasing gas flow to maintain saturation and minimize possible physical stress. The water-sediment test for exposure to clams and brittle starfish involved using in situ-collected sediments at the bottom (2-mm mesh sieved; ~1 cm depth) with 2.5 L overlying in situ seawater.

Water quality monitoring was performed daily for pH and DO using a pH meter (Orion Star, Thermo Scientific, Waltham, MA) and YSI multi-parameter meter (Yellow Springs, OH), respectively. Total alkalinity (TA) was measured by 5 pH point titration method (Moosbrugger et al. 1993). Other parameters were calculated using CO2SYS program (Pierrot et al. 2006) with dissociation constant from Mehrbach et al. (1973) with refit by Dickson and Millero (1987) and KSO4 using Dickson (1990) (data present in Table S1). Experimental setup could affect the results, say tank effect onto pH values etc., thus, such artificial effects on pH levels were tested by applying a two-way ANOVA (Table S2). Factors induced in the analysis were the supplied CO_2 gas concentrations, the measured pH values, and three types of tank system (Fig. 1). The CO_2 gas concentrations significantly contributed to the pH values in the treatment. However, the pH effect had no significant influence on the types of tank system, even though at the sediment-treated tank.

CO_2 exposure of marine organisms

Intertidal organisms

Ten benthic copepod individuals were randomly placed into each 50-mL beaker containing 25 mL seawater (control) or acidified seawater (treatments), with five replicates per treatment, which were maintained at 23 °C and 30 psu (Table 1 and Fig. 1a). After 96-h exposure, mortality and swimming inhibition were observed under the microscope to identify the lethal and sub-lethal endpoints for each treatment. Individuals not showing the mobilization of appendages by physical stimulation (i.e., bright light) were counted as “dead.” Individuals without directional (i.e., swimming at fixed location) and occasional (more than five times) movements after physical stimulation were considered as “swimming inhibition.”

After 2-day acclimation, 10 clam individuals were randomly placed into each glass chamber containing 2.5 L filtered seawater with sediment on the bottom (~1 cm) for both the control (viz., ambient seawater) and CO_2 treatments (viz., acidified seawater), with three replicates per treatment. Test chambers were placed in a temperature-controlled room, maintained at 20 °C for Manila clams and 15 °C for Venus clams, respectively, during the 96-h exposure period (Table 1 and Fig. 1b). During exposure, about 80 % seawater was replaced with fresh seawater every 2 days. Mortality and siphon releasing rate were measured as lethal and sub-lethal endpoints after 24, 48, 72, and 96 h. Individuals showing disability in shell closing after physical stimulation (e.g., soft touching by forceps) were placed in air, at which point no mobilization was confirmed, and the individuals were counted as dead. For Venus clams, failure of shell closure and response delay was also measured as sub-lethal endpoints, providing varying spectrums of acute daily toxic effects.

Sub-tidal organisms

Ten acclimated brittle starfishes were randomly placed into each glass chamber containing 2.5 L seawater with sediment in the bottom (~1 cm) for both the control (viz., ambient seawater) and treatments (viz., acidified seawater), with three replicates per treatment. Test chambers were placed in a temperature-controlled room, maintained at 10 °C (Table 1 and Fig. 1b). About 80 % of exposure seawater was replaced with fresh seawater or acidified seawater every 2 days. In addition to the 96-h acute exposure test, a chronic exposure test of 240 h (10 days) was implemented for brittle starfish, for which the daily mortality of test organisms was recorded.

For the marine medaka experiment, 10 individuals were placed into each glass chamber containing 2.5 L filtered seawater (seawater only), with three replicates of the control (viz., ambient seawater) and each treatment (viz., acidified seawater). Test chambers were placed in a temperature-controlled room, which was maintained at 26 °C during the exposure period (Table 1 and Fig. 1b′). The mortality of marine medaka was measured daily as the endpoint of the lethal effect of CO_2.

Combined effect of CO_2 and copper on Manila clam

Manila clams were selected for a combined toxicity test, due to exhibiting the lowest sensitivity to CO_2, their wide distribution, and the possible scenario of environmental pollutants co-occurring with CO_2 in intertidal sediments. Ten manila clams were randomly placed in each glass chamber containing seawater (negative control), copper-spiked seawater (50 μg Cu L^{-1}, positive control), and CO_2-copper-treated seawater. All glass chambers were pre-cleaned using acid before use for the CO_2-copper toxicity test. Copper stock solution (50 mg Cu L^{-1}) was prepared by using $CuSO_4$ (Sigma-Aldrich, St. Louis, MO). Then, 2.5 mL of the stock solution was added to each glass chamber (2.5 L seawater) to obtain a nominal concentration of 50 μg Cu L^{-1}. Exposure conditions, durations, and endpoints were the same as those used for the CO_2 exposure test for Manila clams, described above.

Data analysis and statistics

The data were used to calculate the LC_{50} toxicity parameter associated with H^+ concentrations. $L[H^+]_{50}$ was defined as the H^+ concentration that causes lethal effect on 50 % of exposed test organisms. This parameter was estimated from the calculated mortality that provokes mortality in 50 % of the exposed population. It was calculated by the probit analysis for lethal effect and finally expressed as LpH_{50} values. Variation in the mortality rate of each organism among pH treatments was examined using analysis of variance, followed by Dunnet's test, with SPSS 21.0 (IBM, Armonk, NY) to determine significant differences ($p < 0.05$) between the results obtained for the control and each CO_2 treatment (Basallote et al. 2012). The effective pH values from a total of 55 CO_2 toxicity databases, including nine datasets from the present study, were compiled and plotted with respect to cumulative probability to obtain the working and effective pH ranges of the different test organisms.

Results and discussion

Acute effects of intertidal organisms

Significant mortality effects were obtained for benthic organisms at pH <5.70 after 96 h CO_2 exposure ($p < 0.05$) (Fig. 2a). Sub-lethal effects (such as immobilization) were significant at pH values below 6.04 ($p < 0.05$) (Fig. 2a′). The immobilization of benthic copepods was explained by metabolic suppression related to CO_2 narcosis, which has been previously observed in CO_2-tolerent species as a means of adapting to CO_2-rich environments (Kita et al. 2013; Seibel and Walsh 2003). These results indicate that benthic copepods are adversely affected by the acidic seawater (Kita et al. 2013). However, epibenthic copepods were relatively tolerant to elevated CO_2 (with lower lethal CO_2 exposure effects) compared to other organisms. This phenomenon may be explained by their being adapted to dynamic habitats, such as an intertidal rocky shore, where there is extreme variation in wave action, temperature, and salinity. Thus, intertidal organisms seem to be more adapted to environmental stress, including elevated concentrations of CO_2, compared to sub-tidal organisms (Halsband and Kurihara 2013).

For intertidal endobenthic organisms, the Manila clam exhibited significant mortality at pH <5.63 after 96 h CO_2 exposure ($p < 0.05$) (Fig. 2b). Sub-lethal effects (such as siphon releasing) were significantly induced at pH <6.22 ($p < 0.05$) (Fig. 2b′). The siphon releasing rate of Manila clams increased at the pH 6.22 and occurred in over 90 % individuals at pH values of 5.91 and 5.63. At pH 5.2, the siphon releasing rate could not be measured due to lethality (i.e., all individuals were dead). Other sub-lethal endpoints, failed shell closure,

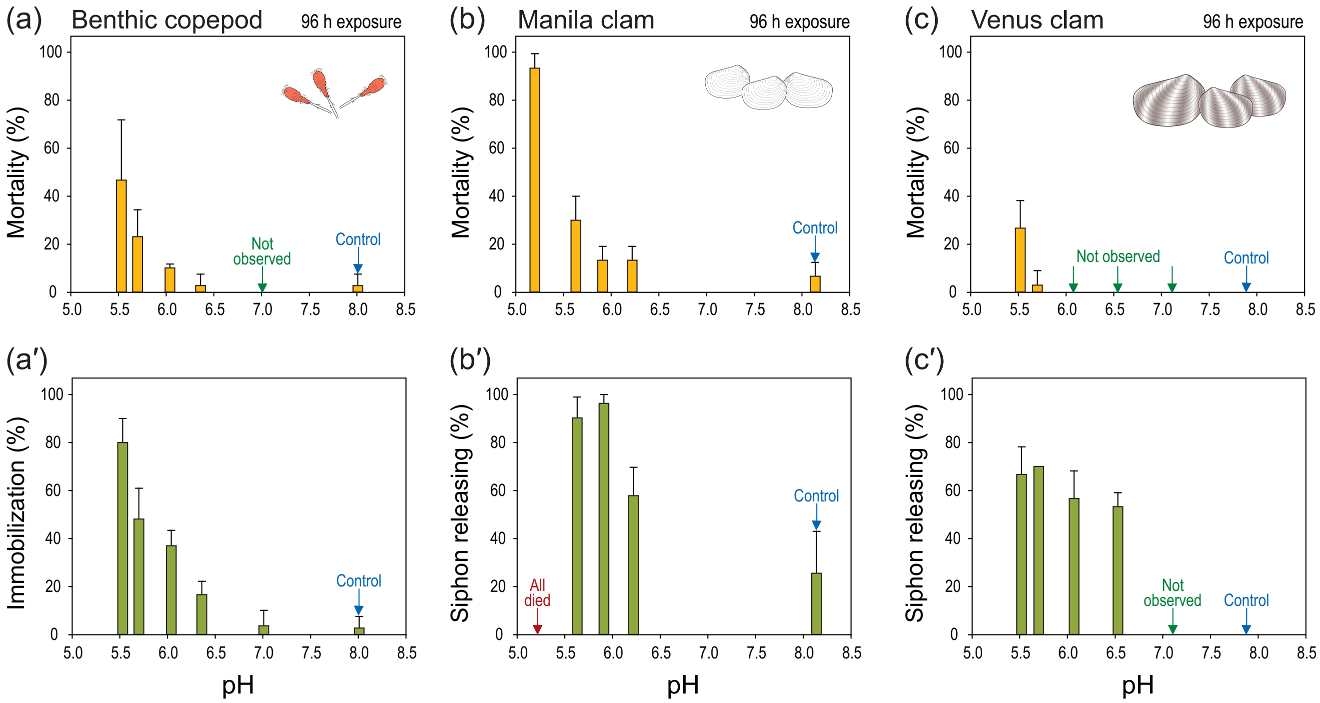

Fig. 2 Effects of elevated CO_2 on intertidal organisms. Lethal and sub-lethal effects of (*a*, *a′*) benthic copepods (*Tigriopus* sp.), (*b*, *b′*) Manila clams (*R. philippinarum*), and (*c*, *c′*) Venus clams (*M. aequilatera*). *Error bars* represent the standard deviation of replicates per treatment

and response delay of Manila clams occurred at pH <5.63 and <5.91, respectively ($p < 0.05$) (Fig. 2b′ and Table S3). In comparison, the Venus clam was relatively tolerant to elevated CO_2 compared to the Manila clam. The Venus clam only exhibited significant mortality at pH <5.52 during 96-h exposure ($p < 0.05$) (Fig. 2c). The siphon releasing rate of the Venus clam increased significantly at pH 6.53 (Fig. 2c′). In addition, failed shell closure and a significant delay in the response of Venus clams occurred at pH <6.53 ($p < 0.05$) (Tables S4 and S5).

Acute mortality of the Manila clam and Venus clam indicated that intertidal clams are relatively tolerant to elevated CO_2 compared to the other test organisms. Both species inhabit the intertidal endobenthic zone, which has unstable temperature, salinity, and turbidity of environmental conditions. In addition to a daily flush of seawater by the tide, varying concentrations of CO_2 in the intertidal area make it a highly dynamic place to live. Consequently, both species are well adapted to elevated CO_2 conditions. Moreover, clams may be tolerant to short-term acidification because their calcareous shells might assist acid-base regulation by dissolution as a buffer against changes in pH (Seibel and Walsh 2003). The siphon activity of clams has been used as a potential behavioral early warning system of water quality (El-Shenawy 2004). Many factors control siphon activity; consequently, it is difficult to conclude which environmental factors influence behavioral changes on siphon activity (Sárkány-Kiss et al. 2012). Overall, individual rates for sub-lethal effects (including the siphon activity of intertidal clams) exceeded the lethal effect at the same pH value in our study, indicating that sub-lethal effects are more sensitive endpoints for assessing the biological effects of elevated CO_2 (Fig. 2).

The sub-lethal effects of elevated CO_2 concentrations on Manila clams and Venus clams clearly varied as a function of time (Tables S3–S5). The siphon releasing rate of the Manila clam noticeably increased within 24 h at pH <6.22 (Table S4). In comparison, the siphon releasing rate, failed shell closure, and response delay of the Venus clam were significantly induced more slowly over 72 h below pH 6.53 (Table S5). Our results indicate that elevated CO_2 adversely affects clams in a time-dependent manner, with variation among species, due to differences in tolerance. Of note, alternatively, the differences in the body size between the Manila clam (25–35 mm) and Venus clam (39–59 mm) may have affected tolerance. Large-sized Venus clams have low surface area to volume ratios, resulting in a slower reaction to CO_2 exposure compared to small-sized Manila clams.

Acute effects of sub-tidal organisms

Brittle starfish exhibited significant mortality at pH <6.37 and 6.44 during 96 and 240-h exposure periods, respectively ($p < 0.05$) (Fig. 3a). Brittle starfish was more sensitive to pH values during longer exposure periods compared to shorter exposure periods (Fig. 3a′). Sub-tidal benthic organisms (such as brittle starfish) appeared to be more sensitive to elevated CO_2 compared to intertidal organisms. This result indicates that sub-tidal organisms have relatively low CO_2 tolerance.

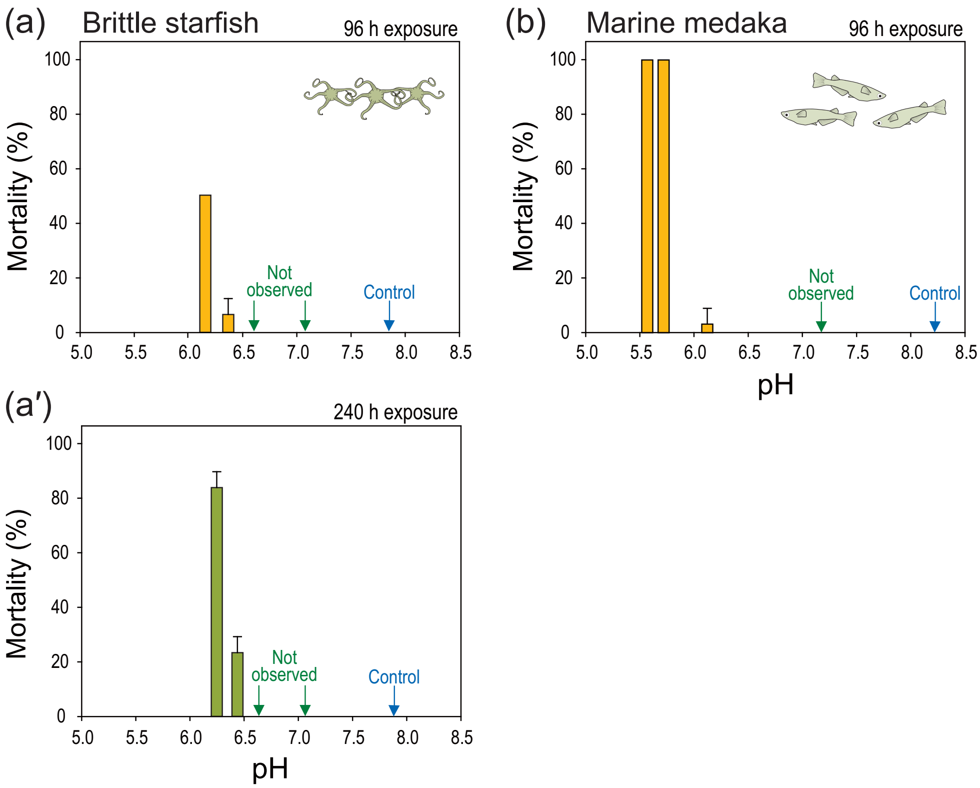

Fig. 3 Effects of elevated CO_2 on sub-tidal organisms. Lethal and sub-lethal effects of (*a*, *a*′) brittle starfish (*Ophiura* sp.) after 96 and 240 h exposure and (*b*) marine medaka (*O. melastigma*) after 96 h exposure. *Error bars* represent the standard deviation of replicates per treatment

The environmental conditions of the sub-tidal area (such as temperature, salinity, and CO_2 concentrations) are more constant compared to the intertidal area. Thus, sub-tidal benthic organisms have low metabolisms and poorer regulation abilities (Collard et al. 2013). Moreover, the skeletons of brittle starfish are composed of magnesium calcite, which is particularly sensitive to elevated CO_2 concentrations (Wood et al. 2008). Consequently, because sub-tidal organisms are adapted to survive in stable environments, they may be sensitive to elevated CO_2. Decreased carbonate saturation was monitored by elevated CO_2 concentration, and it caused a major threat to calcifying organisms for skeletogenesis. The organisms utilizing high-Mg calcite (i.e., brittle starfish) can be more sensitive to seawater acidification.

All free-living water column organisms (marine medaka) survived in the control ($pH = 8.25$), but died within 24 h at pH 5.56 and 5.71 (Fig. 3b). Adult marine medaka was relatively tolerant to elevated CO_2 compared to other test organisms. More than 90 % of all acid-base regulation in fishes occurs via ion transport processes across the branchial epithelium, whereas the intestine and kidney are also involved in ion regulation. In addition, marine fishes have various isoforms of ion exchangers for osmoregulation; therefore, they have strong capacity for regulating acid-base balance (Pörtner et al. 2004). Because fish calcify internal rather than external skeletal elements, ocean acidification is considered to have a negligible effect on fish (Baumann et al. 2012). However, elevated CO_2 conditions cause changes to fish behavior; thus, fish may avoid the effects of CO_2 exposure through avoidance behaviors (Briffa et al. 2012; Nilsson et al. 2012).

LpH_{50} values

The LpH_{50} values of test organisms following CO_2 exposure were calculated based on the lethal effects (Table 2). The units used for proton (H^+) concentrations are moles per kilogram H_2O in the NBS scale (Basallote et al. 2012). A pH value of 5.53 causes mortality in 50 % of benthic copepods. About 77 % of adult benthic copepods survived at pH 5.70, supporting the outcome of a previous study (Kita et al. 2013). The LpH_{50} values of Manila clam and Venus clam were 5.48 and 5.45, respectively. The LpH_{50} values of brittle starfish and marine medaka were 6.16 and 5.91, respectively. The LpH_{50} value of brittle starfish was greater in the treatment of long-term exposure (6.34 for 10 days) compared to that of short-term exposure (6.16 for 4 days). Among the test organisms used in the current study, brittle starfishes were the most sensitive to elevated CO_2, followed by marine medaka, Manila clams, benthic copepods, and Venus clams.

In general, intertidal organisms appeared to be more tolerant to elevated CO_2 compared to sub-tidal organisms. Because most intertidal species are passive or sessile organisms, they are likely to be directly affected by environmental stress (such

Table 2 Summary of the LpH_{50} values for intertidal and sub-tidal organisms exposed to elevated CO_2 based on lethal effects

Target organisms			Experimental conditions			Lethal effects	
Common name	Species name	Life stage	Exposure route	Duration (days)	pH range	LpH_{50}	$L[H^+]_{50}$
Intertidal organisms							
Benthic copepod	*Tigriopus* sp.	Adult	Seawater only	4	5.5–8.0	5.53 (5.43–5.59)	2.95×10^{-6}
Manila clam	*Ruditapes philippinarum*	Adult	Sediment-seawater	4	5.2–8.1	5.48 (5.39–5.57)	3.30×10^{-6}
Venus clam	*Macridiscus aequilatera*	Adult	Sediment-seawater	4	6.0–7.6	5.45 (5.25–5.51)	3.53×10^{-6}
Sub-tidal organisms							
Brittle starfish	*Ophiura* sp.	Adult	Sediment-seawater	4	6.2–7.9	6.16 (6.10–6.20)	6.89×10^{-7}
			Sediment-seawater	10	6.3–7.8	6.34 (6.30–6.38)	4.55×10^{-7}
Marine medaka	*Oryzias melastigma*	Adult	Seawater only	4	5.6–8.3	5.91 (6.01–5.57)	1.22×10^{-6}

as elevated CO_2 condition), whereas mobile animals may avoid exposure by moving to alternative sites (Tamburri et al. 2000). In general, calcifying organisms (such as corals, clams, and starfishes) are more sensitive to seawater acidification than other organisms (De Vries et al. 2013). These organisms have difficulty maintaining their external calcium carbonate skeletons under low pH conditions; thus, they are at risk from CO_2 exposure (Melzner et al. 2009).

Combined effect of CO_2 and copper on Manila clams

While copper is a naturally occurring trace element essential for some biological functions, elevated levels can be toxic to a range of marine organisms. In addition, the common metal contamination such as copper together with CO_2 exposure might stimulate the adverse effects to organisms, generally supporting a synergistic effect scenario (Campbell et al. 2014). In the present study, Manila clams exhibited significant mortality and siphon releasing at pH 6.38 after exposure to a combination of copper (50 μg Cu L^{-1}) and CO_2 ($p < 0.05$) (Fig. 4). Manila clam mortality and siphon releasing did not occur in either the positive (copper-spiked seawater) or negative (seawater) control glass chambers. A previous study reported that Manila clams exhibited a significant reduction in hemocyte superoxide dismutase activity at concentrations ranging from 60 to 110 μg Cu L^{-1} (Matozzo et al. 2001). When only exposed to CO_2 (i.e., in non-copper-spiked chambers), Manila clam mortality and siphon releasing were observed at pH <5.63 and <6.22 (Fig. 2b, b′), respectively. Thus, synergistic lethal and sub-lethal effects of CO_2 and copper exposure were detected for on Manila clams.

Anthropogenic activity has led to significant metal pollution in intertidal sediments (Pascal et al. 2010). Heavy metals may become physically absorbed and chemical bound to the sediment following release in the overlying water. Consequently, the sediment represents a potential sink for pollutants (including copper) that enter the aquatic environment during mining for CCS sites. Thus, the results of this preliminary study indicate that the presence of copper increases the toxic effects of elevated CO_2 concentrations on organisms at CCS sites. In general, the toxicity of copper is dependent on its form in the water column. Cu^{2+} forms strong complexes with carbonates and will be strongly affected by elevated CO_2, because of an increase in competition for binding sites between H^+ and metals (Pascal et al. 2010). Thus, the

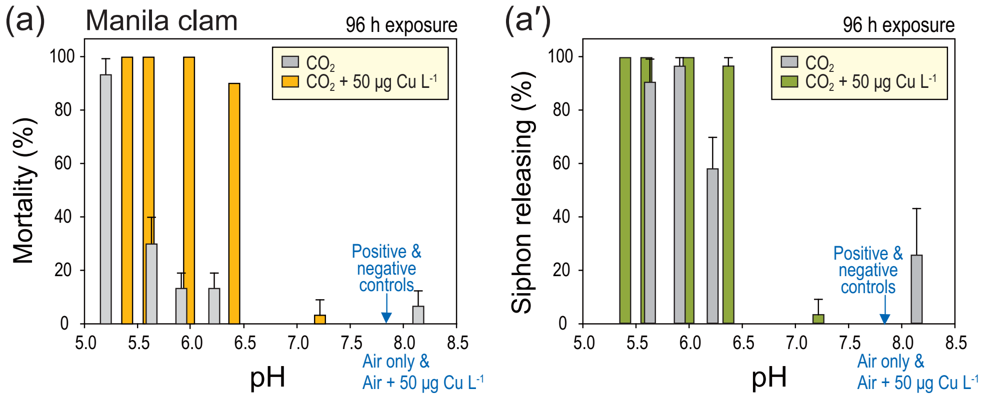

Fig. 4 Combined effects of copper and elevated CO_2 exposure. (*a*) Lethal and (*a′*) sub-lethal effects on Manila clam (*R. philippinarum*). *Error bars* represent the standard deviation of replicates per treatment

concentration of the free ionic copper form will increase, making it more toxic to organisms (Pascal et al. 2010). Until now, the combined effect of CO_2 and metal toxicity on marine organisms has been rarely studied (Millero et al. 2009; Pascal et al. 2010); thus, more complementary studies are urgently required.

Comparison to previous studies

Here, we assimilated and reviewed the effective pH values of various marine organisms associated with elevated CO_2 concentrations from published studies, focusing on the species- and endpoint-specific effects for comparison with our results (Tables 3 and 4). Acute and chronic effects of CO_2 on intertidal organisms (including benthic copepods and clams) are summarized in Table 3 (Basallote et al. 2012; Berge et al. 2006; Duarte et al. 2014; Gazeau et al. 2010; Kita et al. 2013; Michaelidis et al. 2005; Talmage and Gobler 2011). Acute and chronic lethal effects of nauplii on adult benthic copepods (*Tigriopus japonicus* or *Tigriopus* sp.) by elevated CO_2 occur at pH values ranging from 5.70 to 5.85, as demonstrated by Kita et al. (2013) and this study. Delays in hatching, spawning, and development and immobilization of benthic copepods occur at pH values ranging from 6.04 to 6.31 (Kita et al. 2013; this study). These findings seem to be explained by elevated CO_2 having a narcotic effect or causing the metabolic suppression of benthic copepods.

The effective pH values for acute and chronic mortality of adult clams (Manila clam *R. philippinarum* and the Venus clam *M. aequilatera*) ranged from 5.52 to 6.00 (Basallote et al. 2012; this study). Acute sub-lethal effects (such as siphon releasing) of adult Manila clams and Venus clams are induced at pH 6.22 and 6.53, respectively. In comparison, chronic adverse effects (including ammonia excretion, oxygen consumption, and hemolymph ion composition) of adult marine mussels (*Mytilus galloprovincialis*) are induced at pH >7.3 (Michaelidis

Table 3 Effective pH values for acute and chronic effects on intertidal organisms

Target organism		Experimental condition			Lethal and sub-lethal effects	Effective range	References
Species	Life stage	Duration (days)		pH range	Endpoints	(pH)	
Copepod							
Tigriopus japonicus	Nauplii to adult	>20	Chronic	8.16–5.85	Mortality	5.85	Kita et al. 2013
					Development	6.31	
					Mating & Spawning	6.31	
					Hatching	6.31	
Tigriopus sp.	Adult	4	Acute	8.0–5.5	Mortality	5.70	This study
					Immobilization	6.04	
Clam							
Mytilus galloprovincialis	Juvenile	90	Chronic	8.05–7.3	Shell growth	7.3	Michaelidis et al. 2005
	Adult	90	Chronic	8.05–7.3	Hemolymph ion composition	7.3	
					Oxygen consumption	7.3	
					Ammonia excretion	7.3	
Ruditapes philippinarum	Adult	10	Chronic	8.0–5.5	Seawater mortality	6.0	Basallote et al. 2012
				8.0–6.0	Sediment mortality	6.0	
Ruditapes philippinarum	Adult	4	Acute	8.0–5.2	Mortality	5.63	This study
					Siphon releasing	6.22	
Macridiscus aequilatera	Adult	4	Acute	7.6–6.0	Mortality	5.52	This study
					Siphon releasing	6.53	
Mytilus chilensis	Juvenile	60	Chronic	8.0–7.6	Calcification rate	7.6	Duarte et al. 2014
Mytilus edulis	Embryo	2	Acute	8.15–7.58	Developmental period	7.58	Gazeau et al. 2010
					Shell growth	7.81	
					Hatching rate	7.58	
	Larvae	14	Chronic	8.03–7.78	Shell growth	7.78	Berge et al. 2006
	Juvenile	44	Chronic	8.1–6.7	Growth	7.6	
Mercenaria mercenaria	Larvae	36	Chronic	8.17–7.53	Mortality	7.51	Talmage and Gobler 2011
					Metamorphosis	8.05	
					Shell growth	8.05	
					Nutritive condition	7.81	

Table 4 Effective pH values for acute and chronic effects on sub-tidal organisms

Target organism		Experimental condition			Lethal and sub-lethal effects	Effective range	References
Species	Life stage	Duration (days)		pH range	Endpoints	(pH)	
Copepod							
Calanus finmarchicus	Adult	3	Acute	8.23–6.95	Egg production	6.95	Mayor et al. 2007
Acartia steueri	Adult	8	Chronic	8.14–6.84	Egg production	6.84	Kurihara et al. 2004
					Hatching rate	6.9	
Centropages typicus	Adult	1–4	Acute	8.04–6.71	Egg production	6.71	McConville et al. 2013
					Hatching rate	6.71	
Temora longicornis	Adult	1–4	Acute	8.04–6.71	Exposure period	7.8	McConville et al. 2013
Clam							
Argopecten irradians	Larvae	68	Chronic	8.17–7.53	Mortality	7.51	Talmage and Gobler 2011
					Metamorphosis	8.05	
					Shell growth	8.05	
					Nutritive condition	8.05	
Crassostrea virginica	Juvenile	140	Chronic	8.3–7.5	Mortality	7.5	Beniash et al. 2010
					Dry shell mass	7.5	
					Shell hardness	7.5	
					Soft-tissue mass	7.5	
					Oxygen consumption	7.5	
	Adult	14	Chronic	8.3–7.5	ADP concentration	7.5	
Echinoderm							
Ophiura sp.	Adult	4	Acute	7.9–6.2	Mortality	6.37	This study
			Chronic	7.8–6.3	Mortality	6.44	
Fish							
Rachycentron canadum	Egg to Larvae	21	Chronic	8.13–7.04	Body length and shape	7.22	Bignami et al. 2013
					Swimming ability	7.79	
					Otolith deformation	7.79	
Seriola quinqueradiata	Adult	3	Acute	8.25–6.26	Mortality	6.26	Lee et al. 2003
					Heart capacity	6.26	
					Blood pH	6.26	
Sparus aurata	Larvae	3	Acute	8.0–5.5	Seawater mortality	6.0	Basallote et al. 2012
					Sediment elutriate mortality	7.0	
Oryzias melastigma	Adult	4	Acute	8.3–5.6	Mortality	5.71	This study
Sparus aurata	Juvenile	10	Chronic	8.05–7.3	Blood acid-base status	7.3	Michaelidis et al. 2007
					Enzyme activity	7.3	

et al. 2005). In addition, the embryonic, larval, and juvenile stages of various clams (blue mussel *Mytilus edulis*, chilean mussel *Mytilus chilensis*, and hard clam *Mercenaria mercenaria*) are more sensitive to pH values caused by elevated CO_2 compared to adult clams (Berge et al. 2006; Duarte et al. 2014; Gazeau et al. 2010; Talmage and Gobler 2011). Biological adverse effects were significantly induced with respect to shell growth, metamorphosis, nutrient condition, calcification rate, hatching rate, and development of clams by acute and chronic CO_2 exposure (effective pH values 7.51–8.05). Decreasing CO_2 sensitivity during development from the embryonic to adult stages may be due to a decrease in the surface area to volume ratio and increasing ability of adaptation to dynamic intertidal environments (Halsband and Kurihara 2013).

The effects of elevated CO_2 on sub-tidal organisms (including copepods, clams, starfishes, and fishes) are also summarized in Table 4 (Basallote et al. 2012; Beniash et al. 2010; Bignami et al. 2013; Duarte et al. 2014; Kurihara et al. 2004; Lee et al. 2003; Mayor et al. 2007; McConville et al. 2013; Michaelidis et al. 2007; Talmage and Gobler 2011). Sub-tidal organisms generally tend to be more sensitive to elevated CO_2 concentrations compared to intertidal organisms. Acute mortality of adult fish, gilt-head sea bream (*Sparus aurata*), marine medaka (*O. melastigma*), and yellowtail (*Seriola quinqueradiata*) occurs at pH values ranging from 5.71 to 6.26 (Basallote et al. 2012; Lee et al. 2003; this study). Lethal pH values for brittle starfish (*Ophiura* sp.) occur at 6.37 and 6.44 for acute and chronic exposure, respectively, based on the results of the present study, providing the first report on how CO_2 affects in situ living starfish.

Hatching rate and egg production of marine planktonic copepods (*Acartia steueri*, *Calanus finmarchicus*, and *Centropages typicus*) are affected by acute and chronic exposure to CO_2 at pH values ranging from 6.71 to 6.95 (Kurihara et al. 2004; Mayor et al. 2007; McConville et al. 2013). In particular, exposure duration regulates the extent to which copepod egg production and hatching rates are inhibited (*C. typicus* and *Temora longicornis*) (McConville et al. 2013). Fish eggs, larvae, and juveniles (corbia, *Rachycentron canadum*) are more sensitive to elevated CO_2 concentrations than adults, with body length and shape, enzyme activity, blood acid-base status, and swimming ability being affected by pH values ranging from 7.22 to 7.79 (Bignami et al. 2013). The larvae and juvenile of sub-tidal clams (such as bay scallop *Argopecten irradians* and the eastern oyster *Crassostrea virginica*) are relatively sensitive to chronic CO_2 exposure, with biological endpoints being influenced by oxygen consumption, soft-tissue mass, shell hardness, and nutrient condition at pH ranges of 7.50 to 8.05 (Beniash et al. 2010; Talmage and Gobler 2011).

Conclusion

In conclusion, ocean acidification associated with CO_2 leakage at CCS sites could have lethal and/or sub-lethal effects on various marine organisms. CO_2 leakage from CCS sites could cause pH values to decrease to between 4.0 and 6.0, which would produce significant acute and chronic lethal and sub-lethal effects on most intertidal and sub-tidal organisms. In summary, our results support that CO_2 exposure shows species-specific biological effects on marine organisms, with these effects being strongly dependent on (1) pH value, (2) exposure duration, and (3) CO_2 tolerance of organisms in different habitats. Additionally, previous findings have suggested that biological effects were strongly associated with (4) the developmental stages of organisms (Basallote et al. 2012) and (5) the animals' skeletal composition (Ries et al. 2009). The results of the present study provide a valuable contribution to the existing database about the biological effects for elevated CO_2 concentrations on marine organisms. More complementary studies are recommended to obtain more precise results for the use in the ecosystem management of specific CCS site.

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<Supplemental Materials>

Lethal and sub-lethal effects of elevated CO_2 concentrations on marine benthic invertebrates and fish

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Table S1. Summary of carbonate system speciation in the test seawater.

Temp (˚C)	**Sal (psu)**	**pH (NBS scale)**	**A_T (mmol kg^{-1} SW)**	**TC (mmol kg^{-1} SW)**	**HCO_3^- (mmol kg^{-1} SW)**	**CO_3^{2-} (mmol kg^{-1} SW)**	**CO_2 (mmol kg^{-1} SW)**	**pCO_2 (µatm)**	**Ω_{Cal}**	**Ω_{Arag}**
Benthic copepod										
23(±1)	30(±1)	8.01(±0.12)	2301.50	2135.76	1983.5	131.85	20.41	667.3	3.29	2.13
23(±1)	30(±1)	7.01(±0.03)		2513.47	2265.3	15.05	233.12	7620.6	0.38	0.24
23(±1)	30(±1)	6.36(±0.06)		3351.50	2293.7	3.41	1054.39	34467.0	0.09	0.06
23(±1)	30(±1)	6.04(±0.04)		4507.64	2298.5	1.63	2207.51	72161.3	0.04	0.03
23(±1)	30(±1)	5.70(±0.05)		7138.78	2301.7	0.75	4836.33	158094.8	0.02	0.01
23(±1)	30(±1)	5.53(±0.03)		9462.11	2303.3	0.51	7158.30	233997.7	0.01	0.01
Manila clam										
20(±1)	30(±1)	8.14(±0.01)	2286.42	2080.85	1910.1	155.46	15.29	460.9	3.85	2.47
20(±1)	30(±1)	6.22(±0.02)		3803.42	2281.6	2.23	1519.59	45794.3	0.06	0.047
20(±1)	30(±1)	5.91(±0.03)		5393.32	2285.0	1.10	3107.22	93638.9	0.03	0.02
20(±1)	30(±1)	5.63(±0.04)		8214.87	2287.4	0.58	5926.89	178612.3	0.01	0.01
20(±1)	30(±1)	5.20(±0.01)		18279.26	2292.3	0.21	15986.75	481775.5	0.01	0.00
Venus clam										
15(±1)	30(±1)	7.82(±0.10)	2315.67	2253.89	2144.0	70.67	39.22	1021.8	1.74	1.10
15(±1)	30(±1)	7.14(±0.18)		2492.70	2277.6	15.68	199.42	5195.5	0.39	0.25
15(±1)	30(±1)	6.53(±0.28)		3133.08	2306.5	3.90	822.68	21433.7	0.10	0.06
15(±1)	30(±1)	6.07(±0.19)		4694.14	2313.2	1.36	2379.58	61996.5	0.03	0.02
15(±1)	30(±1)	5.70(±0.09)		7902.54	2316.3	0.58	5585.66	145525.9	0.01	0.01
15(±1)	30(±1)	5.52(±0.16)		10777.97	2317.8	0.38	8459.79	220407.0	0.01	0.01

A_T: Total alkalinity; TC: total inorganic carbon content; Ω_{Cal}: saturation state of calcite; Ω_{Arag}: saturation state of aragonite.

Table S1. (Continue).

Temp (°C)	**Sal** (psu)	**pH** (NBS scale)	**A_T** (mmol kg^{-1} SW)	**TC** (mmol kg^{-1} SW)	**HCO_3^-** (mmol kg^{-1} SW)	**CO_3^{2-}** (mmol kg^{-1} SW)	**CO_2** (mmol kg^{-1} SW)	**pCO_2** (µatm)	**Ω_{Cal}**	**Ω_{Arag}**
Brittle starfish **(acute)**										
10(±0.1)	35(±1)	7.86(±0.02)	2311.93	2237.47	2126.4	74.39	36.68	839.1	1.77	1.13
10(±0.1)	35(±1)	7.06(±0.01)		2541.34	2280.5	12.64	248.20	5678.2	0.30	0.19
10(±0.1)	35(±1)	6.56(±0.01)		3098.47	2302.1	4.04	792.33	18126.8	0.10	0.06
10(±0.1)	35(±1)	6.37(±0.01)		3537.56	2305.8	2.61	1229.15	28120.3	0.03	0.04
10(±0.1)	35(±1)	6.16(±0.01)		4306.05	2308.6	1.61	1995.84	45660.5	0.04	0.02
Brittle starfish **(chronic)**										
10(±0.1)	35(±1)	7.78(±0.02)	2319.44	2270.38	2162.6	62.93	44.85	1026.0	1.50	0.95
10(±0.1)	35(±1)	7.06(±0.01)		2549.59	2287.9	12.69	249.00	5696.7	0.30	0.19
10(±0.1)	35(±1)	6.62(±0.01)		3004.62	2308.1	4.65	691.87	15828.6	0.11	0.07
10(±0.1)	35(±1)	6.44(±0.01)		3364.21	2312.1	3.08	1049.03	23999.6	0.07	0.05
10(±0.1)	35(±1)	6.25(±0.01)		3943.89	2315.1	1.99	1626.80	37217.8	0.05	0.03
Marine medaka										
25(±0.1)	35(±1)	8.25(±0.06)	2276.7	1937.77	1690.9	237.71	9.16	323.7	5.72	3.77
25(±0.1)	35(±1)	7.19(±0.01)		2374.99	2210.4	27.07	137.52	4859.2	0.65	0.43
25(±0.1)	35(±1)	6.11(±0.04)		3973.41	2271.8	2.31	1699.30	60043.2	0.06	0.04
25(±0.1)	35(±1)	5.71(±0.03)		6554.43	2276.4	0.92	4277.11	151127.5	0.02	0.01
25(±0.1)	35(±1)	5.56(±0.01)		8324.06	2277.9	0.65	6045.51	213612.3	0.02	0.01
Manila clam **(copper)**										
20(±1)	35(±1)	7.85(±0.09)	2312.86	2198.76	2069.4	98.98	30.38	940.5	2.37	1.54
20(±1)	35(±1)	7.78(±0.07)		2224.55	2102.4	85.89	36.26	1122.6	2.05	1.33
20(±1)	35(±1)	7.23(±0.04)		2412.96	2249.5	25.81	137.65	4261.9	0.62	0.40
20(±1)	35(±1)	6.38(±0.09)		3305.98	2304.1	3.73	998.15	30904.9	0.09	0.06
20(±1)	35(±1)	5.98(±0.11)		4825.73	2310.3	1.49	2513.94	77837.0	0.04	0.02
20(±1)	35(±1)	5.56(±0.04)		8938.7	2314.3	0.57	6623.83	205087.8	0.01	0.01
20(±1)	35(±1)	5.36(±0.12)		12824.61	2316.4	0.36	10507.85	325345.4	0.01	0.01

A_T: Total alkalinity; TC: total inorganic carbon content; Ω_{Cal}: saturation state of calcite; Ω_{Arag}: saturation state of aragonite.

Table S2. Two-way ANOVA of pH values in the treatment versus experimental setup.

Variable	**Degrees of freedom**	**Sum of squares (% of total)**	***F* value**	**Pr(>*F*)**
CO_2 (%)	1	45.63	170.711	<0.05
Tank (1, 2, 3)	2	0.41	0.761	0.467
Residuals	92	24.86		

Table S3. Sub-lethal effects of elevated CO_2 on the manila clam (*Ruditapes philippinarum*).

Endpoint	**Exposure duration (h)**	**pH values exposed to $CO_{2\ gas}$**				
		8.14	**6.22**	**5.91**	**5.63**	**5.2**
Siphon releasing (%)	96	O	O	O	O	X
Response delay (%)	96	X	X	O	O	O
Shell closed failure (%)	96	X	X	X	O	O

X: not significant; O: significantly observed.

Table S4. Time-dependent lethal and sub-lethal effects of elevated CO_2 on manila clams (*Ruditapes philippinarum*) measured at 24 h intervals.

Experimental condition		**Individual rate (%)**			
End points	**pH**	**24 h**	**48 h**	**72 h**	**96 h**
Siphon releasing	8.14	13.3 (±5.8)	24.1 (±15.9)	31.1 (±10.2)	25.6 (±17.5)
	6.22	60.0 (±10.0)	70.0 (±10.0)	60.0 (±10.0)	57.9 (±11.8)
	5.91	93.3 (±5.8)	96.7 (±5.8)	96.7 (±5.8)	96.3 (±6.4)
	5.63	100.0 (±0.0)	100.0 (±0.0)	100 (±0.0)	90.3 (±8.7)
	5.20	70.0 (±10.0)	46.7 (±11.5)	0	0
Mortality	8.14	0	3.3 (±5.8)	3.3 (±5.8)	6.7 (±5.8)
	6.22	0	0	0	13.3 (±5.8)
	5.91	0	3.3 (±5.8)	3.3 (±5.8)	13.3 (±5.8)
	5.63	0	3.3 (±5.8)	3.3 (±5.8)	30.0 (±10.0)
	5.20	0	0	0	100.0 (±0.0)

Table S5. Time-dependent lethal and sub-lethal effects of elevated CO_2 on venus clams (*Macridiscus aequilatera*) measured at 12 h intervals.

Experimental condition		**Individual rate (%)**							
End points	**pH**	**12 h**	**24 h**	**36 h**	**48 h**	**60 h**	**72 h**	**84 h**	**96 h**
Siphon releasing	7.82	0	0	0	0	0	0	0	0
	7.14	0	0	0	0	0	0	0	0
	6.53	6.7 (±11.6)	13.3 (±11.6)	13.3 (±11.6)	16.7 (±5.8)	26.7 (±5.8)	40.0 (±0.0)	50.0 (±0.0)	53.3 (±5.8)
	6.07	16.7 (±11.6)	20.0 (±10.0)	20.0 (±10.0)	20.0 (±10.0)	26.7 (±5.8)	46.7 (±11.6)	53.3 (±5.8)	56.7 (±11.6)
	5.70	30.0 (±10.0)	40.0 (±10.0)	40.0 (±10.0)	43.3 (±5.8)	43.3 (±5.8)	66.7 (±5.8)	66.7 (±5.8)	70.0 (±0.0)
	5.52	3.3 (±5.8)	3.3 (±5.8)	13.3 (±11.6)	26.7 (±5.8)	40.0 (±10.0)	53.3 (±15.3)	56.7 (±11.6)	66.7 (±11.6)
Response delay	7.82	0	0	0	0	0	0	0	0
	7.14	0	0	0	0	0	0	0	0
	6.53	6.7 (±11.6)	13.3 (±11.6)	13.3 (±11.6)	16.7 (±5.8)	26.7 (±5.8)	40.0 (±0.0)	50.0 (±0.0)	53.3 (±5.8)
	6.07	16.7 (±11.6)	20.0 (±10.0)	20.0 (±10.0)	20.0 (±10.0)	26.7 (±5.8)	46.7 (±11.6)	53.3 (±5.8)	56.7 (±11.6)
	5.70	30.0 (±10.0)	40.0 (±10.0)	40.0 (±10.0)	43.3 (±5.8)	43.3 (±5.8)	66.7 (±5.8)	66.7 (±5.8)	70.0 (±0.0)
	5.52	3.3 (±5.8)	3.3 (±5.8)	13.3 (±11.6)	26.7 (±5.8)	40.0 (±10.0)	53.3 (±15.3)	56.7 (±11.6)	66.7 (±11.6)
Shell closed failure	7.82	0	0	0	0	0	0	0	0
	7.14	0	0	0	0	0	0	0	0
	6.53	6.7 (±5.8)	10.0 (±10.0)	10.0 (±10.0)	10.0 (±10.0)	26.7 (±11.5)	36.7 (±11.5)	43.0 (±5.8)	43.0 (±5.8)
	6.07	20.0 (±10.0)	30.0 (±20.0)	30.0 (±20.0)	40.0 (±20.0)	40.0 (±20.0)	60.0 (±20)	63.0 (±15.3)	63.0 (±15.3)
	5.70	43.0 (±5.8)	43.0 (±5.8)	43.0 (±5.8)	46.0 (±11.5)	46.0 (±11.5)	76.7 (±5.8)	76.7 (±5.8)	76.7 (±5.8)
	5.52	40.0 (±0.0)	43.0 (±5.8)	43.0 (±5.8)	60.0 (±10.0)	60.0 (±15.3)	63.3 (±15.3)	66.7 (±11.5)	66.7 (±11.5)
Mortality	7.82	0	0	0	0	0	0	0	0
	7.14	0	0	0	0	0	0	0	0
	6.53	0	0	0	0	0	0	0	0
	6.07	0	0	0	0	0	0	0	0
	5.70	0	0	0	0	0	3.3 (±5.8)	3.3 (±5.8)	3.3 (±5.8)
	5.52	0	0	0	0	0	26.7 (±11.5)	26.7 (±11.5)	26.7 (±11.5)

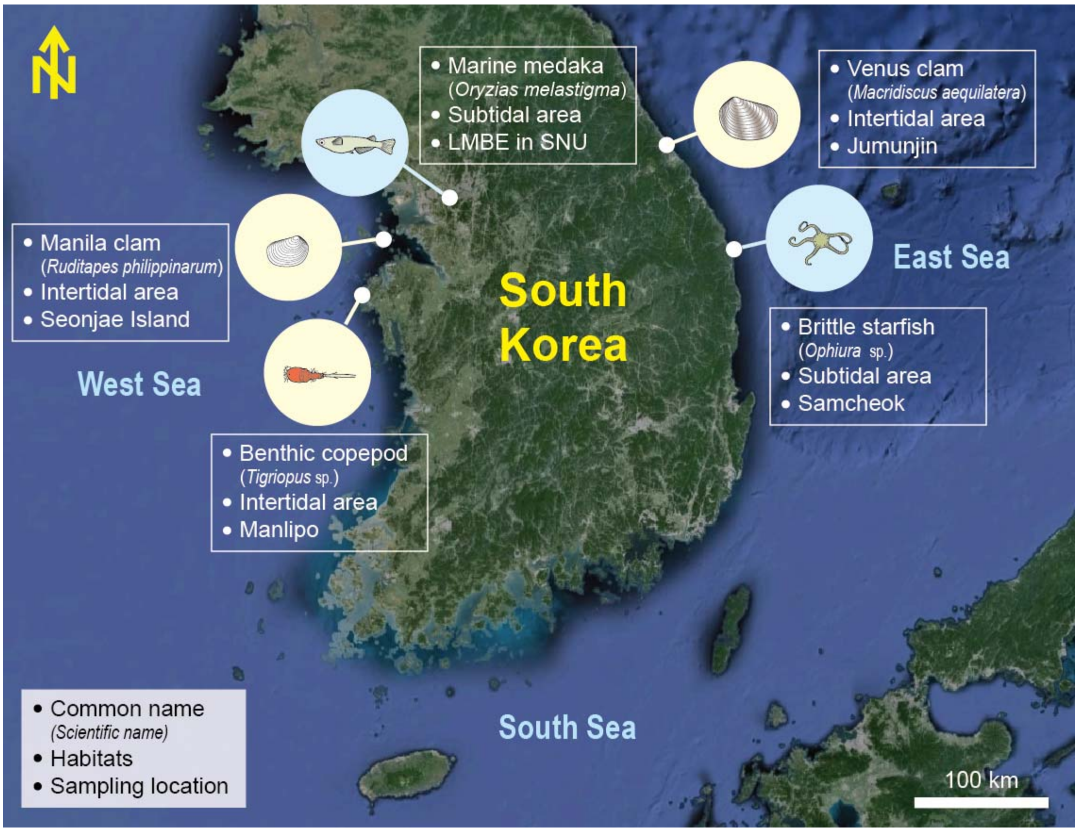

Fig. S1. Collection sites of test organisms in Korean coastal waters.