



RESEARCH PAPER



# The Potential Impact of Underwater Exhausted CO<sub>2</sub> from Innovative Ships on Invertebrate Communities

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## Abstract

Liquefied natural gas (LNG) powered ships equipped with an underwater exhaust system to reduce the ship's water resistance could form a future generation of energy-efficient ships. The potential consequences of the underwater exhaust gas to the local ecosystems are still unknown. Especially, the CO<sub>2</sub> levels may locally exceed estimated future global levels. The present study exposes marine communities to a wide range of CO<sub>2</sub> dosages, resulting in pH 8.6–5.8 that was remained for 49 days. We found that the zooplankton and benthic community were adversely affected by high CO<sub>2</sub> exposure levels. In detail, (1) between pH 6.6 and 7.1 polychaete worms became the dominating group of the benthic community and their larvae dominated the zooplankton group. (2) Due to the reduced grazing pressure and the flux of nutrients from decaying organic material planktonic microalgae (phytoplankton) started blooming at the highest exposure level. The periphyton (fouling microalgae) community was not able to take advantage under these conditions. (3) Marine snails' (periwinkle) shell damage and high mortality were observed at pH < 6.6. However, the growth of the surviving periwinkles was not directly related to pH, but was positively correlated with the availability of periphyton and negatively correlated with the polychaete worm density that most likely also used the periphyton as food source. Our result indicates that the impact of underwater exhaust gasses depends on various factors including local biological and abiotic conditions, which will be included in future research.

## Article Highlights

- A marine mesocosm study was performed with pH levels ranging from 8.6 to 5.8 as a result of CO<sub>2</sub> injection.
- Planktonic algae bloomed in pH 5.8 mesocosms, while low biomass of sessile algae was produced.
- Polychaete worms dominated the benthic community and their larvae dominated the zooplankton between pH 6.6 and 7.1.
- High CO<sub>2</sub> levels/low pH resulted in shell damage and increased mortality of periwinkles (marine snails).
- The biomass of periwinkle showed no direct relation with pH, but positive linear correlated with the availability of sessile algae and their major food source.

**Keywords** Underwater exhaust · Periphyton · Plankton · Benthos · Mesocosm · Ocean acidification

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## Introduction

The marine transport sector has been a fast-growing sector of the global economy and it contributes significantly to anthropogenic air pollution. In the period of 2007–2012, on average, shipping emission accounted for about 2.8% of annual greenhouse gas emission (IMO 2014). The emitted air pollution contributes to climate change and adverse impacts on human and environmental health in several ways (Corbett et al. 2007; Fuglestad et al. 2009). The International Maritime Organization (IMO), thus, implemented several regulations to reduce the sulfur oxides' ( $\text{SO}_x$ ) content in the fuel and nitrogen oxides' ( $\text{NO}_x$ ) emissions from shipping operations to the air (IMO ed. 2013; IMO 2016). The European Commission's white paper 2011 "Roadmap to a Single European Transport Area" described the goal of reducing shipping carbon emissions by 40% by 2050 in the European Union area (Commission 2011).

To comply with the regulations of reducing  $\text{NO}_x$ ,  $\text{SO}_x$ , and  $\text{CO}_2$  emissions all together, the interest in using Liquefied Natural Gas (LNG) engines in ships has increased, especially when the price of conventional marine fuels is rising while the regional price of natural gas declines (Schinas and Butler 2016). LNG consists mainly of methane, which seems promising to meet the gas emissions limits (except carbon), and may also reduce fuel consumption up to 15% (Burel et al. 2013). Besides shifting to LNG powered ships, to lower the direct emissions to the atmosphere, the maritime industries tend to apply underwater exhaust systems to minimize pollution on working decks (Sapra et al. 2017; Van Biert et al. 2016). In addition, the potential of using an underwater exhaust system to reduce the ship's water resistance is under study. However, the impact of underwater exhaust gas from LNG powered ship on the marine ecosystems is largely unknown. Apart from inert nitrogen gas, hydrocarbons, and nanoparticles, exhaust gas from LNG powered engines contains substantial amounts of  $\text{CO}_2$  (Anderson et al. 2015).

The underwater  $\text{CO}_2$  emission may significantly increase the locally dissolved  $\text{CO}_2$  level and could exacerbate local ocean acidification. Ocean acidification is predicted to have both direct and indirect effects on habitat-forming organisms, which, in turn, mediates shifts in community production (Sorte and Bracken 2015) and biodiversity, such as lowering the species diversity in coral reefs, mussel beds, and some macroalgal habitats (Sunday et al. 2016).

Besides the direct influence of elevated  $\text{CO}_2$  concentrations on marine organisms, species interaction via the food web is included in more recent studies (Isari et al. 2016; Sorte and Bracken 2015; Taucher et al. 2017). However, these studies mainly focus on plankton communities. For example, Isari et al. (2016) showed that elevated  $\text{CO}_2$

concentrations (up to 1296  $\mu\text{atm } p\text{CO}_2$ ) have no significant effects on the nutritional composition of dinoflagellates, and, in turn, have minimal impact on the fecundity and hatching success of the calanoid copepods that feed on them. In addition, Taucher et al. (2017) found that the biomass of copepods increased with 30–40% in high  $\text{CO}_2$  concentrations (up to 760  $\mu\text{atm } p\text{CO}_2$ ) as a result of increased primary production. Therefore, to take into account the indirectly effects of underwater exhausted  $\text{CO}_2$  on local marine invertebrate, a more complex food web system with multi-communities should be studied under elevated  $\text{CO}_2$  concentrations.

In spite of a significant amount of studies that concern climate change, with expected future atmospheric  $\text{CO}_2$  levels and rising temperature (Currie et al. 2017; Sorte and Bracken 2015; Vaqué et al. 2019), studies that cover a wide range of  $\text{CO}_2$  concentrations under current environmental conditions remain rare (de Vries et al. 2013; Wittmann and Pörtner 2013). A complete  $\text{CO}_2$  dose–response relation is not only crucial to quantify a (no) effect level (de Vries et al. 2013; Wittmann and Pörtner 2013), but also necessary to assess the potential impact of extremely low pH levels that may locally result from underwater exhausted  $\text{CO}_2$ . Therefore, we present a mesocosm study including plankton and benthic communities under a wide range of  $\text{CO}_2$  dosages, which resulted in a pH ranging from 8.6 (control) to 5.8. The latter being the lowest pH level that can be reached by directly injecting  $\text{CO}_2$  into seawater. The aim of the study was to achieve more information about the direct and indirect responses of marine communities to this wide range of  $\text{CO}_2$  levels.

**Table 1** The mesocosm number with treated fixed  $\text{CO}_2$  flow rate, the calculated  $p\text{CO}_2$  values, and the mean pH level of the treatments. The mean pH levels were used to define different treatments in the result figures

Mesocosm nr.	Fixed $\text{CO}_2$ flow rate (ml/min)	Mean $p\text{CO}_2$ ( $\mu\text{atm}$ )	Mean pH level
M6 M13	0	114	8.6 (control)
M2 M14	1	1410	7.8
M5 M11	5	3032	7.4
M8 M10	10	6678	7.1
M1 M7	20	29,653	6.6
M3 M12	73	79,954	6.2
M4 M9	310	179,013	5.8

## Materials and Methods

### Experimental Setup

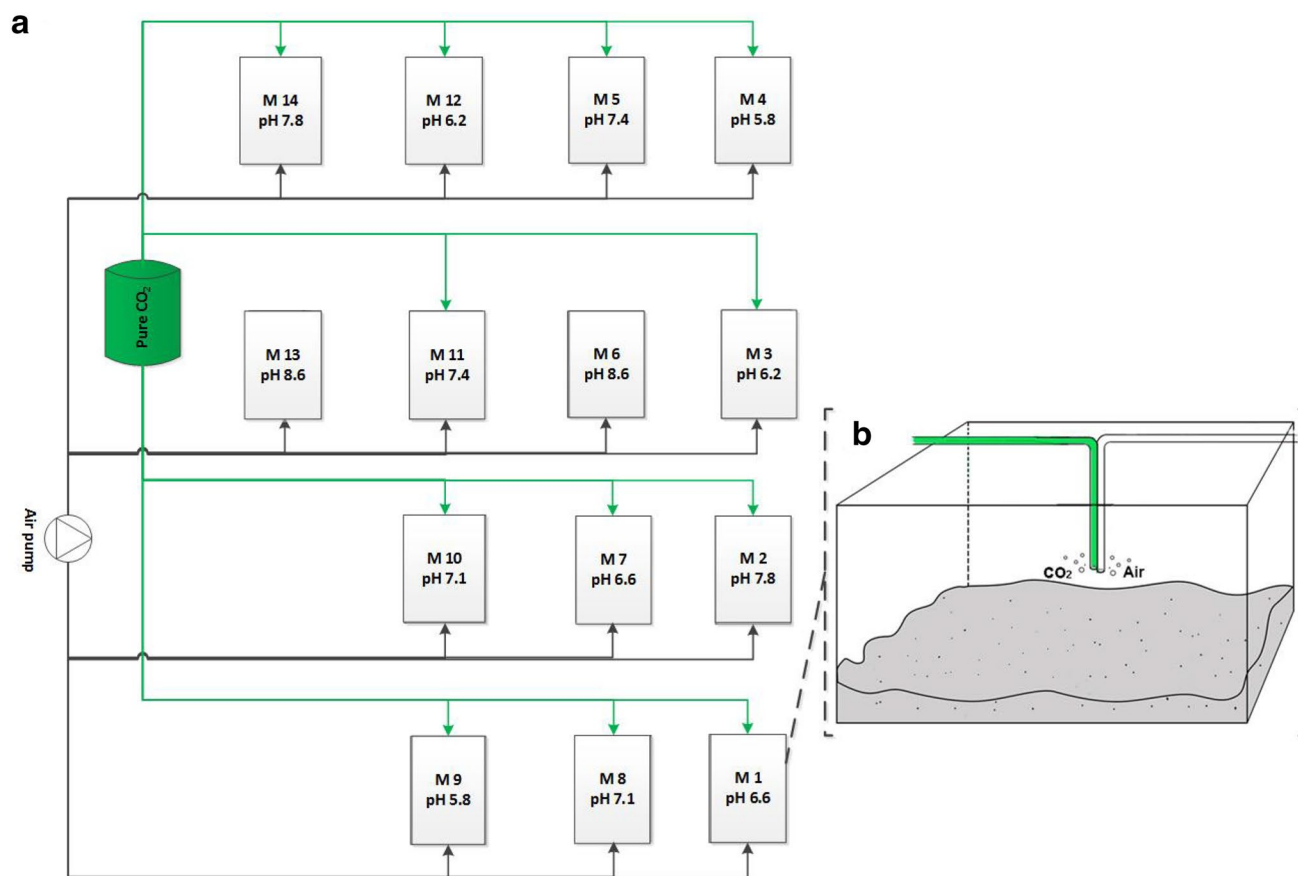
Fourteen mesocosms were provided with fixed CO<sub>2</sub> fluxes, resulting in seven pH levels ranging from 8.6 to 5.8 (Table 1) for 49 days (June 12<sup>th</sup>–July 31<sup>st</sup> 2017). The mesocosms (600 l) were filled with about 8 cm natural sandy sediment that was collected from the coastal North Sea and natural seawater collected from the Eastern Scheldt, The

Netherlands. This site is often used as a reference site in marine ecotoxicological studies in The Netherlands [e.g., (Foekema et al. 2012; Kuiper et al. 2007)]. After installation, the mesocosms were allowed 28 days to stabilize, while the water was circulated through all mesocosms until the start of the CO<sub>2</sub> exposure. Each mesocosm was covered with a transparent lid as a defense against rainfall, birds and litter. Evaporation losses were replenished with demi-water to maintain salinity at  $32 \pm 1$  throughout the study.

Phyto- and zooplankton and small benthic invertebrate species were naturally introduced to the mesocosm with the water and sediment. In addition, from relatively pristine field locations, identical number of macroinvertebrates, periwinkle (*Littorina littorea*), mud snail (*Peringia ulvae*), and mud shrimp (*Corophium volutator*) were introduced into each mesocosm (Table 2) during the first days of the establishment phase. They were representatives from various taxonomic classes that are commonly present in shallow soft sediment coastal ecosystems. Of each macroinvertebrate species that was introduced, a representative sub-sample was

**Table 2** Species and number of macroinvertebrate introduced to each mesocosm

Group	Species	Common name	Nr. per mesocosm
Mollusc	<i>Littorina littorea</i>	Periwinkle	20
Mollusc	<i>Peringia ulvae</i>	Mud snail	40
Crustacean	<i>Corophium volutator</i>	Mud shrimp	300



**Fig. 1** Schematic of the experiment setup. Fourteen mesocosms (M) are connected with both air pump and CO<sub>2</sub> tank (a). The treatment of each mesocosm is randomly selected. The mesocosms are filled with

about 8 cm natural sandy sediment and natural seawater (b). Both CO<sub>2</sub> and compressed air are continuously injected in the center of the mesocosm at about 10 cm above the sediment

stored at  $-20^{\circ}\text{C}$  for determination of initial size and biomass at a later stage.

## CO<sub>2</sub> Application

The water column of each mesocosm was continuously aerated with 1300 ml/min compressed air in the center of the mesocosm at about 10 cm above the sediment, to ensure gas exchange and sufficient mixing of the water column. At the start of the exposure phase, the treatments were created by additional bubbling of CO<sub>2</sub> (Foodgrade, supplier Linde Gas BV, The Netherlands) from the same position at six different fixed flow rates (Table 1 and Fig. 1). Subsequently, a range of pH in steps of about 0.4 could be attained in the water column from the lowest attainable pH of 5.8 to a natural pH level of 8.6. Treatments were assigned randomly over the mesocosms (Fig. 1).

## Water Analysis

Water temperature, oxygen saturation level, salinity, and alkalinity were measured weekly starting at 9 a.m. (local time) by submerging electrodes (Hach LDO101, LDO101, and CDC401, respectively) and alkalinity test kit (Hach model AL-DT). The pH level was measured daily by electrode pH meter (Hach PHC101) at 10 a.m. (local time) at half water depth. In the present study, the so-called Seacarb package (Lavigne and Gattuso 2010) in R (Team 2013) was used to express all exposures as  $p\text{CO}_2$  in microatmosphere ( $\mu\text{atm}$ ) based on alkalinity, pH, salinity, and temperature values. On days 0 and 49, water samples were collected for analyzing nitrite, nitrate, ammonia, silicate, and orthophosphate level by means of a Aquakem Autoanalyzer with spectrophotometric detection, following Netherlands standard NEN-6604 (NEN 2007).

## Sampling and Analysis

Phytoplankton samples were taken on every Monday at 10 a.m. (local time) at approximately 30 cm below water surface. Phytoplankton biomass was measured in these samples as chlorophyll-*a* concentration by means of a 1 Hz-kuvetten Fluorimeter (BBE-Moldaenke AlgaeLabAnalyser BG43000).

The development of sessile algae (periphyton) was monitored on four glass microscope slides (76 × 26 mm). These slides were introduced in vertical position facing south at ca. 10 cm below the water surface in the mesocosm on the day that the CO<sub>2</sub> application started. To avoid grazing by periwinkles, the microscope slides were located about 3 cm away from the wall of the mesocosms. The chlorophyll-*a* fluorescence on these slides was measured during the experiment period, on days 2, 8, 17, 31, 43, and 49, by a microtiter

plate reader (BioteK FLx800). As a proxy of the availability of periphyton throughout the study, the area under the curve of the periphyton development was calculated.

For determination of the zooplankton community, a core sampler was used to collect ten sub-samples of 1 l each at ten different positions in each mesocosm. From these sub-samples, zooplankton was collected using a 55  $\mu\text{m}$ -plankton net and the composite sample was preserved in a formaldehyde solution until visual microscopic analyses. This sampling was performed four times during the experiment period, on days 0, 7, 15, and 49.

To avoid disturbance of the systems, the introduced macroinvertebrates were only sampled on Day 49. The alive and dead periwinkles were counted separately. As usual, not all introduced periwinkles could be recovered from the mesocosms at the end of the study. It cannot be avoided that some individuals climbed out of the mesocosms during the experiment period. Due to the space between individual mesocosms, it is not possible for periwinkles to move from one mesocosm to another. The mortality of the remaining periwinkles was calculated based on the ratio of dead (empty shells) and total (dead and alive) recovered periwinkles per mesocosm. The recovered periwinkles from each mesocosm were rinsed with demi-water to wash away the sand, and then grouped and pictured to be able to compare the color and condition of the shells between treatments. The living periwinkles were dried overnight at  $103^{\circ}\text{C}$  to determine the dry weight, and subsequently transferred to  $450^{\circ}\text{C}$  oven for 4 h to determine the ash weight. The flesh dry weight was calculated by subtracting the ash weight from the dry weight, while the ash weight was used as a proxy for shell weight.

The benthic invertebrates were sampled also on day 49. For this, two PVC tubes each with a surface of  $0.07\text{ m}^2$  (30 cm diameter) were pressed in the sediment surface before the water was fully pumped off. The sediment and water within each tube was collected and sieved (500  $\mu\text{m}$ ). The sediment sample on the sieve was preserved in a formaldehyde solution until further analysis.

The relation between treatment and abundance on day 49 of total zooplankton, adult polychaetes, and planktonic polychaetes larvae was analyzed with a Generalized Additive Model [GAM, (Wood 2006)]. A GAM fits a smoothing function of potentially relevant explanatory variables (in this case, CO<sub>2</sub> enrichment causes pH reduction) to the density data to describe the number of organisms in relation to the variables. All GAM analyses in our study were implemented using the Mixed GAM Computation Vehicle (mgcv) package (Wood 2011) in R (Team 2013).

GraphPad Prism 7 was used to draw graphs and perform statistical analysis using one-way or two-way ANOVA with Dunnett's multiple comparison post-test. The result were considered significant when  $p < 0.05$ .

## Results and Discussion

### Water Parameters

The study was conducted during early summer (June 12th and July 31st 2017). Water temperatures in the mesocosms ranged between 16 °C at the start and 22 °C maximum. The pH in the untreated controls was 8.3 at the start and increased to over 9 at the end of the study. These values are relatively high compared to the global open ocean mean of pH 8.07 (in 2010) (Hofmann et al. 2011). In more shallow and productive areas, however, pH levels can increase to over 9, as well, as a result of primary production (Verspagen et al. 2014). The high pH in our controls can also be attributed to primary production. In the CO<sub>2</sub>-treated mesocosms, the pH rapidly decreased after the start of the CO<sub>2</sub> application, and then stabilized within the next 2 days at the target value (Supplementary Material S1a). The treatments followed a mean pH level gradient and were significantly different from each other: pH mean values of 8.6 (control), 7.8, 7.4, 7.1, 6.6, 6.2, and 5.8 (Table 1 and Supplemental Material S 1b). The mean pH level of each treatment was used to define different treatments in the results. The salinity of all the mesocosms remained in the range of 31–34‰ (Supplementary Material S 2). The alkalinity level increased along with the pH level decrease, from about 2.7 meq/l in the controls to the highest 6.4 meq/l in the pH 5.8 mesocosms (Supplementary Material S2).

The solubility of CO<sub>2</sub> is dependent on the temperature, pressure and the composition of the aqueous solution (Al-Anezi et al. 2008; Duan and Sun 2003). Besides the widely studied and modelled CO<sub>2</sub> solubility in the CO<sub>2</sub>–H<sub>2</sub>O system and in single or a mixed-salt aqueous system, CO<sub>2</sub> solubility in natural seawater is hard to predict due to the complicated chemical composition of the natural seawater (Duan and Sun 2003; Zhao et al. 2015). Therefore, we could not calculate the exact solubility level of CO<sub>2</sub> in our mesocosms. In our study, the lowest pH level that could be reached by continuously adding CO<sub>2</sub> was pH 5.8. From this, we assume that the maximum CO<sub>2</sub> saturation level was reached and maintained in our highest treatment level.

As the temperature, depth, pressure, and composition of the seawater were identical in all mesocosms, the solubility of CO<sub>2</sub>, and thus, the maximum saturation level must have been similar, as well. The CO<sub>2</sub> particle pressure in water (*p*CO<sub>2</sub> in µatm) was different between mesocosms due to the different CO<sub>2</sub> flow rates (Table 1). *p*CO<sub>2</sub> values were estimated based on pH, total alkalinity, salinity, and temperature, and were 114 µatm in the controls and extremely high (up to  $1.7 \times 10^5$  µatm) in the highest treatment groups. The low *p*CO<sub>2</sub> in the controls were in accordance with the high pH levels that increased during the study period from 8.6 to

over 9, due to primary production, as can also be expected in shallow productive areas (Verspagen et al. 2014). The *p*CO<sub>2</sub> in seawater of global oceans is higher and varies seasonally about 60% below and above the current atmospheric *p*CO<sub>2</sub> level of about 360 µatm (Takahashi et al. 2002).

In the presence of sufficient carbonate sources, increasing CO<sub>2</sub> levels will result in increasing alkalinity. In the marine aquarium technology, a calcium reactor is used to raise alkalinity for coral cultivation (Brockmann and Janse 2008). Our mesocosms contained calcium carbonate (shell fragments)-rich sediments. This explains the rapid increase in alkalinity with elevated CO<sub>2</sub> concentration, resulting in a total alkalinity that was 1–5 times higher in the treated mesocosms than in the controls (Supplementary Material S2). This process was facilitated by the relatively high sediment surface water–volume ratio and lack of water replacement or exchange. These conditions represent a worst case situation that could occur in isolated shallow water ecosystems. In a real situation where underwater release of exhaust gas is applied, CO<sub>2</sub> injection will appear near the water surface. This will probably lead to less harmful exposure conditions, especially in deeper water where only the upper part of the water column will be affected. On the other hand, the mesocosms did not contain early life stages of calcifying species that are regarded most sensitive to elevated CO<sub>2</sub> concentrations (Kurihara 2008).

The oxygen saturation level in the controls remained over 96% during the whole study. The lowest oxygen saturation level in all CO<sub>2</sub>-treated mesocosms was observed in the replicated pH 5.8 treatments, namely 75% (Supplementary Material S2). This probably was related to high oxygen consumption by biodegradation of dead organisms (see next section). Other treatments remained above 85% (Supplementary Material S2). These values are all well above the levels where biological effects due to lack of oxygen will occur. Nutrient levels for P (PO<sub>4</sub>) and N (NO<sub>3</sub> + NH<sub>4</sub>) were not significantly different between treatments on the last day of the experiment, while SiO<sub>2</sub> levels were below detection limits (0.3 mg/l) in all treated mesocosms, but around 0.8 mg/l in control groups (Supplementary Material S2).

### Phytoplankton and Zooplankton

The chlorophyll-*a* concentration, a proxy for phytoplankton biomass in the controls, remained below 20 µg/l during the whole experiment. The CO<sub>2</sub>-treated groups showed similar development as the controls, except for the pH 5.8 treatment. The presence of planktonic grazers (e.g., zooplankton) at the moderate pH levels could explain why the chlorophyll-*a* concentrations in these mesocosms remained relatively low (Table 3). In the pH ≥ 7.1 mesocosms, the total abundance of zooplankton was increased at the beginning of the experiment. This increase was slightly slower

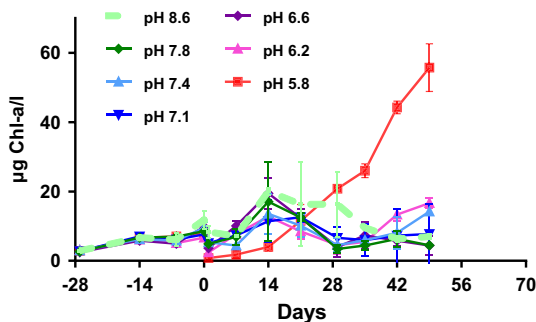


**Table 3** The average density of total zooplankton (ind./l) in the duplicate mesocosms under seven different pH conditions (pH 5.8–8.6) on days 0, 7, 15, and 49

Ind./l	pH 8.6	pH 7.8	pH 7.4	pH 7.1	pH 6.6	pH 6.2	pH 5.8
Day 0	111 (55)	372 (81)	102 (33)	105 (74)	177 (45)	59 (53)	517 (116)
Day 7	702 (604)	396 (310)	507 (381)	432 (356)	86 (72)	14 (11)	12 (8)
Day 15	733 (591)	427 (285)	754 (382)	288 (131)	43 (22)	17 (11)	11 (7)
Day 49	208 (199)	264 (248)	185 (148)	237 (133)	177 (5)	6 (4)	3 (2)

CO<sub>2</sub>-application started on Day 0

The number in the bracket indicates the average density of copepod (ind./l). The average density of copepod was calculated by sum of the density of Nauplii, Calanoide, Harpacticoide, and Cyclopoide in the mesocosms



**Fig. 2** Development of phytoplankton under seven different pH conditions (pH 5.8–8.6) from 28 days before starting the treatment to 49 days after. The result is expressed as chlorophyll-*a* concentration (µg Chl-*a*/l). Error bars represent the standard deviation of the two duplicate mesocosms. CO<sub>2</sub>-application started on day 0

in the CO<sub>2</sub>-treated groups compared to the control (most cases > 500 ind./l) (Table 3). In addition, the development of chlorophyll-*a* concentration could have been restricted by phosphorous availability, since the dissolved phosphorous concentrations in all our mesocosms were below the detection limit on Day 49. Although the inorganic nutrients were limited, the chlorophyll-*a* concentration of the pH 5.8 mesocosms increased up to about 60 µg/l, which is more than nine times higher than in the controls (Fig. 2). Similarly, Bach et al. (2017) observed a positive CO<sub>2</sub> effect on chlorophyll-*a* build-up in their 50 m<sup>3</sup> mesocosms when the inorganic nutrient concentrations were close to the detection limit. This indicates that fluxes of inorganic nutrients that became available from decaying organic material were immediately used by developing phytoplankton, resulting in a continuously low nutrient concentration in the water column. In our pH 5.8 mesocosms, degradation of dead zooplankton and benthic organisms that did not survive the high CO<sub>2</sub> exposure must have resulted in a flux of inorganic nutrients to the water column. For example, in pH 5.8, the total zooplankton abundance decreased from over 500 individuals per liter (ind./l) on days 0–3 ind./l on Day 49 (Table 3). The high mortality amongst organisms (e.g. zooplankton) that feed on phytoplankton subsequently reduced grazing

pressure and provided inorganic nutrients, which resulted in the algae bloom that was observed in the pH 5.8 mesocosms.

The biodegradation of the organic material that becomes available in the high-dosed mesocosms due to the increased mortality of invertebrates leads to higher oxygen consumption. This result in a concentration of dissolved oxygen in our pH 5.8 mesocosms that was about 15% lower than in the controls. The lowest observed oxygen concentration of 75% of the maximum saturation level is not that low as to have caused additional mortality among the organisms living in these mesocosms. The indications for reduced abundance or increased mortality of the zooplankton and benthic organisms in the mesocosms with higher treatment levels are, therefore, considered as a direct effect of the elevated CO<sub>2</sub> levels.

The high tolerance of phytoplankton to elevated CO<sub>2</sub> conditions may result from the utilization of different forms and amount of dissolved inorganic carbon. For example, microalgae are able to take up HCO<sub>3</sub><sup>−</sup>, the dominant carbon form when pH is between 6.5 and 10, as well as free CO<sub>2</sub> and H<sub>2</sub>CO<sub>3</sub>, the predominant form when the pH is lower than 6.5 (Markou et al. 2014). Since the utilization of the carbon form is species-specific (Camiro-Vargas et al. 2005), it is likely that there were different dominant microalgae species in the different treated mesocosms in our study. The fluorescent data indicated that green algae dominated the phytoplankton community throughout the whole study in each treatment (Fig. 2 and Supplementary Material S3), but further identification of algae species was not performed.

At the start of the exposure, the zooplankton community was already dominated by copepods, and the relative and absolute abundance of these crustaceans strongly increased during the first week application in all mesocosms with pH ≥ 7.1 (Table 3 and Supplementary Material S4). In these mesocosms, the copepods remained the dominant zooplankton group until the end of the study on day 49. In mesocosms with pH 6.2 and 5.8, a strong decline of the copepod density was observed during the first week of CO<sub>2</sub> application, and the densities further declined until the end of the study. Such a clear decline was also observed in mesocosms with

pH 6.6, although here the copepod population still showed some increase during the first week. It is clear from these data that the copepod community suffered directly from the elevated  $\text{CO}_2$  concentrations. In our mesocosms, it was thus not the copepods that took advantage of the elevated  $\text{CO}_2$  concentrations as was described by Taucher et al. (2017), but it was the polychaete worms. The planktonic larvae of polychaete worms were almost absent in the control mesocosms, but reached high numbers and were the dominant zooplankton group in the pH 7.1 and 6.6 mesocosms at the end of the study (Supplementary Material S4). At the highest two treatment levels, pH 6.2 and 5.6 even the polychaete could not maintain their position, and at the end of the study, zooplankton was only present in extremely low numbers in these mesocosms.

The positive impact of the intermediate  $\text{CO}_2$  levels on the development of the polychaete worms was also reflected by the numbers of adult worms, and affected other organisms, as will be discussed in the following sections.

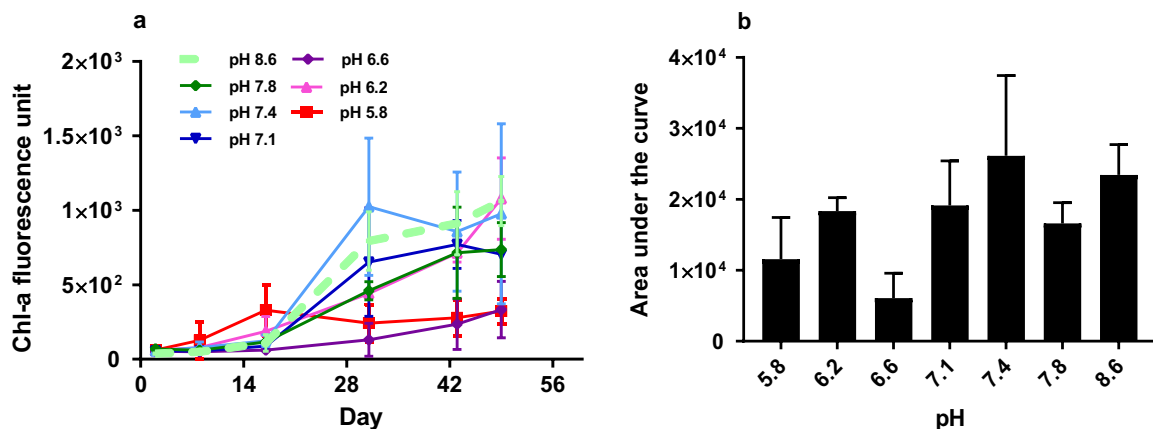
### Polychaete Worms and Periphyton

Development of sessile algae (periphyton) biomass on the glass slides that were introduced on Day 0 was first detectable after 14 days. This time lag can be explained by the fact that the colonization of a new surface takes time, since the algae adherence depends on the bacterial colonization and its secreted organic matrix which may take from days to weeks (Azim and Likens 2009). After 14 days, a strong development of periphyton was observed in the control and most  $\text{CO}_2$ -treated groups, except at pH 6.6 and 5.8 (Fig. 3a). In the pH 6.6 treatment, the chlorophyll fluorescence, a proxy for periphyton biomass, stayed below 300 units for the entire experiment period. It was similar to or lower than (e.g., on

day 15) in the pH 5.8 treatment (Fig. 3a). The integrated chlorophyll concentrations, a proxy for the general presence (availability) of periphyton biomass during the course of the study was lower in the pH 6.6 and 5.8 treatments than in the control. However, in the intermediate treatment (pH 6.2), the periphyton was able to develop and reached levels similar to the controls (Fig. 3b). Thus, it is unlikely that the elevated  $\text{CO}_2$  concentration on itself was responsible for the poor periphyton development in the pH 6.6 treatments. The main drivers in the development of periphyton biomass are growth (primary production) and grazing.

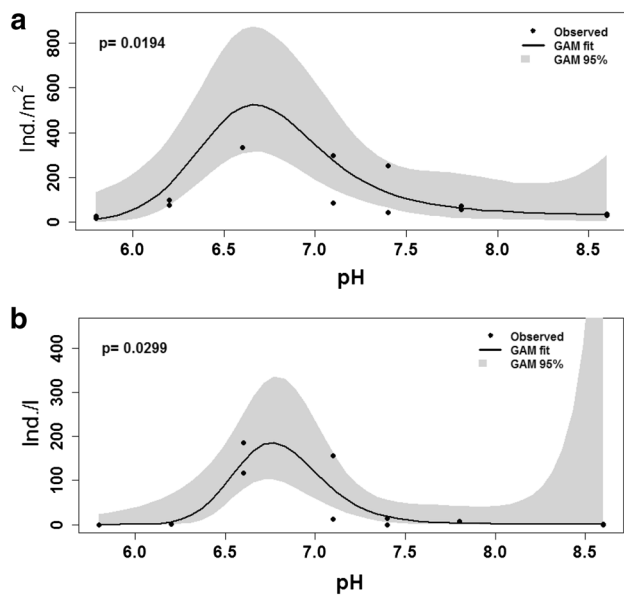
In our study, the glass slides that were used for the monitoring of the periphyton development were exposed out of reach of gastropods (periwinkles and mud snails) and the benthic invertebrates that all consume (benthic) periphyton. However, it cannot be excluded that planktonic polychaetes were able to colonise the glass slides as small juveniles and feed on the sessile algae. These planktonic polychaete larvae became especially abundant in the pH 6.6 mesocosms where the polychaete worms became the most dominant benthic group, reaching significantly ( $p=0.0194$ ) higher densities than in the controls on day 49 (Fig. 4a). They produced substantial amounts of offspring as reflected by the significant ( $p=0.0299$ ) higher numbers of pelagic polychaete larvae in the plankton samples in pH 6.6 (Fig. 4b). With decreasing pH, the relative abundance of the polychaete larvae increased until it comprised over 85% of the total zooplankton community in treatments pH 7.1 and pH 6.6 (Fig. 4b and Supplementary Material S4). The reduced periphyton biomass in the pH 6.6 mesocosms might, thus, be the result of additional grazing by larvae of polychaete worms.

This hypothesis is also corroborated with the finding of increasing biomass of periphyton in the pH 6.2 treatment,



**Fig. 3** Development of periphyton on glass slides under seven different pH conditions (pH 5.8–8.6) in the mesocosms from day 0 to day 49, expressed as chlorophyll-*a* fluorescence units (a).  $\text{CO}_2$ -application started on Day 0. The area under the curves of periphyton develop-

ment in Fig. 3a is calculated and represent the presence (availability) of periphyton biomass in the mesocosms under different pH conditions (b). Error bars represent the standard deviation of the two duplicate mesocosms



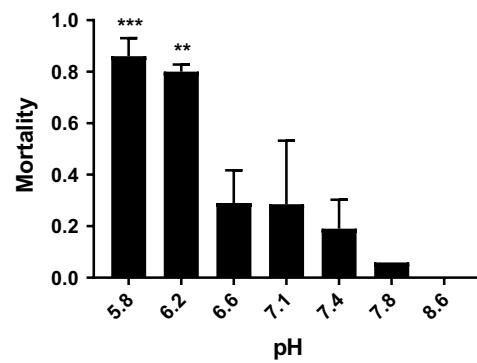
**Fig. 4** Generalized Additive Modelling results: the observed adult population (black dots) of polychaetes in sediment (ind./m<sup>2</sup>) (a) and the observed density (black dots) of planktonic polychaetes (ind./l) (b) under seven different pH conditions (pH 5.8–8.6) on day 49 in all the mesocosms. CO<sub>2</sub> application started on day 0. The solid line represents a fit smoothing function of potentially relevant explanatory variables (in this case, enriched CO<sub>2</sub> caused pH reduction) to the observed density data. The grey area indicates the 95% confidence interval of the smooth function

where the polychaetes were no longer able to take advantage and their larvae were only present in low numbers, as were the other primary consumers. As the glass slides were only analyzed for fluorescence to determine periphyton biomass, no observations of other organisms were made that could test this hypothesis.

In the highest CO<sub>2</sub> treatment, pH 5.8, the development of periphyton was inhibited despite the virtual absence of polychaete larvae, and potential other grazers. These results indicate that sessile algae (periphyton) are less resilient to extreme CO<sub>2</sub> concentrations than planktonic algae (phytoplankton) that were able to bloom in the pH 5.8 mesocosms. The acidic condition may disturb the colonization process of bacteria and/or the attachment of the periphyton itself. Currently, little is still known about how exactly physical disturbance events in the environment influence periphyton attachment and development (Azim and Likens 2009).

### Periwinkles and Other Benthic Organisms

During the final sampling, the *Peringia ulvae* (mud snail) and *Corophium volutator* (mud shrimps) numbers showed a high variation between duplicated mesocosms, which hampered the detection of subtle effects. Nonetheless, it was clear that survival of both benthic species was



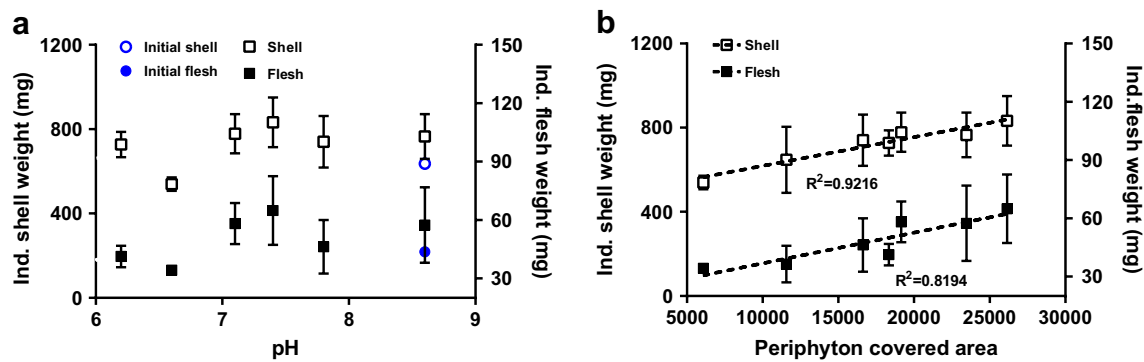
**Fig. 5** Mortality of recovered periwinkles under seven different pH conditions (pH 5.8–8.6) in the mesocosms on day 49. Error bars represent the standard deviation of the mortality between the two duplicate mesocosms. Statistical difference between the CO<sub>2</sub>-treated groups and control (pH 8.6): \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ . CO<sub>2</sub> application start on day 0

strongly reduced to almost absent in treatments pH 6.2 and 5.8 (Supplementary Material S 5).

The appearance of the periwinkle's shells showed the direct impact of the elevated CO<sub>2</sub> concentrations. A clear trend of the shell color changing with increasing CO<sub>2</sub> concentration was observed. Starting from a normal dark shell (pH 8.6–7.4) via half shell dark and half shell bleached in the pH 7.1 treatments, to the entire shell bleached in the pH 6.6 treatment, eventually with shell damage (pH 6.2–5.8) (Supplementary Material S6). The shell calcification rate of periwinkles is reported to be negatively affected by elevated CO<sub>2</sub> concentrations (Ries et al. 2009). When the organisms are not able to compensate the dissolution rate of shells in acidic condition with a higher calcification rate, shell bleaching and damage occur. Although the major part of the shell is covered by an external organic layer, the protection is not sufficient to fully protect against to elevated CO<sub>2</sub> (Ries et al. 2009). It is possible that the first bleaching indicates the erosion of the external organic layer.

Besides the color changes, the mortality of periwinkles also significantly increased ( $p = 0.0011$  in pH 6.2;  $p = 0.0007$  in pH 5.8) with increasing CO<sub>2</sub> concentrations. In pH 6.2 and 5.8 treatments, where the shell damage occurred, the mortality was as high as 85% (Fig. 5 and Supplementary Material S7). However, the CO<sub>2</sub> induced pH decrease had no significant effect on the biomass of surviving periwinkles (Fig. 6a). In comparison to the periwinkles at introduction, both flesh and inorganic weight (shell weight) in the control groups increased with 31% and 20%, respectively. While, in the most extreme treatment, pH 5.8, the flesh, and shell weight did not change relative to the initial weights (Fig. 6a and Supplementary Material S7). In addition, both weights showed no direct relation with CO<sub>2</sub> concentration. The





**Fig. 6** The average initial shell (empty blue circle) and flesh (solid blue dot) weights of periwinkle and the average shell (empty black square) and flesh (solid black square) weight of surviving periwinkles under seven different pH conditions on day 49 (a); the average shell

(empty square) and flesh (solid square) weight of surviving periwinkles under different periphyton availabilities (b). Error bars represent the standard deviation of the mortality between the two duplicate mesocosms. CO<sub>2</sub> application starts on day 0

weights, however, showed a significant positive correlation with the total periphyton biomass available (Fig. 6b), which forms the periwinkles main food source. As discussed above, the development of the periphyton biomass was indirectly affected by the CO<sub>2</sub> concentration through the increasing abundance of polychaete larvae at intermediate CO<sub>2</sub> levels (pH 6.6), and directly affected by higher CO<sub>2</sub> levels. The periwinkles are, thus, indirectly affected by these complex interactions that consider their main food source.

## Conclusion

During the 49 days with elevated CO<sub>2</sub> concentrations, several effects were observed. In the highest CO<sub>2</sub> treatment, the invertebrate community collapsed and phytoplankton bloomed. In contrast to their planktonic equivalent, the sessile microalgae (periphyton) were not able to increase biomass. At lower concentrations of CO<sub>2</sub>, more subtle changes were observed. In pH 7.1 and 6.6 treatments, high numbers of planktonic polychaete larvae appeared, corresponding with high numbers of sedentary adult polychaetes in these treatments. The periwinkles clearly suffered directly from the treatments as was illustrated by affected shells at pH 7.1 and below, and increasing mortality with higher CO<sub>2</sub> dosage. The development of their individual total biomass, however, was not directly related to the CO<sub>2</sub> treatment level, but more indirectly via the availability of their major food source, periphyton. As the periwinkles shared this food source with the polychaete worms, their biomass was affected by the increasing densities of these worms with increasing CO<sub>2</sub> levels.

This mesocosm study shows the importance of species interactions in the response of an ecosystem to elevated CO<sub>2</sub> concentrations.

It illustrates that the environmental impact of underwater release of exhaust gasses depends on various factors including the composition of the gas, the shipping intensity, the volume of the receiving water body, and local environmental conditions. The relative importance of these factors is subject of future research that will be used to assess the boundaries where the advantages of underwater release of exhaust gasses can be applied with minimum adverse impact on the local marine ecosystem.

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## Compliance with Ethical Standards

**Conflict of interest** On behalf of all authors, the corresponding author states that there is no conflict of interest.

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