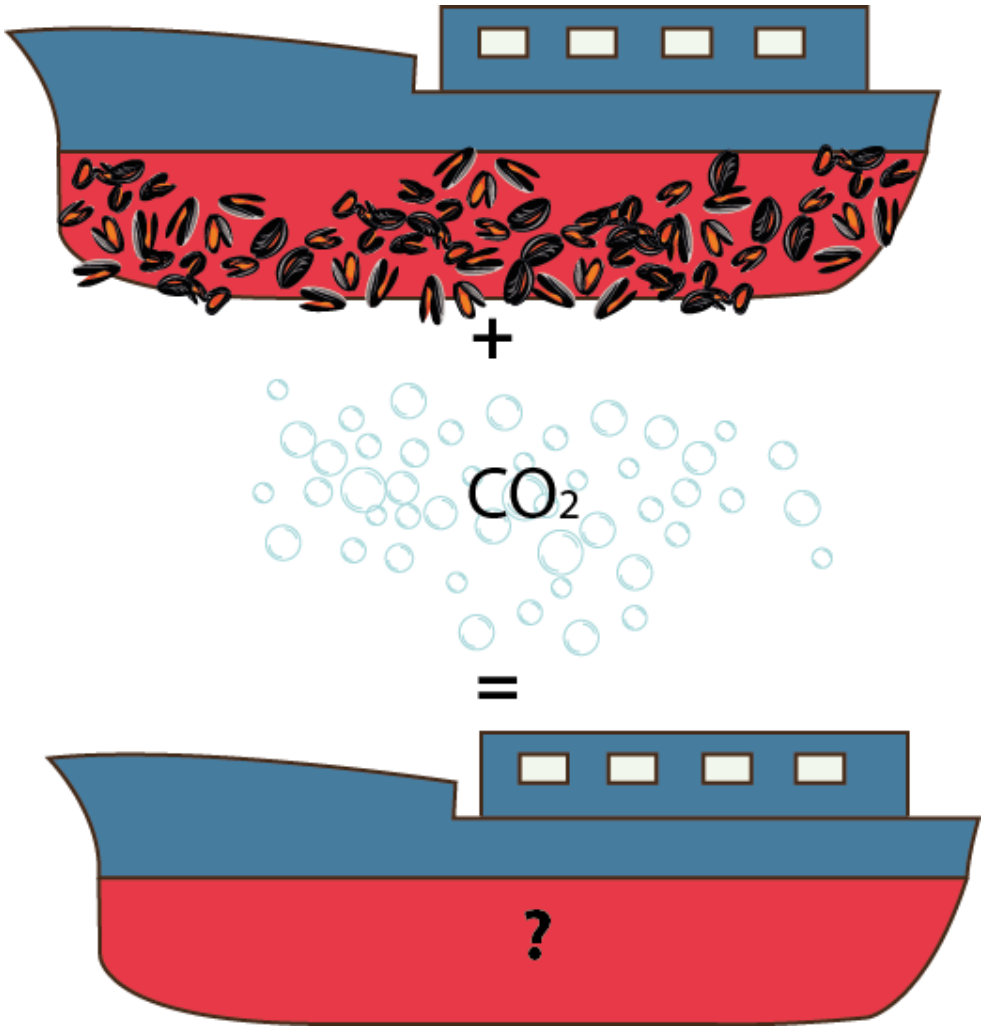
The effect of CO_2 concentrations on blue mussel (*Mytilus edulis*) behaviour, health, mortality and detachment rates: a new potential way of anti-fouling



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Abstract

Fouling organisms on ship's hulls increase hydrodynamic drag of the vessel and decrease speed and fuel efficiency which has large economic impacts for shipping companies. The GasDrive project is developing an innovative ship, with a more than 50% reduced fuel demand compared to traditional ships. The exhaust, mainly consisting of water and CO_2, will be released under the ship's hull. A great deal of research has been conducted of the negative effects of future CO_2 levels on calcifying organisms due to anthropogenic carbon emission. However, a locally elevated CO_2 concentration has never been suggested as a means of anti-fouling. The combination of CO_2 exhaust gasses on the ship's hull and the water forces past the moving vessel, may turn out detrimental to fouling organisms, causing mortality and detachment. Therefore, GasDrive may prove a novel anti-fouling strategy.

As we aim to discover whether the GasDrive CO_2 output may have anti-fouling properties, we must understand the effect of both CO_2 and water velocities on fouling bivalves. CO_2 can cause detachment of bivalves from a vessel in three ways: Through mortality, lowered attachment strength leading to a reduction in detachment velocity (the maximum water velocity an organism can resist before it detaches from the substrate) or avoidance behaviour. Mytilus edulis was chosen as a model organism for marine fouling bivalves. This brings us to the following research question: How do elevated CO_2 concentrations affect M. edulis activity, health, mortality and detachment velocities? We hypothesised that elevated CO_2 concentrations will decrease M. edulis health and detachment velocities and increase mortality and movement activity.

M. edulis was exposed to several degrees of CO_2 induced acidified water conditions at pH 6.5, 7.5 and 8 (control) for 4 days. Twice a day, mussel displacement was recorded in order to determine movement activity. In a second experimental study, M. edulis was exposed to several degrees of CO_2 induced acidified water conditions at pH 6.2, 6.8, 7.2, 7.6 and 8 (control) for 21 days. Mortalities were recorded throughout the experiment and a novel experimental setup was designed to determine detachment velocities. Mussel health was assessed through the following factors: Shell strength, growth rates, reaction time (the time it takes a mussel to close its shell in response to a disturbance) and feeding rates.

CO_2 induced acidification to pH 6.2 resulted in a 35% reduction in M. edulis shell strength and a 5 fold increase in reaction time. A trend was seen between mortality and pH, with a 10% increased mortality rate at pH 6.2 and 6.8. Also, a trend was visible with lower feeding rates at reduced pH. No significant effect of elevated CO_2 was found on movement activity, growth rates and detachment velocities.

In conclusion, if around a ship hull that is covered by mussels the pH is decreased to as low as 6.2, it would take more than a month for the mussels to show significant mortality rates. However, if CO2 was to be used in combination with toxic paints, mortality rates may probably drastically increase. We therefore recommend the use CO_2 as an anti-fouling strategy when dealing with marine bivalves in combination with pre-existing methods to increase effectiveness. Thereby reducing the required concentrations of toxic compounds in paints, and lowering environmental impact.

Also, shell strength and reaction time are both important features that protect mussels from predation and they are negatively affected by increased CO_2 concentrations. This may greatly affect vulnerability to predation. If there is a sufficient amount of predators around a GasDrive ship, the abundance of mussels on the hull may be greatly reduced.

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Introduction

Bio-fouling is defined as "the colonisation of aquatic organisms on ships and platforms" [1]. Two groups of bio-fouling organisms can be distinguished, micro-foulers (e.g. Bacteria, Algae and Fungi) and macro-foulers (e.g. Barnacles, Bivalves, Algae and Bryozoans). Bivalves are one of the major groups of macro-fouling organisms [1]. Fouling organisms on ship's hulls increase the friction of the vessel and have been estimated to decrease speed by approximately 2% and reduce fuel efficiency up to 45% [2, 3]. This costs shipping companies enormous amounts of money [3]. In addition to the economic impacts due to increased fuel demand, biofouling on ships is one of the most important vectors for the invasion of nonindigenous species [4]. These non-native species occur globally, they are associated with negative impacts that affect ecosystem structure and functioning and cause environmental impacts and economic damage [5]. Anti-fouling can reduce the spread of these species.

Anti-fouling paints are currently the main anti-fouling strategy in the marine shipping industry [2, 3]. Most of these coatings used to contain tributyl tin (TBT) biocides that also impact non-target species, which has resulted in a ban by the International Maritime Organization in 2001 [3]. Current principle anti-fouling paints employ copper and/or co-biocides [6]. These biocides have received increased research attention towards their environmental impacts and this has boosted interest in the development of novel environmentally friendly anti-fouling strategies [3, 7].

The GasDrive project is developing an innovative ship, powered by liquid natural gas (LNG), with a more than 50% reduced fuel demand compared to traditional ships. The exhaust, mainly consisting of water and CO_2, will be released under the ship's hull. The hull is covered with a nanostructure that captures the gas bubbles, reducing the ships resistance in the water [8]. When CO_2 dissolves in water, it reacts with H_2O to form carbonic acid (H_2CO_3), which in turn results in titration of carbonate ions (HCO_3^-) [9]. As pH in seawater is largely determined by the ratio of carbonic acid to carbonate ions and Ω_{CaCO3} (calcium carbonate saturation state) is driven by the total amount of carbonate ions, this leads to a reduction in seawater pH and Ω_{CaCO3} [9]. Ω_{CaCO3}, is a measure of the potential for calcium carbonate to dissolve [9]. A large group of marine organisms utilize calcium carbonate as the primary mineral for the formation of shells and skeletons (calcifying organisms). As Ω_{CaCO3} decreases, shell dissolution starts to occur, which impacts shell strength [10] and requires higher allocation of energy to calcification [11]. A great deal of research has been conducted of the negative effects of future CO_2 levels on calcifying organisms due to anthropogenic carbon emission [11]. However, a locally elevated CO_2 concentration has never been suggested as a means of anti-fouling. The combination of CO_2 exhaust gasses on the ship's hull and the water forces past the moving vessel, may turn out detrimental to fouling organisms, causing mortality and detachment. Therefore, GasDrive may prove a novel anti-fouling strategy.

Due to increased anthropogenic carbon emission, expected future CO_2 conditions will result in a drop of 0.77 pH units at most by the year 2300 [12]. Therefore, the majority of experimental studies towards the effects of elevated CO_2 on marine organisms have been tested at a pH of 7.0 or higher [11]. Locally elevated CO_2 concentrations as caused by the GasDrive may lower the pH to a level below 7.0 around the ship's hull. And thus, to predict its effects on fouling organisms, further research was required. The aim of this study is to explore whether CO_2 can be used as a means of anti-fouling and predict the influence of GasDrive on marine bivalves on a ship hull.

A common benthic organism, that shows a widespread distribution with representatives in both the Northern and Southern hemisphere, are blue mussels of the genus

Mytilus. Mussels utilize byssus threads to attach to hard substrates [7] and are regularly exposed to water velocities that are generated by breaking waves of up to 10 $m \cdot s^{-1}$ in the rocky intertidal coastal zone [13]. These characteristics make them a successful fouling organism [14]. *Mytilus edulis,* a species common to the Northern Atlantic coast, is used in this experimental study as a model organism for fouling bivalves [15].

Fouling organisms are regularly exposed to strong hydrodynamic currents, with cargo and passenger vessels reaching speeds of up to 25 knots (12.9 $m \cdot s^{-1}$) [16]. However, the impact of water velocities on detachment of fouling organisms have received little attention in the past. As we aim to discover whether the GasDrive CO_2 output may have anti-fouling properties, we must understand the effect of both CO_2 and water velocities on fouling bivalves (Figure 1). This brings us to the following research question: How do elevated CO_2 concentrations affect *M. edulis* activity, health, mortality and detachment velocities?

Modelling studies have revealed that mussel movement can mostly be explained by local abundance of other mussels [17–20]. Mussel movement activity increases when close-by mussel density (within 1-2cm distance) is too low, or when the long range density (within 3-7.5cm distance) of mussels is too high [18, 20]. This is because mussels benefit from mussels close-by, through byssal attachment, but experience disadvantages from mussels that are relatively far away though competition in feeding [18, 20]. To the authors knowledge, it is unknown how these mussels "know" what the mussel density is. We therefore hypothesised that CO_2 concentration is associated with mussel density because respiration of mussels locally increases CO_2 concentration. And this may thus be a cue for them to increase movement when local mussel densities are too high. Additionally, it is possible that mussels display avoidance behaviour in order for them to displace to a better suited environment. Increasing mussel movement when exposed to unfavourable environmental conditions like increased CO_2 concentrations.

In previous studies elevated CO_2 concentrations have revealed several negative impacts on *M. edulis*. It causes shell dissolution and reduced shell formation [21, 22], decreases byssal attachment strength [23] and increases mortality [24]. It is therefore hypothesised that elevated CO_2 concentrations will decrease *M. edulis* health and increase mortality and detachment.

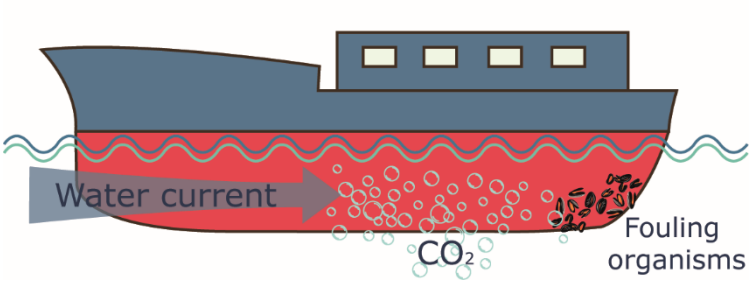

Figure 1; Schematic drawing of factors that influence the fouling community on a GasDrive ship.

To test these hypotheses, we exposed mussels to several degrees of CO_2 induced acidified water conditions at pH 6.2, 6.8, 7.2, 7.6 and 8 (control) for 21 days. Mortalities were recorded throughout the experiment and mussel health was assessed by investigating the following factors: Shell strength, growth rates, reaction time and feeding rates. Furthermore, mussels were exposed to a parallel water flow in order to determine detachment velocities.

Materials & Methods

Sampling and preparation

In April 2018 100 mussels were collected from a mussel stock at the Wageningen Marine Research institute in Yerseke, the Netherlands. In July 2018 another 300 mussels were collected from the same location. Two water

samples were taken to establish the salinity and alkalinity required for the experimental controls. The mussels were stored in plastic bags with cooling elements and transported by car to the Carus research facility at the Wageningen University, the Netherlands. The first 100 mussels were used for the behavior experiment to test whether CO_2 induces avoidance behaviour in *Mytilus edulis*. The experiment ran in the period 28.05.2018-03.06.2018. The 300 mussels were used for the long-term exposure experiment to test whether mussels will detach from a moving ship when exposed to high CO_2 concentrations. This experiment ran in the period 06.08.2018-27.08.2018.

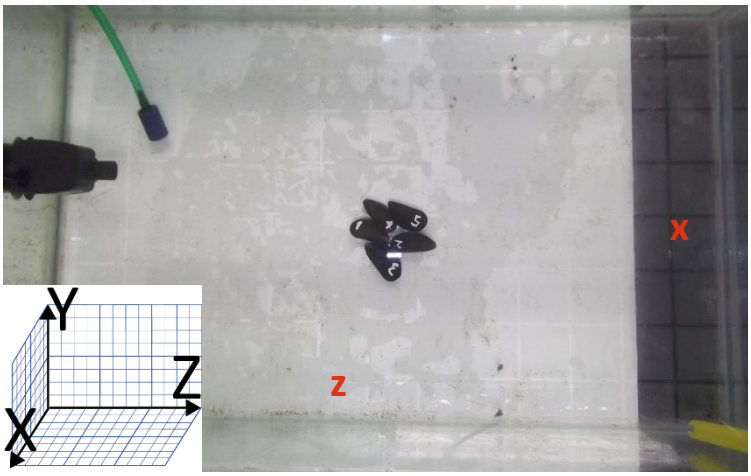

Figure 2; Top view of the experimental setup, five mussels were placed in the middle of the tank, the tank contains an aquarium pump, a CO2 gas supply (top left corner) and a pH electrode (bottom right corner). The bottom of the tank is gridded, bottom right corner (x, y, z) is (0, 0, 0), top left corner is (8, 0, 13).

Experimental procedure behaviour experiment

The 100 mussels were acclimatized in 25 L tanks with artificial seawater of salinity 31.5 ppt and alkalinity of 2.67 $mEq \cdot L^{-1}$ and supplied with an air pump and an EHEIM® EH-300 aquarium pump. They were fed daily with 2 ml Easyreefs, Easybooster 25 liquid phytoplankton feed. Phytoplankton composition of the feed is 33% Isochrysis T-ISO, 31% Nannochloropsis, 18% Tetraselmis, 18% Phaeodactylum.

In order to record the position of mussels, a LineaFix® gridded static window film was put on the sides and bottom of each tank. The width (x), height (y), and depth (z) of each tank consisted of 8, 5 and 13 grid cells respectively (Figure 2). The cells were square with sides of 30 mm. The pH in the tanks was controlled with a Milwaukee® MC125 pH controller. The controller sends a signal to open a gas valve when the pH is above the specified value. When the valve opens, a CO_2 gas flows into the bottom of the tank through the open valve, lowering the pH. As the pH drops below the set pH value, the pH controller closes the valve, thereby constantly maintaining the pH at the set value. CO_2 pressure was turned down as low as possible (<0.1 bar) to avoid overshooting of the pH. To keep the water well mixed, all tanks contained an EHEIM® EH-300 aquarium pump (Figure 2). All tanks received the same feeding regime as during the acclimatization.

Five randomly selected mussels were placed middle of each tank (x = 4, y = 0, z= 7) and numbered from 1 to 5 using a Edding® 780 paint marker. The experiment was conducted in duplicate and mussels were exposed to six different treatments in total. There were three different pH treatments: 6.5, 7.5 and 8 (control) and one half of the mussels received the "Pre-settled" treatment while the other half received the "Detached" treatment (Figure 3). The "Pre-settled" treatment means that the mussels had three

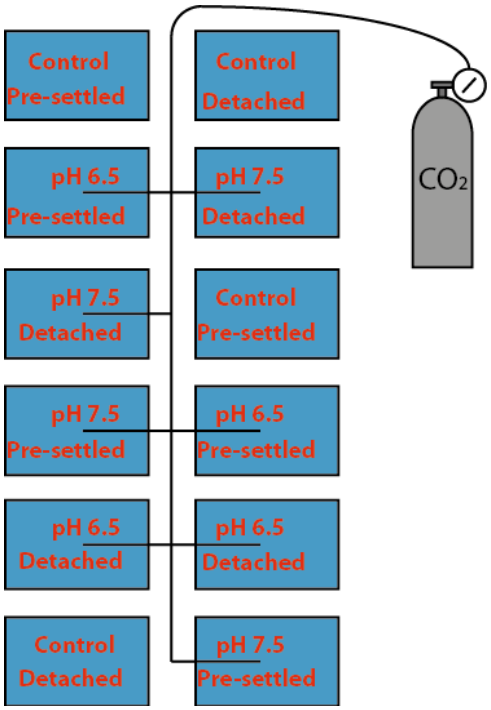

Figure 3; Setup of the experimental treatment groups in a 2x3 experimental design. Groups received "pre-settled" or "detached" treatment and a pH of 6.5, 7.5 or 8 (control) in duplicate. Location of treatments were allocated randomly across the lab.

days before the start of the pH treatment to move around and attach to the tanks and to each other. While the "detached" treatment means that at the start of the pH treatment, the mussels were detached from their position and placed back in the middle of the tank (x= 4, y = 0, z =7). Twice a day, pictures were taken and the location of the mussels were recorded in x, y and z values rounded to the nearest 0.5. After three days, half of the mussel treatment groups received the "detached" treatment, the other half, received the "pre-settled" treatment and the pH treatment was started and the pH exposure lasted for four days.

Experimental procedure long term exposure

The 300 mussels collected in July 2018 were stored in 25L tanks with artificial seawater of salinity 31.5 ppt and alkalinity of 2.67 $mEq \cdot L^{-1}$. All tanks were aerated and well mixed with an air supply and an EHEIM® EH-300 aquarium pump. Twice a week, fecal matter was siphoned out using a plastic tube and half of the seawater in each tank was replenished. The feeding regime was different from the behaviour experiment. Mussels were fed an algal diet (Easyreefs, Easybooster 25 liquid phytoplankton feed), diluted to optimal algal feeding concentration of around 6000 $cells \cdot ml^{-1}$ in the tanks [25]. Food was applied using an automatic feeding pump for nine time periods of 30 minutes each day (total of 4.5 hours per day). The required algal concentration of the feeding stock was determined through a feeding simulation in R version 3.3.2 (Appendix 1 for the R script).

After one week of acclimatization to the new lab environment, 180 randomly selected mussels were color coded using several colors of nail polish in order to distinguish individuals. The rest of the mussels were used for try-out experiments and kept as backups. Prior to the start of the experiment, length, width and height of each individual mussel was measured using a Toolland digital caliper. Eighteen PVC tubes of 105 mm long and a 50 mm diameter were filled with 10 randomly selected mussels per tube. Both ends of the tubes were blocked using a small net and a tie-wrap (Figure 4) and each tube was placed in a different 25 L tank. Mussels were left in their new environment for one more week before the start of the experiment and half of the water in each tank was replenished once.

Figure 4; A PVC tube was filled with 10 mussels, the mussels were color coded with nail polish and both ends of the tubes were blocked off with a small net.

At the start of the experiment, two tubes with mussels were removed to measure the initial shell volume at the start of the experiment and one was kept as a backup in another 25L tank that received the same treatment as the controls. The remaining 15 tubes were assigned, in triplicate, to four treatment groups and a control group. The different treatments were kept at a pH of 6.2, 6.8, 7.2, 7.6 and control group (pH 8) (Figure 5Figure 5). The pH values were maintained using the same setup as in the behavior experiment (Figure 2). Twice a day, the pH values were measured and each tube was checked for mortalities by taking the tube out of the water to check whether all mussels closed their shells. As mussels need to actively close their shells, so when dead, the adductor muscles do not function anymore and the shell remains open. In case of mortality, the mussels color code, and time and date was recorded and the mussel was stored in the freezer at -20°C. Half of the water in each tank was replenished once a week and before and after replenishment, the pH, temperature,

oxygen, salinity, alkalinity, ammonia and nitrite values were measured.

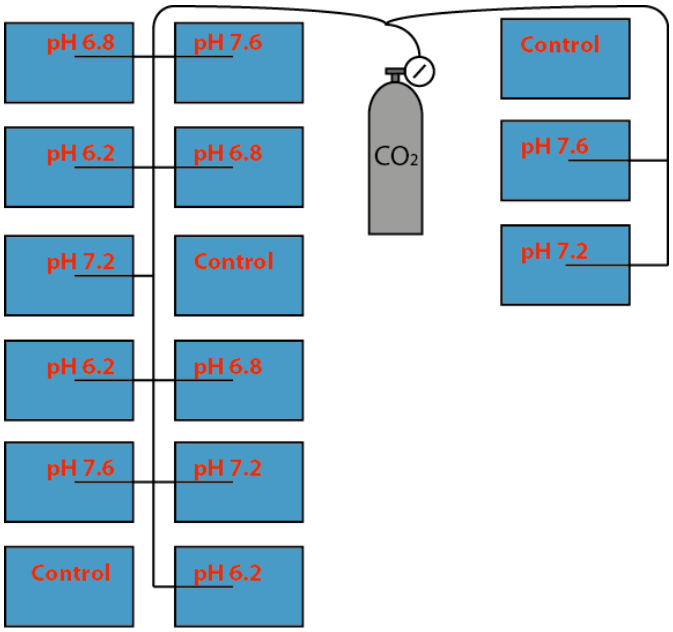

Figure 5; Setup of the long term exposure experiment. Groups were exposed to pH 6.2, 6.8, 7.2, 7.6 or 8 (control) in triplicate. Location of the treatments were allocated randomly across te lab.

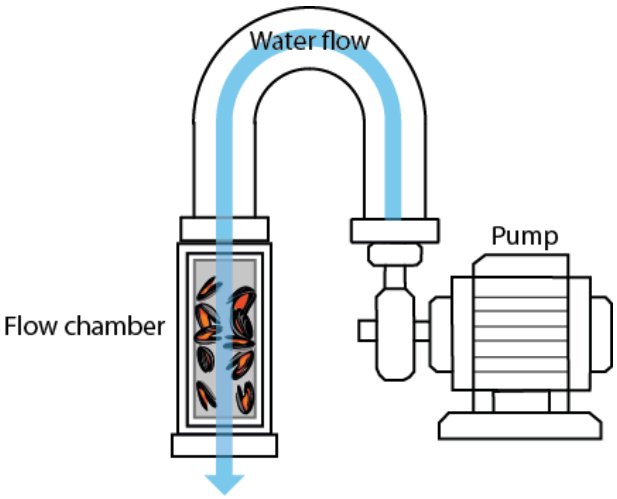

Figure 6; Detachment experiment setup. PVC tubes with mussels are placed in a flow chamber. A pump creates a water flow through the flow chamber. The water velocity is measured behind the flow chamber and pump power is increased in steps.

Mussels were exposed to treatment conditions for three weeks. Then, mussel detachment velocities were determined for all experimental units. To determine detachment velocity the net was removed from one of the tube ends and the tube was placed into a flow chamber and water was pumped through the flow chamber using an Aqua Force DM vario 10000 pump with pump controller (Figure 6). Water velocity was increased in steps of 0.1 $m \cdot s^{-1}$. The color codes of detached mussels were identified and detachment velocity was recorded. Afterwards, mussel length, width, and height were determined and they were put back into their tanks.

In order to determine shell strength, five mussels from each tank were randomly selected for a strength test. A crushing device was placed on top of a live mussel. Weights were applied until the shell cracked. Total weight required to crack the shell was measured using a scale.

On day 18 of the experiment, average mussel feeding rates were determined for each tank. 1 ml dead algal feed was pipetted in each tank. The water was left to mix by means of the aquarium pump for 15 minutes. Then, a 10 ml water sample was taken to determine algal starting concentration. The mussels were left to feed for 30 minutes and after this feeding period another 10 ml water sample was taken to determine the algal concentration after feeding. Both samples were mixed with 10 ml formalin and kept at 5°C for preservation. Afterwards, the algal concentrations of the samples were determined. Firstly, the samples were homogenized for 5 seconds using a vortex mixer. Then, 20 µl sample was pipetted on both sides of a Fuchs-Rosenthal counting chamber. Pictures of each sample were taken using a Leica DMi8 inverted light microscope. Pictures were analyzed using ImageJ, contrast of the pictures was enhanced by 8% and algae cells in eight 1x1 mm squares were counted by hand using the multi-point tool. To determine the algal concentration, total nr of algae is divided by the total volume of the eight squares ($1.6 \cdot 10^{-6}$ L) and multiplied by 2 (dilution of formalin). Afterwards, the feeding rates (in $L \cdot min^{-1}$) were calculated using the following equation:

$$Feeding\ Rate = \frac{1}{n \cdot t}\left(\frac{C_{start} - C_{end}}{C_{start}} \cdot V_{tank}\right) \quad (1)$$

Where n is the number of mussels in the tank, t is the feeding period in minutes, C_{start} is the algae concentration before the feeding period, C_{end} is the algae concentration after the feeding period and V_{tank} is the volume of the tank in L. This equation is based on the assumption that mussels feed with a 100% efficiency.

Data analysis

All statistical analysis was carried out in R version 3.3.2.

Behaviour experiment

The activity of each individual mussel was calculated from the x and z values, as the minimum distance moved between each time step was calculated using the following equation:

$$d_{t,t+1} = \sqrt{(x_t - x_{t+1})^2 + (z_t - z_{t+1})^2} \quad (2)$$

During the experiment it occurred only twice that a mussel had a non-zero y value, in those cases the y value was added to either the x or z value, depending on the side of the tank to which the mussel was attached. A measure of the spread between mussels was also calculated daily for all treatments. The spread is defined as the average distance between all mussels and is given by the equation:

$$Spread = \frac{1}{n} \cdot \sum_{i=1}^{n} \frac{1}{n-1} \cdot \sum_{j=1}^{n} d_{i,j} \quad (3)$$

Where the distance between mussel i and mussel j is given by the formula:

$$d_{i,j} = \sqrt{(x_i - x_j)^2 + (z_i - z_j)^2} \quad (4)$$

To correct for individual variation between mussels (active and inactive mussels), the difference between mussel activity in the three days before the treatment started, and activity during the three days after the treatment started was calculated.

A two-factor mixed design ANOVA was used to test whether there were significant differences in activity and spread between the pH treatment groups.

Long term exposure experiment

In order to compare detachment rates of mussels for each pH treatment, the detachment velocity at which 50% of the mussels detached (DV_{50}) was calculated for each tank. In order to determine DV_{50}, a logistic regression model was fit to the detachment data of each tank with % of detached mussels as the dependent variable and water velocity in ms^{-1} as the independent variable. This was done by using the R generalized linear model (glm) function with a binomial distribution. Afterwards, the DV_{50} was calculated from the intersection of the model with the 50% detachment line.

All variables (growth, mortality, reaction time, DV50, shell strength and feeding rate) were tested for homogeneity of variances using Levene's test for Homogeneity of variances and normality was checked using Shapiro-Wilk's normality test. If model assumptions were met, a one way ANOVA was carried out to test for significant differences between the pH treatment groups, followed by a post-hoc Tukey HSD test in case of significance. If model assumptions were not met, a non-parametric Kruskal-Wallis test was conducted to test for between group differences.

Results

Behaviour experiment

The pH of the tanks in the behaviour experiment before the start of the treatment was slightly lower than expected (7.87 ± 0.052), this was the same for the control treatments during the CO_2 treatment period (7.76 ± 0.068). This was probably caused by a leak in one of the CO_2 tubes, which increased the CO_2 concentration in the room and resulted in a lowered pH of the control tanks. This was later remedied for the long term exposure experiment. The pH of the 6.5 and 7.5 treatments (7.61 ± 0.049 and 6.64 ± 0.042 respectively) were a bit higher than expected due to the sometimes unresponsiveness of the pH sensor.

There were no significant effects of pH and Pre-settlement on ΔActivity (Figure 7). The initial activity of mussels was relatively high for all treatments (0.48 ± 0.29 cm·h^{-1} for the Pre-settlement treatment and 0.41 ± 0.28 cm·h^{-1} for the Detached treatment), but then gradually decreased towards zero with time

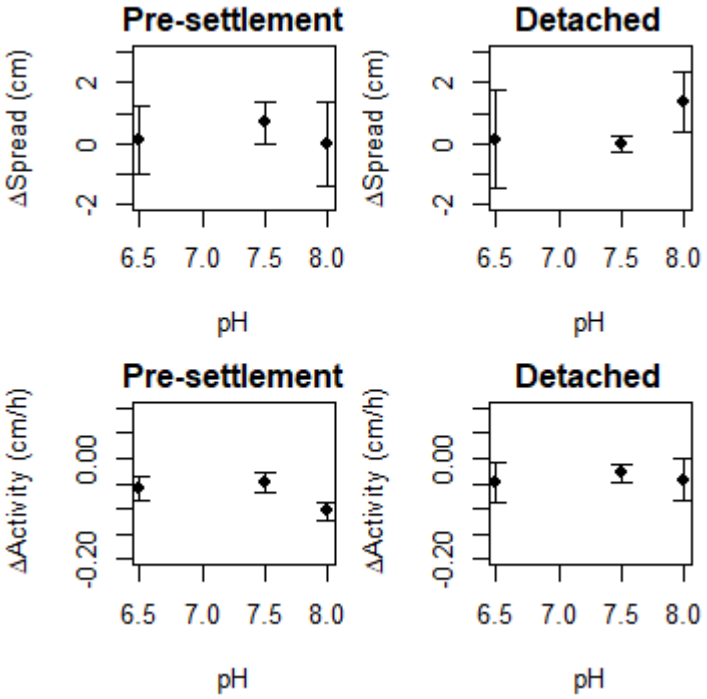

Figure 7; Mean difference in spread and activity before and after the CO2 treatment, error bars are Standard deviation.

(Figure 8 C,D). For the Pre-settlement treatment, after 48 hours activity was close to zero (0.036 ± 0.059 cm·h^{-1}). However, for the detached treatment, activity increased again (.51 ± .22 cm·h^{-1}) when detached and placed

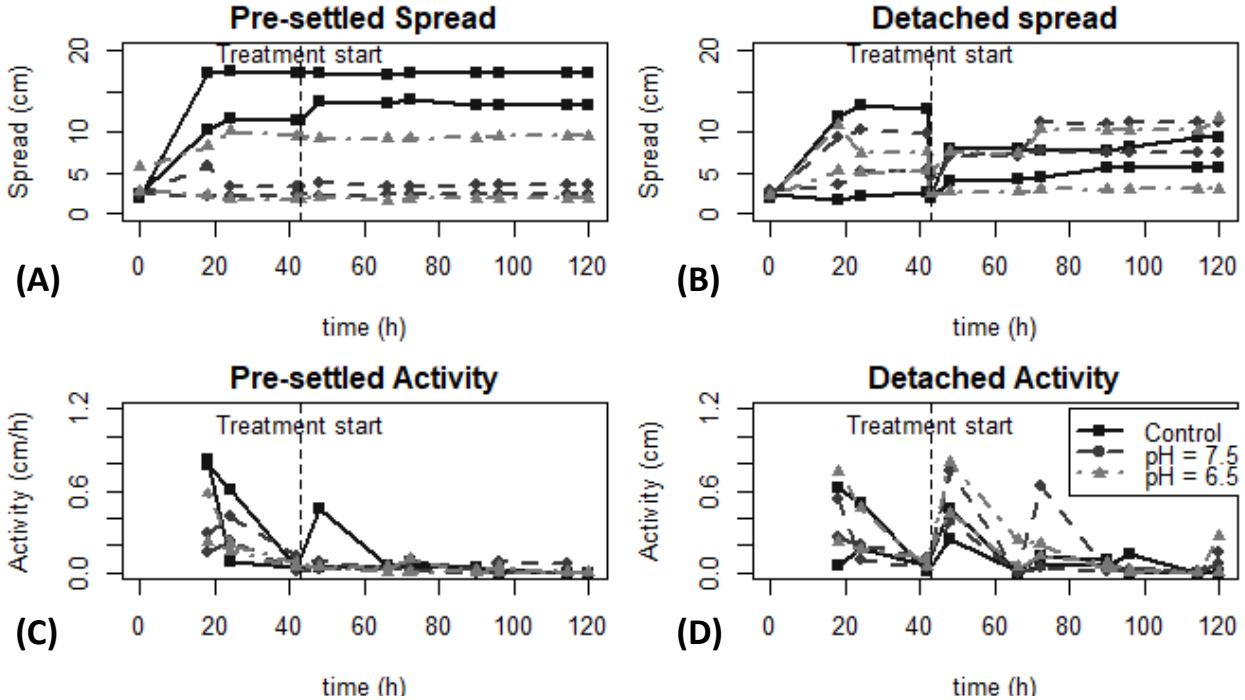

Figure 8; Mussel spread and activity over time. Spread is a measure of the average distance between all mussels in a tank. Activity is the average distance moved by all mussels in a tank per hour. The mussels in the detached treatment were detached at the start of the CO_2 treatment (striped vertical line) and placed back together, therefore the spread decreases at t= 42, mussels in the pre-settled treatment were left as they were. Colours and line types indicate the pH treatment (see legend in the bottom right graph).

back in the middle of the tank at the start of the CO_2 treatment (t = 42 h) and again gradually decreased to zero with time (Figure 8 D).

There were no significant effects of pH and Pre-settlement on ΔSpread (Figure 7). As mussels were placed together, the initial spread was 2.80 ± 0.47 cm, as time progressed, the Pre-settlement and detached treatment spread increased to 7.86 ± 0.059 cm and 7.26 ± 0.21 cm respectively and after being placed back in the middle, the detached mussel spread again increased to 7.16 ± 0.48 cm (Figure 8 A,B). As the activity decreased with time, the spread showed almost no change after 48 h.

Long term exposure experiment

The pH, salinity, alkalinity and oxygen values were maintained close to their target values (Salinity: 31.5‰, Oxygen: 100%, Alkalinity: 2.67 meq·L^{-1}) (Appendix 2). On day 9 of the experiment there was a malfunction in a CO_2 valve in one of the 6.8 treatment tanks, which meant the valve kept bubbling CO_2 into the tank and did not shut off, dropping the pH to 5.6. The mussels from this tank were placed in one of the other pH 6.8 treatment tanks as soon as possible. The valve was replaced with a new one and the pH was restored by applying an air pump to the tank. The mussels were then placed back in the tank but two out of 10 mussels had died. There was almost no growth in either length, width or height in any of the tanks, therefore there was no significant effect of pH on growth of mussels (Figure 9).

During the experiment there were only 7 mortalities out of 150 mussels, these 7 mortalities all occurred in the pH 6.2 and 6.8 treatments. There were two tanks in the pH 6.2 treatment groups that had 1 and 3 mortalities and two tanks in the pH 6.8 treatment groups that had 2 and 1 mortalities (Figure 10). However, a Kruskal-Wallis test revealed that there was no significant effect of pH on mortality.

There was a significant effect of pH on mussel reaction time ($F(4, 10) = 36.4$, $p = 6.25 \cdot 10^{-6}$). Reaction time is the amount of time it takes for a mussel to close when it is taken out of the water. Post-hoc comparisons using the Tukey HSD test indicated that the pH 6.2 treatment group had a significantly different reaction time of 14.6 ± 1.91 s compared to all other groups (which had an average of 4.11 ± 3.86 s) (Figure 11).

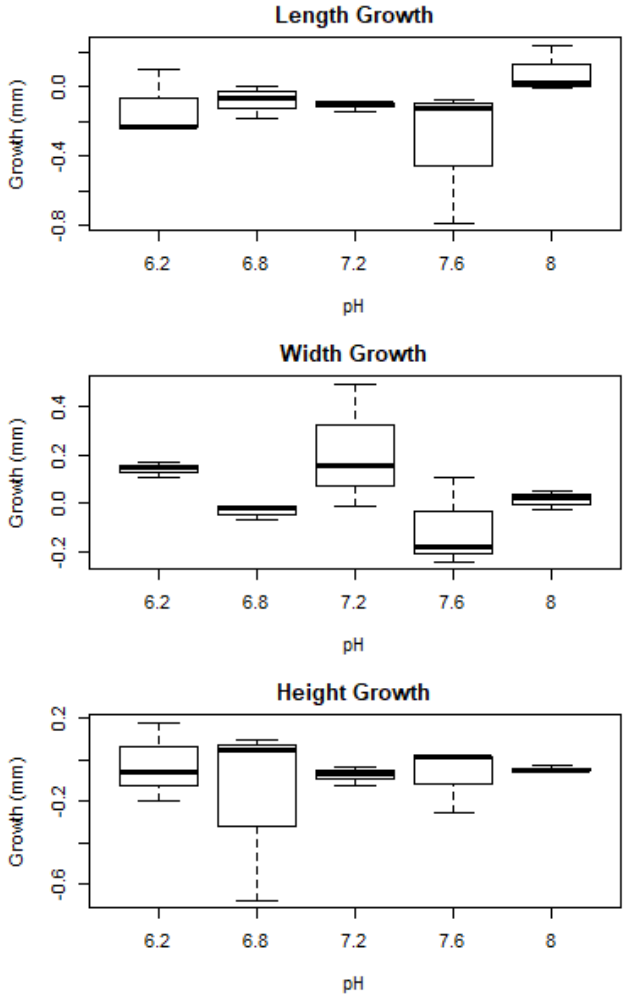

Figure 9; Growth of mussels in length, width and height in mm since the start of the experiment.

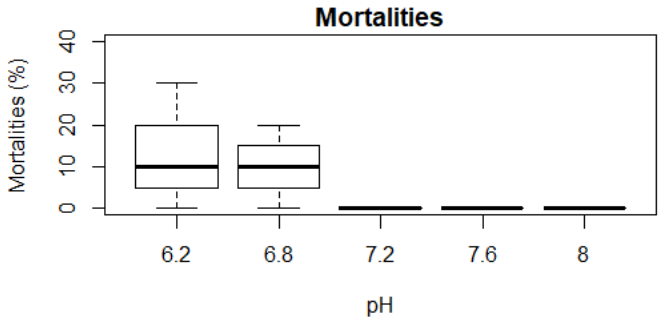

Figure 10; Percentage of mussels that died during the experiment per treatment.

The logistic models that were plotted through the detachment experiment data fit quite accurately (Appendix 3). The water velocity at which 50% of the mussels detached (DV50) was calculated from these models. There was no significant effect of pH on detachment velocity (Figure 12). DV50 was on average 0.89 ± 0.18 m/s. There was also no significant relationship between mussel shell size and DV50.

There was a significant effect of pH on mussel shell strength ($F(4, 10) = 4.424$, $p = 0.0257$). Shell strength was measured by the amount of weight that is required to crack a mussel shell. Post-hoc comparisons using the Tukey HSD test indicated that the mean shell strength of the pH 6.2 treatment group (8.10 ± 0.93 kg) was significantly different from the mussels in pH 6.8 and the Control group (11.84 ± 0.58 kg and 12.46 ± 2.42 kg respectively) (Figure 13).

A Kruskal-Wallis test revealed no significant effect of pH on mussel feeding rates (Figure 14). However, a trend is seen and a Pearson's correlation test revealed there was a significant correlation between feeding rate and pH ($r = 0.612$, $p = 0.015$).

Discussion

Elevated CO_2 levels are known to cause negative effects on the health and fitness of marine shelled molluscs. But whether CO_2 can be used to remove undesired marine fouling bivalves had not yet been investigated. The GasDrive project [8] is currently developing a ship that utilizes the exhaust gasses produced by the engine. The exhaust gas, which consist mainly of CO_2, is released under the ship's hull in order to reduce hydrodynamic drag in the water. If the exhaust were to have additional anti-fouling properties, it would reduce drag caused by fouling organisms even further. Moreover, it would reduce the costs of maintenance and may prove a more environmentally friendly alternative to the widely used anti-fouling paints [6].

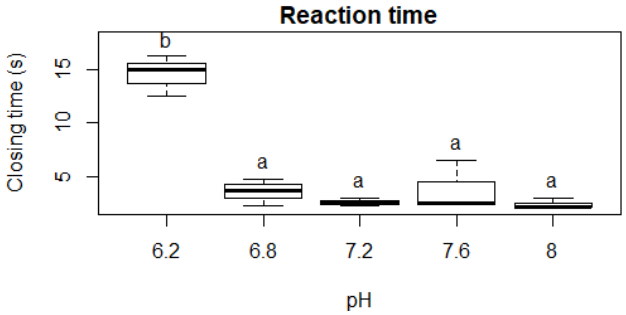

Figure 11; Reaction time of mussels per treatment (the amount of time it takes for a mussel to close when it is taken out of the water). Letters a and b signify which groups differ significantly from each other.

Behaviour

In this study we conducted a behavioural study to test whether elevated CO_2 concentrations

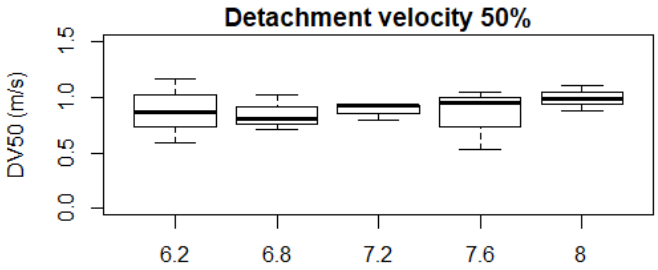

Figure 12; Water velocity in m/s at which 50% of the mussels detach for each different treatment pH.

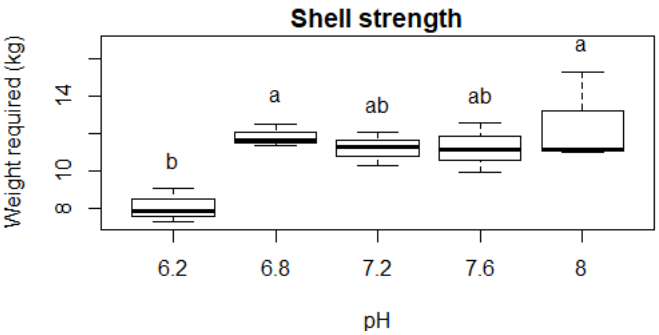

Figure 13; The shell strength of the mussels in each treatment group. It is measured as the amount of weight that is required to crack the mussel shell in kg. Letters a and b signify which groups differ significantly from each other.

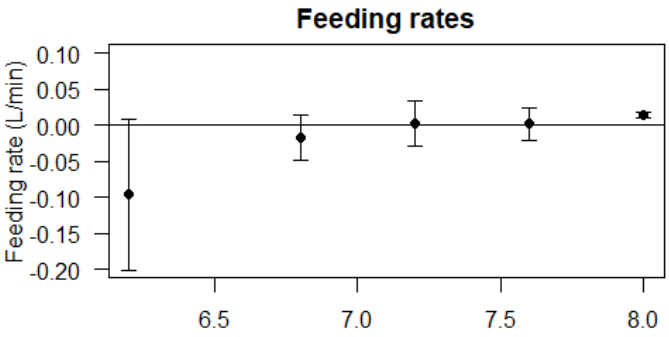

Figure 14; Mussel feeding rates in Lmin-1 for each treatment group, error bars are standard deviations.

would induce avoidance behaviour in *Mytilus edulis*, which would ultimately result in detachment from a vessel. We exposed *M. edulis* to CO_2 induced lowered pH conditions (pH 6.2, 6.8, 7.2, 7.6 and Control) and recorded movement during 7 days of exposure. We found that CO_2 did not induce a significant change in activity of *M. edulis*. Thus, it is unlikely that CO_2 is the cue that drive mussels to increase movement when local mussel densities become too high [18, 20]. Why mussel movement increases with high mussel densities in the surrounding area remains a mystery. It is possible that waterborne cues from mussels in the area induce increased movement of mussels, similar to how waterborne cues from crabs induce increased shell thickening in *M. edulis* [26] or that it is caused by low algae concentrations due to feeding competition. Furthermore, we can conclude that CO_2 is unlikely to induce avoidance behaviour and as a result, mussels will probably not detach from a GasDrive ship on their own accord.

Attachment strength

In a second, longer experimental study, we tested whether elevated CO_2 concentrations reduce attachment strength in *M. edulis*. We exposed *M. edulis* to CO_2 induced lowered pH conditions (pH 6.2, 6.8, 7.2, 7.6 and Control) for 21 days. Afterwards, we measured at which velocity the different *M. edulis* treatment groups detached when exposed to a parallel water flow. No significant reduction in detachment velocity was found. This is in contrast with findings from two other experimental studies on Mytilid species. They found that 1 week of exposure to elevated CO_2 concentrations at a pH of 7.4-7.5 reduced mussel attachment strength by 40-65% [23, 27]. There were a few differences in experimental setup. They used a pulling force on individual mussels to determine the force required to detach the mussel, while we used a parallel water flow on groups of 10 mussels. Also, they used 8 and 10 replicates in their studies, while we only used 3 [23, 27]. Variation in detachment may have increased due to interaction between mussels (attaching to each other), because detachment from the substrate of one mussel in a mussel clump influences the detachment rates of other mussels [28]. This increases complexity of the interactions and may thus increase variation of the results. Increased variation reduces the chance of revealing a significant effect. It is also possible that *M. edulis* attachment strength is less affected by acidification than other Mytilid species. Another difference is that mussels in this experimental study were allowed to attach before the start of the CO_2 treatment. As the process of byssal secretion requires a number of pH dependent chemical functions [29], there is a chance that byssal attachment strength is lower for threads that were produced in an acidified environment.

Detachment velocities found in this experimental study were much lower than what was previously found in field studies. Detachment starts at velocities greater than 0.5 ms^{-1} in the current study (Appendix 1), compared to velocities greater than 7 ms^{-1} in field studies [28]. Mussel attachment strength is known to decrease when exposed to lower hydrodynamic forces [30], apart from a small aquarium pump the mussels in this experimental study were not exposed to any hydrodynamic disturbances. Which may have resulted in lower detachment velocities when they were suddenly exposed to an increased water velocity, than what is seen under natural circumstances.

Mortality

This experimental study shows that Mytilus edulis adults have the ability to survive exposure to elevated CO_2 conditions (up to 37711 ppm leading to a pH of 6.2) for as long as three weeks. More mortalities were observed in the higher exposure groups, 13.3% and 10% mortality at pH 6.2 and 6.8 respectively by day 21 and no mortalities in higher pH groups, but this did not lead to a statistically significant effect. In a similar study, mussels were exposed to elevated CO_2 concentrations at a pH of 6.7

and no mortalities were observed within 23 days [24]. However, within the next week (by day 30), about 20% mortality occurred [24]. In another study, at a pH of 6.2, *M. edulis* showed only about 15% mortality by day 20, which is similar to our findings [31]. However, by day 30, about 70% mortality had occurred and at a pH of 5.8, 75% mortality was already observed by day 20, with a 100% mortality rate by day 30 [31]. Therefore, it is likely that longer exposure times and slightly lower pH conditions will lead to greatly elevated mortality rates.

Overall health

Mussels are well equipped with physiological mechanisms that protect them from acidified water conditions [32]. Even though these mechanisms allow them to survive in acidified water conditions, they come at energetic costs [32]. These energetic costs reduce the mussel health, negatively impacting other important functions . Many negative effects have been reported in prior studies, like reduced growth, metabolism, calcification rate, feeding rate and immune response [22, 24, 31–33]. In addition, we found a reduction in shell closing speed and shell strength when *M. edulis* was exposed to a pH of 6.2. Also, a trend is seen between feeding rate and pH, where mussels exposed to lower pH values had lower feeding rates than those exposed to higher pH.

As the pH decreases, the saturation state of Calcium Carbonate increases, which causes shell dissolution and reduced shell growth [9]. Therefore it is likely that shell strength was reduced through shell dissolution. Why a negative effect of reduced shell closing time is seen is uncertain, but it may be an indication that overall mussel health is reduced. Because the mussels experience stress from the increased CO_2 concentrations, they must invest more energy in the maintenance of their internal pH and Calcium concentrations. And thus they have less energy available to them to invest in muscle function.

The mussel shell is an important feature that blocks predators from reaching the vulnerable tissue inside the shell. When the shell does not close quickly enough, predators will be able to reach the nutritious tissue before the mussel is closed. Furthermore, when shell strength is reduced, predators require less force and or shorter handling times to crack the mussel shell. Therefore it is likely that mussels that are exposed to increased CO_2 concentrations, at pH 6.2, will be more vulnerable to predation.

A reduced health from one stressor, may lead to an increased vulnerability to other stressors. In another study, *M. edulis* was exposed to 3 different heavy metals (cu, pb & cd) at CO_2 induced reduced pH conditions. Heavy metal exposure at control pH led to a 10-15% mortality rate, while a reduction of the pH to 6.2 caused an increase to 40-60% mortality in 21 days [34]. We found that *M. edulis* exposed to a pH of 6.2 without heavy metals had a mortality rate of 10%. Currently, the most widely used anti-fouling paints employ copper as a biocide [6]. Whether the increased toxicity is the result of reduced mussel health due to CO_2, or due to the increased solubility of metals when pH is decreased, is uncertain. But if CO_2 from the ship exhaust were to be used in combination with copper paints or other biocides, this would most likely lead to a greater reduction of biofouling. It might also reduce the amount of copper required in the paints to reduce Bio-fouling and thus reduce negative environmental impacts of these paints.

Method reflection

No significant growth or shrinkage was recorded in any of the treatment groups after 21 days. This is in contrast with a similar study, in which *M. edulis* length increased by 1.42 mm in 44 days at control pH, with no increase at a pH 6.7 [24]. Why there was no growth during the experiment is uncertain. Even though *M. edulis* growth is influenced by a variety of factors like temperature, salinity and density, the most important factor is food availability [35–38]. We measured feeding rates in the control group that was similar to those recorded in previous studies [25, 39]. Also,

algae concentrations were calculated to be constantly kept between 2000 and 9000 cells/ml (Appendix 1), which is around the optimal concentration for *M. edulis* feeding [25] and all algae species in the feeding stock were in the 3-20µm *M. edulis* adult particle size selection range [40]. However, it is possible that the nutrient composition of the algae stock was not sufficient for mussel growth. Salinity was kept at optimal growth conditions [36, 37]. The temperature used during the experiment was kept similar to the temperature at the mussel collection site, but this was sub-optimal for *M. edulis* growth, which may explain the lack of growth in the control group [38].

During the second experimental study, we tested whether elevated CO_2 concentrations reduce feeding rates of *M. edulis* on dead algal cells, after 18 days of exposure. A trend is seen between feeding rate and pH, where mussels exposed to lower pH values had lower feeding rates than those exposed to higher pH. However, some of the treatment groups were calculated to have negative feeding rates. This means that when the algae concentration was measured, the amount of algae was higher after the 30 minute feeding period than before. This is quite unlikely, in 30 minutes of feeding, the concentration of dead algal cells should either remain the same or decrease. It is possible that after adding the dead algal feed, the algae were not given enough time to be thoroughly mixed when we took the first water sample.

The hydrodynamic forces that mussels are exposed to are different in a PVC tube than the hydrodynamics around a ship hull. However, determining detachment velocities by placing mussels on a ship hull, is hard to accomplish in practice. Prior to this study, we conducted a couple of try-out experiments in which we attempted to make mussels attach to a PVC plate, that could be placed on a boat. Unfortunately, mussels were able to move from the PVC plate and attached to the glass walls and floor of the tank instead. We

Figure 15; Mussels are placed between two PVC plates, surrounded with chicken fence.

Figure 16; Mussels are kept in place on a PVC plate using a small net. The net is fixed by using a tie-wrap at the back.

therefore developed two techniques to keep mussels attached to the plates: 1. Tying them to the plate using a small net and tie-wraps (Figure 16) and 2. Placing mussels in between two PVC plates and placing a chicken fence around the plates (Figure 15). Unfortunately there were two problems with these techniques:

1. The mussels also attached themselves to the nets and fences. This meant that if the fences and nets were removed, some of the mussels would already

detach. For other mussels, some of their byssus threads would break, reducing their attachment strength.

2. Even when all the mussels remained attached to the PVC plates, 15-80% of the mussels would detach themselves and move away from the plate in less than two days.

We therefore developed a new technique; by using PVC tubes blocked off at both ends by nets (Figure 4). Only one of the two nets needs to be removed just before the detachment experiment, allowing you to select the side that has the least mussels attached to the net. This greatly reduced the risk of affecting the experimental results by the two previously mentioned problems.

Using PVC tubes as containers for the mussels also made it much easier to expose the mussels to a parallel water flow by using a water pump. In general, this was an easy and cheap way of testing detachment rates at different water velocities. However, the water velocities you are able to produce with an affordable pump (up to 1.8 ms^{-1} in this study) are much lower than the velocities that are reached by a moving vessel (up to 13 ms^{-1}) [16]. As stated before, attachment strength of the mussels in this study were low, because they were exposed to negligible hydrodynamic forces prior to the detachment experiment. Therefore more than 95% of the mussels in this study detached before 1.8 ms^{-1}. Even though this makes it possible to use an affordable water pump in the experimental setup, in order to see the effect of CO_2 on detachment, it decreases the range of velocities over which mussels detach, making it harder to discover significant differences between treatment groups.

Conclusion

In conclusion, if the pH around a ship hull that is covered by mussels is decreased to a pH as low as 6.2, it would take more than a month for the mussels to show significant mortality rates. However, if CO2 were to be used in combination with toxic paints, mortality rates may drastically increase. I therefore recommend not to use CO_2 as the sole anti-fouling strategy when dealing with marine bivalves, but rather in combination with pre-existing methods to increase effectiveness.

Also, our findings show that at a pH of 6.2, two important features that protect mussels from predation are negatively affected by increased CO_2 concentrations. The time it takes for the organism to close its shell in reaction to a threat, is increased by 500%. And the shell's resistance to a cracking force is reduced by 35%. This may greatly affect vulnerability to predation. If there is a sufficient amount of predators around a GasDrive ship, the abundance of mussels on the hull may be greatly reduced.

Suggestions

If the detachment setup we developed in this study were to be used again, it would be best to either permanently apply a strong water pump in each aquarium during the CO2 exposure, or to increase the water velocity in the tanks for specific periods of the day. This would drive the mussels to invest more energy in the production of byssal threads and could induce a bigger difference in attachment strength between treatments. However, it would probably require a stronger pump for the detachment experiment to make all mussels detach at maximum pump velocity.

I suggest a study of the effect of CO2 in combination with (copper) anti-fouling paints to research whether it increases anti-fouling efficiency. As of yet, it is uncertain whether anti-fouling paints can be used on a GasDrive ship. It might decrease the effectiveness of the microstructure that is used to capture gas bubbles on the hull of the ship. It would be useful to investigate whether copper or other biocides can processed into the microstructure material.

It would be useful to test whether byssal threads that are produced by mussels in an

acidified environment are weaker than threads produced under normal circumstances, but later exposed to a reduced pH. I assume that most mussels on ship hulls grow there when the ship is not moving and thus the mussels on a GasDrive ship would have produced the most Byssus threads before being exposed to increased CO_2 conditions. However, it would give insight into the underlying mechanisms of byssal attachment and it would explain why we found no effect of CO_2 on mussel attachment strength while other studies did show significant differences.

In this study we investigated *M. edulis* as a model organisms for marine fouling bivalves. There are however many other major groups of marine fouling organisms such as Bryozoa, Arthropoda (mainly barnacles), Coelenterata and Algae [1]. The effect of CO_2 on fouling barnacles (the most abundant group of fouling organisms) was investigated in a sister study. These two groups were chosen because their Calcium Carbonate skeletons make them more vulnerable to CO_2 induced acidification. However, there are still more important calcifying fouling organisms such as Bryozoa and some Polychaete species like *Pomatoceros triqueter* that may be negatively affected. Furthermore, it is important to realize that non-calcifying fouling organisms may be less affected by elevated CO_2 concentrations. Some organisms, such as Algae may even profit from an increase in available CO_2. Also, a study of the relative importance of the different fouling organism groups on the production of hydrodynamic drag may provide valuable information.

This experimental study revealed that elevated CO_2 concentrations negatively affected closing time and shell strength in *M. edulis*. As these aspects provide the mussel protection from predators I would suggest a field study of mussels exposed to increased CO_2 concentrations. In which one group is protected from predation by means of a protective cage, while for the other group, natural predation may occur. To see whether the increased vulnerability of CO_2 exposed mussels to predation, would lead to higher mortalities in a more natural environment.

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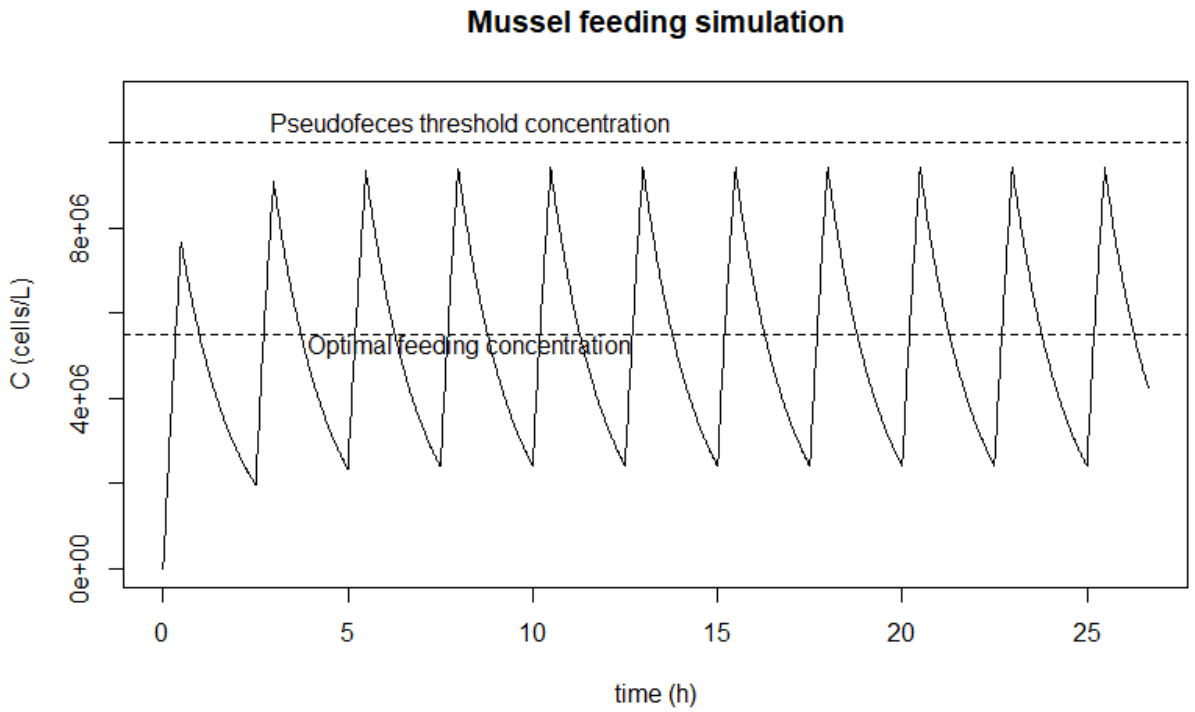
Appendix 1: Mussel feeding simulation R script

```
rm(list = ls())

#variables in algal cells, Liters & minutes

#Tank Volume (L)
Tvol = 25

#Pseudofeces threshold (cells/L) (Above this value algae are wasted)
PsThresh = 10e6

#Optimal concentration for mussels (cells/L)
OptC = 5.5e6
#Optimal nr. of cells in tank
Optcells = Tvol*OptC

#Feeding rates per mussel (L/min)
Frate = 0.014
#Nr of mussels in the tank
Mussels = 20
#Ratio of cells removed /min
RatioRem = Frate*Mussels / Tvol

#Speed of the feeding pump (L/min)
Prate = 0.0039
#Stock concentration (feeding pump input) (cells/L)
SC = 2e9
#Total Cells/min added by pump
CellAdd= Prate*SC

#time window
t = 1:1600
#Starting concentration (cells/L)
C0 = 0

#create an empty data sheet
df = data.frame(rep(NA, tail(t, n=1)),rep(NA, tail(t, n=1)))
df[1,1] = 0
df[1,2] = 0
colnames(df) = c("Concentration (cells/L)", "Time (min)")

#The cells model (computes the concentration the next minute in the aquarium (cells/L))

Cnext = function(C0, t){
  Cells = C0*Tvol
  #The times at which the feeding pump is on
  if(t <30 | t>150 & t<180 | t>300 & t<330| t>450 & t<480 | t >600 & t<630| t>750 & t<780| t>900 &
t<930 | t >1050 & t<1080 | t >1200 & t<1230 | t >1350 & t<1380| t>1500 & t<1530){
    Cells = Cells + CellAdd
  }
```

```
 Cells = Cells* (1-RatioRem)
 C0 = Cells/Tvol
 return(C0)
}

#run the simulation for the specified amount of time
for(i in t){
 df[i+1,2] = i
 C0 = Cnext(C0,i)

 df[i+1,1] = C0
}

#plot the graph
plot((df$`Time (min)`)/60,df$`Concentration (cells/L)`, type = "l",xlab = "time (h)", ylab = "C (cells/L)",
main = "Mussel feeding simulation", ylim = c(0,11e06), lwd = 1.5)
abline(a =OptC, b = 0, lty = 2)
text(500/60, OptC*0.95, labels = "Optimal feeding concentration")
abline(a =PsThresh, b = 0, lty = 2)
text(500/60, PsThresh*1.05, labels = "Pseudofeces threshold concentration")
```



Appendix 2: Water parameters of the long term exposure experiment

Table 1; Water quality parameters of the different pH treatments, pCO_2 values were calculated from the measured pH, Temperature, Salinity and alkalinity, with the R package seacarb version 3.2.10 [41].

	Treatment pH									
	6.2		**6.8**		**7.2**		**7.6**		**8**	
Water quality parameters	**Mean**	**St. dev**	**Mean**	**St. dev**	**Mean**	**St. dev**	**Mean**	**St. dev**	**Mean**	**St. dev**
pH	6.22	± 0.02	6.79	± 0.05	7.18	± 0.05	7.62	± 0.02	7.97	± 0.01
Temperature (°C)	13.58	± 0.15	13.39	± 0.16	13.32	± 0.32	13.36	± 0.45	13.34	± 0.42
Salinity (‰)	32.03	± 0.06	32.14	± 0.06	32.02	± 0.07	32.05	± 0.06	32.03	± 0.07
Oxygen (%)	93.62	± 2.03	95.98	± 0.41	95.79	± 0.56	96.60	± 0.61	96.56	± 0.31
Oxygen ($mg \cdot L^{-1}$)	7.97	± 0.02	8.15	± 0.04	8.06	± 0.24	8.21	± 0.08	8.21	± 0.12
Alkalinity ($meq \cdot L^{-1}$)	2.70	± 0.02	2.69	± 0.03	2.68	± 0.02	2.69	± 0.01	2.68	± 0.02
pCO2 (ppm)	37711	± 1613	10058	± 1302	4033	± 486	1419	± 69	591	± 17.4

Appendix 3: Ratio of detached mussels with increasing water velocity.

With the corresponding logistic regression model plotted through the data. Striped lines indicate the velocity at which 50% of mussels detach (DV_{50}).