Influence of sea water acidification on benthic bivalve communities

Master thesis

Hanna Schade

Supervisor: Prof. Dr. Frank Melzner

Mathematisch Naturwissenschaftliche Fakultät der
Christian-Albrechts-Universität zu Kiel

Helmholtz-Zentrum für Ozeanforschung Kiel │GEOMAR

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Summary

Marine organisms have to cope with increasing CO_2 partial pressures in the oceans. The ocean acidifies as an effect of ongoing pCO_2 increase and as a possible result of leakage at carbon capture and sequestration (CCS) storage sites. Leakage at CCS storage sites has the potential to cause a localized very strong increase in pCO_2.

This is the first study testing the effects of moderately to highly elevated pCO_2 on three marine infauna bivalve species during a 12 week period using six different pCO_2 levels (900, 1,500, 2,900, 6,600, 12,800, 24,000 µatm) were applied. In this study the effects of high pCO_2 on mortality, shell free dry mass, corrosion, behavior and tissue malondialdehyde (MDA) content of different size classes of the cockle *Cerastoderma edule* were investigated. For the same treatment levels, mortality and behavior of *Mya arenaria* and *Macoma balthica* were assessed.

C. edule was found to be highly sensitive to possible leakage as pCO_2 had a significant influence on mortality for levels > 6,600 µatm. Mortality decreased with size, larger animals were less susceptible than smaller animals. In the highest treatment even larger specimens were strongly impacted by elevated pCO_2 and mortality was high. *C. edule* was found to be influenced at lower pCO_2 as corrosion of the shell, visualized using SEM analysis, occurred at a level of 1,500 µatm. This value will occur due to general CO_2 increase, even without the added influence of potential CCS leakage. Visible macroscopic corrosion could be shown for 2,900 µatm. Shell free dry mass decreased in the highest pCO_2 level indicating starvation and tissue metabolization. MDA content and filtration rate tended to increase until a pCO_2 of 2,900 µatm and decrease at higher levels. Significant correlation between both parameters suggest a decrease in metabolism at the highest pCO_2 levels tested. At very high levels of pCO_2, cockles move to the sediment surface prior to death and are easily visible. This behavior is applicable as a monitoring technique for possible leakages. *C. edule* was found to be highly sensitive towards acidification within the water column. Future ocean acidification scenarios (< 3000 µatm) were shown to be not lethal but did influence shell morphology, while leakage scenarios (> 6,000 µatm) led to increased mortality.

Mortality and behavior were not significantly influenced by pCO_2 at any of the applied levels for *M. arenaria* and *M. balthica,* suggesting a very high resistance of both species towards increasing pCO_2 in the ocean.

Zusammenfassung

Marine Organismen müssen in der Zukunft erhöhten CO_2 Partialdrücken standhalten. Der Ozean versauert durch den Anstieg der atmosphärischen CO_2 Konzentration, außerdem kann es aufgrund potentieller Leckagen aus Kohlenstoffspeicherstätten (CCS) zu lokal sehr stark erhöhtem pCO_2 Werten kommen. In dieser Studie wurden erstmalig während eines 12 Wochen Experiments die Effekte von schwach bis stark erhöhtem pCO_2 auf drei marine Infauna Muscheln untersucht. 6 unterschiedliche pCO_2 Stufen (900, 1.500, 2.900, 6.600, 12.800, 24.000 µatm) wurden angewandt. Die CO_2 Effekte auf Mortalität, schalenfreie Trockenmasse, Schädigung der Schale, Verhalten und MDA Konzentration in verschiedenen Größenklassen von *Cerastoderma edule* wurden gemessen.

Zusätzlich wurden die Effekte der unterschiedlichen CO_2 Stufen auf Mortalität und Verhalten von *Mya arenaria* und *Macoma balthica* untersucht.

C. edule reagierte höchst sensitiv auf erhöhte pCO_2 Werte. Erhöhter pCO_2 führte zu signifikant höherer Mortalität für Level > 6,600 µatm. Die Mortalität nahm mit der Größe der Tiere ab; größere Individuen waren weniger anfällig als kleinere. Die größeren Individuen waren in den hohen Behandlungslevel dennoch stark beeinflusst und die Mortalitätsrate war hoch. Eine Schädigung der Schale konnte durch die REM Analyse bereits in niedrigeren Stufen > 1.500 µatm gezeigt werden. Dieses Level würde auch bei Ozeanversauerung durch einen zukünftigen CO_2 Anstieg, ohne zusätzlichen Einfluss von Leckagen, auftreten. Makroskopisch sichtbare Schädigung der Schale trat ab einem Level von 2.900 µatm auf. Die schalenfreie Trockenmasse war geringer im höchsten pCO_2 Level und wies damit auf eine Verstoffwechslung von Gewebe und Hungersstress hin. MDA Konzentration und Filtrationsrate zeigten einen zunehmenden Trend bis zu einem pCO_2 Wert von 2.900 µatm und nahmen in den höheren Stufen ab. Beide Parameter deuten auf eine Abnahme des Metabolismus für die höchsten Level hin. Muscheln kamen vor ihrem Tod auf die Sediment Oberfläche und konnten dadurch einfach gezählt werden. Dieses Verhalten kann als Überwachungstechnik für mögliche Leckagen genutzt werden. *C. edule* wurde als äußerst sensitive Art im Bezug auf erhöhten pCO_2 klassifiziert. Die Szenarien der Ozeanversauerung (< 3.000 µatm) durch den prognostizierten pCO_2 Anstieg unabhängig von Leckagen aus CCS Lagerstätten wirkten nicht tödlich, beeinflussten

aber die Schalenmorphologie. Szenarien für den lokalen Anstieg von pCO_2 durch Leckagen (> 6.000 µatm) führten zu erhöhter Mortalität für C. edule.

M. arenaria und *M. balthica* wurden als sehr resistente Arten im Bezug auf Ozeanversauerung eingestuft. Weder Mortalität, noch Verhalten wurde signifikant von erhöhtem CO_2 Partialdruck beeinflusst.

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List of Abbreviations

A. excavata:	*Acesta excavata (*Fabricius, 1779)
A_T	Total Alkalinity
$CaCO_3$:	Calcium carbonate
CCS:	Carbon Capture and Sequestration
C. edule:	*Cerastoderma edule* (Linnaeus, 1758)
CO_2:	Carbon dioxide
CO_3^{2-}:	Carbonate
C. gigas:	*Crassostrea gigas* (Thunberg, 1793)
C. virginica:	*Crassostrea virginica* (Gmelin 1791)
C_T:	Dissolved inorganic carbon
Gt:	Giga tonnes
HCO_3^-:	Bicarbonate
M. arenaria:	*Mya arenaria* (Linnaeus, 1758)
M. balthica:	*Macoma balthica* (Linnaeus, 1758)
M. edulis:	*Mytilus edulis* (Linnaeus, 1758)
M. galloprovincialis	*Mytilus galloprovincialis* (Lamarck, 1819)
M. mercenaria:	*Mercenaria mercenaria* (Linnaeus, 1758)
M. truncata	*Mya truncata* (Linnaeus, 1758)
MDA:	Malondialdehyde
pCO_2:	Partial pressure of carbon dioxide
µatm:	Micro atmosphere
R. sp.	*Rhodomonas* sp. (Karsten, 1898)
S. spirobis:	*Spirobis spirobis* (Linnaeus, 1758)

1. General Introduction

1.1 Ocean Acidification

Atmospheric carbon dioxide (CO_2) is the most important green house gas. CO_2 concentrations have been increasing since the beginning of the industrial revolution due to combustion of fossil fuels (Körtzinger, 2010). CO_2 concentrations remained relatively stable prior to the industrial revolution and are increasing exponentially since then (Fig.1.1). A number of scenarios assembled by the International Panel on Climate Change (IPCC) predict a rise from today's 380 µatm in atmospheric CO_2 concentrations to between 650 µatm and 970 µatm until the year 2100 (IPCC 2007). Business as usual scenarios of carbon emission predicted a rise of up to 1,900 µatm until the year 2300 (Caldeira and Wickett, 2005). The differences in scenarios are based on different estimates of anthropogenic CO_2 emissions, which are strongly dependent on social and technological development. A rise in atmospheric CO_2 will alter the climate system as it leads to global warming and an increase in dissolution of CO_2 in the ocean surface water. Additional CO_2 through ocean acidification will increase CO_2 partial pressure which causes a threat also to systems with high seawater pH variability like the Western Baltic (Thomsen et al., 2010). The ocean has already taken up about 50% of the emitted CO_2 and this process is continuing (Sabine et al, 2004).

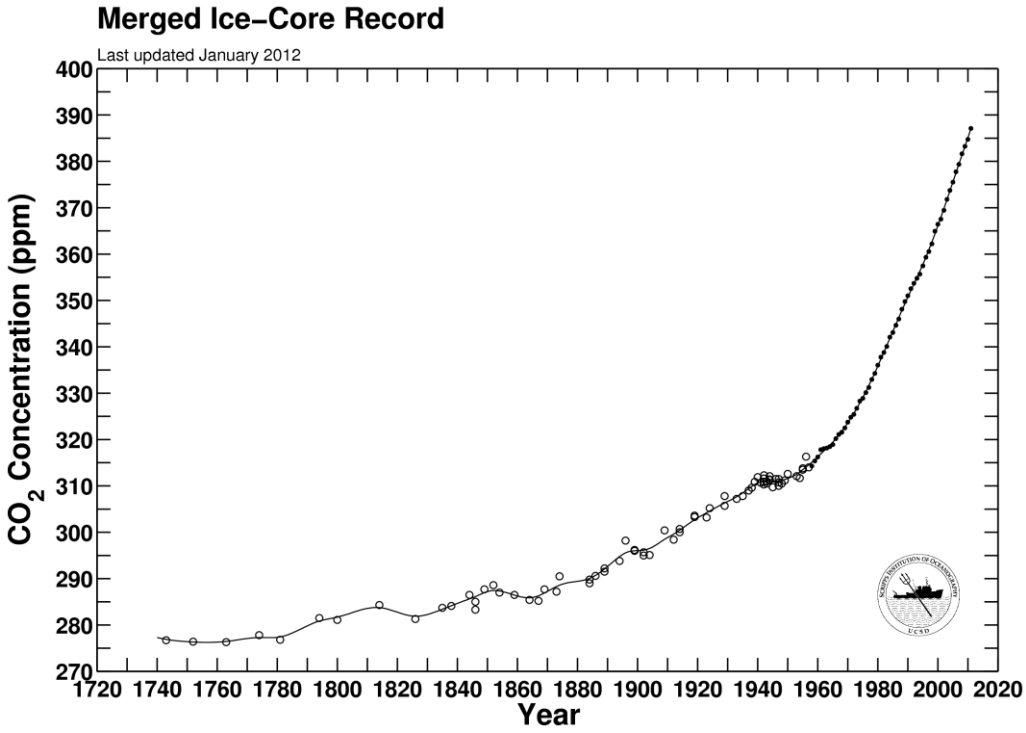


Fig.1.1: Atmospheric CO_2 concentrations over the last 300 years. Concentrations from ice cores and measured atmospheric values are combined in the data set. (http://scrippsco2.ucsd.edu/images/graphics_gallery/originalpng/merged_ice_core_record.png)

1.2 Carbonate system

When CO_2 is taken up by the ocean it reacts with water which results in unstable carbonic acid (H_2CO_3) (Equ.1.1). Bicarbonate (HCO_3^-) is formed by the dissociation of carbonic acid and the release of one proton. One further proton is released when bicarbonate dissociates to carbonate (CO_3^{2-}). All carbonate species are in chemical equilibrium.

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \leftrightarrow 2H^+ + CO_3^{2-} \qquad \text{(Equ.1.1)}$$

The sum of the concentrations of those three carbonate species is called dissolved inorganic carbon (C_T). CO_2 uptake by the oceans, leads to an increase of C_T and accordingly to a continuous decrease in pH. Ocean surface pH has already decreased to 8.1, compared to preindustrial values of 8.2. Future increases in atmospheric and oceanic CO_2 concentrations will lead to a further decrease in pH by approximately -0.46 units until 2100 (Caldeira and Wickett, 2005).

The seawater is buffered against pH changes by conversion of the carbon species into each other. As the chemical conversion is highly pH dependant, a change in pH has a strong influence on the composition of carbonate species as depicted in the Bjerrum plot (Fig.1.2). At current pCO_2, C_T consists of about 1% [CO_2], 91% [HCO_3] and 8% [CO_3^{2-}]. With decreasing pH, [CO_3^{2-}] will decline, while [CO_2] and [HCO_3] will increase (Fig.1.2; Körtzinger 2010).

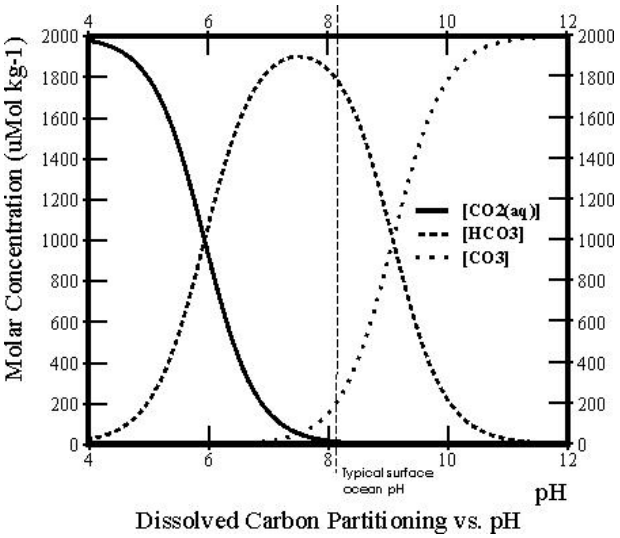


Fig.1.2: The Bjerrum plot depicts the pH dependency on concentrations of the three carbonate system species CO_2, HCO_3^- and CO_3^{2-}
http://www.noc.soton.ac.uk/jmodels/wiki/images/thumb/a/a3/Bjerrum_DIC_plot.png/300px-Bjerrum_DIC_plot.png

Depending on the rise of atmospheric CO_2, the concentration of carbonate in the surface water of the ocean will decrease by 60% (atmospheric CO_2 of 800 µatm) by the year 2100. C_T will rise by about 12% (Feely et al., 2004).
As a consequence of lowered [CO_3^{2-}], the calcium carbonate saturation state omega (Ω) is going to decrease. Omega is the product of [Ca^{2+}] and [CO_3^{2-}] divided by the stoichiometric solubility coefficient (K_{sp}) (Equ.1.2).

$$\Omega = \frac{[Ca^{2+}][CO_3^{2-}]}{K_{sp}} \qquad \text{(Equ.1.2)}$$

Biogenic calcium carbonate mainly exists in two polymorphs, the relatively stable calcite and the less stable and more soluble aragonite (Cao et al., 2007). The calcium content of seawater is relatively stable; $CaCO_3$ saturation is mainly dependent on the

carbonate concentration. Rising surface ocean CO_2 concentrations will lead to a decrease of carbonate ion concentrations and thus of calcium carbonate saturation values (Fig.1.3; Orr et al., 2005). High latitude oceans are expected to be undersaturated with respect to aragonite by the end of this century (Fig.1.3; Orr et al., 2005).

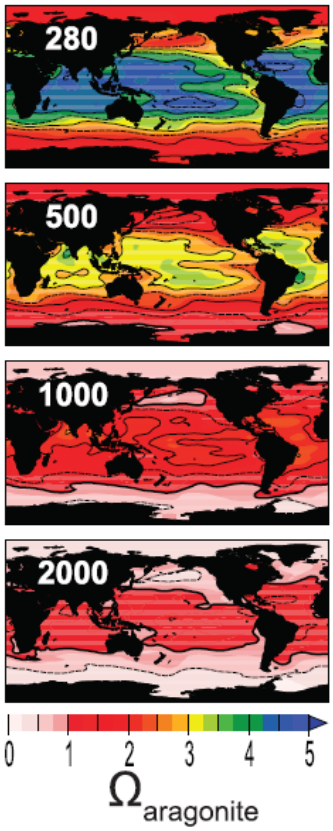


Fig.1.3: Aragonite saturation state (Ω) of the surface ocean modeled for atmospheric CO_2 concentrations between 280 (pre-industrial), 1,000 (end of this century) and 2,000 µatm. Aragonite undersaturation in the surface water is expected for CO_2 concentrations of 1,000 µatm (Cao and Caldeira 2008).

1.3 Carbon Capture and Sequestration

In order to limit dangerous anthropogenic influence on the climate system, greenhouse gases need to be kept at a certain level. To achieve a limit of global climate change temperature increase to 2°C, the emission of greenhouse gases needs to be reduced by 30 % in industrial countries until 2020; global emission by 70 % compared to 1990 values (EU Directive 2009). To achieve a reduction of CO_2 emission to stabilize greenhouse gas concentrations in the atmosphere at the targeted level, an application of a combination of different technologies is necessary. Different mitigation measures can achieve abatement to varying degrees. Abatement can be achieved by applying energy efficiency improvements, suitable less carbon

intensive fuels, nuclear power, renewable energy sources, enhancement of biological sinks and the reduction of non-CO_2 greenhouse gas emissions. The world energy outlook (2010) suggests an application of energy efficiency improvement of 73 %, renewable energy of 14 %, biofuels of 2 % and nuclear power of 4 % by 2020.

One additional way to reduce CO_2 emissions into the atmosphere is carbon capture and sequestration (CCS). Application of 3 % by 2020 is suggested for this technique. CCS is the capture and long-term storage of CO_2 in geological formations (Steeneveldt, 2006). It involves the capture of gaseous CO_2 from point sources (e.g. coal power plants), compression and injection of liquid CO_2 into porous reservoir rocks (Pires et al., 2011; Holloway, 2007; Johnston and Santillo, 2002). The potential of these strategies is scientifically well recognized (Widdicombe and Needham, 2007; Johnston and Santillo, 2002), however the acceptance of this technique depends for instance on successful management of the risks of storage. This technique will contribute to reducing anthropogenic influence on climate change. Even though CCS represents a promising and powerful technology to stabilize the CO_2 concentrations in the atmosphere (Pires et al., 2011) and is an important building block for future climate politics, the development of CCS techniques should not lead to a decrease of efforts to support other CO_2 mitigation techniques. 20-40 % of global fossil fuel CO_2 emissions could be technically suitable for capture by 2050 (IPCC, 2005). Storage capacity in geological formations is estimated to range between 200 $GtCO_2$ and 2,000 $GtCO_2$ worldwide.

Sub-seabed CO_2 sequestration is already utilized on industrial scales, i.e. in the framework of the operation of the Sleipner and Snøhvit gas fields in Norway. Sleipner is an active gas field in the North Sea (58° 22' 2.33'' N, 1° 54' 31.01'' E). Since 1996 14 Million t of CO_2 have been pumped into the saline aquifer, at a rate of 1 Million t CO_2 a^{-1}. CO_2 is separated from natural gas and stored at a water depth of 90m, under a sediment layer of 800m of gas tight cap rock. 0.7 Million t CO_2 a^{-1} have been injected into Snøhvit (70°41'13"N 23°35'55"E), a saline sandstone formation where CO_2 hydrates are formed as a consequence of high pressure and low temperature. Even though Sleipner is the best-studied offshore storage site, risks of leakage from CO_2 sequestration are not well understood at present. Sub-seabed leaks are possible and might cause damage of the marine ecosystem (Hawkins, 2004; Gibbins and Chalmers, 2008; Bibby et al., 2008). Leakages would lead to localized pH decreases

of varying magnitude. It is thus important to assess possible consequences of leakage on the marine ecosystem (Bibby et al., 2008; Pires et al., 2011, Donohue et al., 2012).

Storage of CO_2 is an important part of the European CCS and climate policy. Policy and regulatory frameworks are needed for management. Risk assessment for CO_2 leakage is the ground for regulatory frameworks. As there is limited experience with the monitoring, verification and reporting of actual leakage rates, there is an urgent need for further research.

This present research was conducted within the framework of the EU FP7 project ECO_2 (ECO_2 - Sub-seabed CO_2 Storage: Impact on Marine Ecosystems). The ECO_2 project proposes to investigate the likelihood of leakage, assess the risks and aims to develop a monitoring strategy and to define guidelines for the best environmental practices. The project wants to establish the guidelines as an addendum to the EU directive on “Geological Storage of CO_2” and to guide the management of offshore CO_2 injection and storage. The EU directive agrees on aims which must be achieved by all member states. The directive applies to storage of CO_2 within the territories of all member states. Some environmental risks of CCS are regulated by legislative instruments, but an establishment of a legal framework for environmentally safe application of CCS is important.

ECO_2 involves different work packages regarding fluid and gas flux, modeling, public perception, risk assessment and others. One work package covers effects of leakage on benthic organisms and the marine ecosystem. It aims to investigate to investigate at which levels of increasing pCO_2 and decreasing pH organisms would be affected to be able to prevent negative effects on ecosystems at potential leakage sites. ECO_2 aims to generate knowledge on the question whether CCS is the promising technique some scientists believe (i.e. Pires et al, 2011) and to assess potential new environmental risks of CCS.

1.4 Biological Impacts

1.4.1. Effects on benthic organisms

Marine organisms are expected to be affected negatively by ocean acidification (Hale et al., 2011, Widdicombe et al., 2009, Hall-Spencer, 2008, Kroeker et al., 2011). The diversity of marine organisms will also lead to diverse responses making any predictions on the ecosystem level difficult. Acidification at leakage sites will lead to a restructuring of communities as low pH zones will lack less tolerant species, causing a reduction of diversity in extremely low pH zones (Kroeker et al., 2011, Widdicombe et al., 2009). Generally studies including community experiments found a reduction in abundance of calcifying species in low pH zones, as those show higher susceptibility towards acidification (Widdicombe et al., 2009; Hall-Spencer et al., 2008).
Responses to ocean acidification by marine organisms include negative effects on survival, calcification, growth and reproduction, feeding and motility (Kroeker et al., 2011; Turley et al, 2006). Studies on different species show different responses, reduction in growth rates and higher mortality being mostly observed within other responses. A recent study by Saderne and Wahl (2012) showed a significant influence on growth rates and reproduction of the worm *Spirorbis spirobis* under higher pCO_2. Reduced calcification, growth and survival were shown in a study by Ries et al (2009). Responses to acidification may be diverse depending on different mechanisms, including i.e. reduced calcification, metabolic change or reduced growth, used to respond to stress (Fabry et al., 2008; Guinotte and Fabry, 2008).

1.4.2. Mechanisms of CO_2 sensitivity in bivalves

Changes in seawater CO_2 influence extracellular pH within animals. A rise in pCO_2 leads to an increase in haemolymph pCO_2. While intracellular regulation of pH is common among organisms, extracellular pH is seldom regulated in bivalve mollusks (Thomsen et al., 2010). Reduction in extracellular pH and intracellular regulation might have different effects depending on the level of acidification. Two effects might be observed, the depression of metabolism (Pörtner et al., 2005) or upregulation of

metabolism as energy demand for pH homeostasis, cellular homeostasis and calcification might be increased (Thomsen and Melzner, 2010).

Reduction in extracellular pH leads to cellular stress and might lead to metabolic depression (Pörtner et al. 2004). Metabolic depression is defined as the downregulation of metabolic energy turnover. The energy demand is downregulated, as well as the aerobic energy supply and is thereby closely linked to a reduction of the oxygen consumption rate. Metabolic depression is seen as an adaptive strategy in unfavorable environmental conditions (Diaz and Rosenberg, 1995; Boutilier, 2001; Guppy et al., 2005). Metabolic depression limits the depletion of internal energy reserves during environmental stress and thereby enables longer survival times and more efficient recovery after favorable conditions are restored. One target of metabolic depression is a reduction of ATP demanding processes (Boutilier, 2000). A study by Hand and Hardewig (1996) argued a direct relation between ability to reduce energy demand and survival time of organisms during environmental stress. A reduction in metabolic rate during environmental hypercapnia was also suggested in a study of Michaelidis et al. (2005) as the oxygen consumption rate fell significantly with increasing CO_2. Reduction of metabolic rate in environmentally stressful conditions is argued to be an adaptation of mussels to very high CO_2 (Thomsen and Melzner, 2010). In intertidal habitats hypoxic/ anoxic conditions occur regularly, leading to a rise in extracellular CO_2 (Burnett, 1997). Reduction of metabolism could thus be an evolutionary adaptation.

Down regulation of metabolism does not seem to occur generally with increasing pCO_2 in bivalve species. The contrary response is no change or the upregulation of metabolism. Thomsen and Melzner (2010) tested different levels of pCO_2 on *M. edulis* and showed an upregulation of metabolism up to a certain pCO_2. Higher standard metabolic rates for elevated pCO_2 were also found in a study by Beniash et al. (2010). However, an increase of metabolic rate was only significant in juveniles, although the same trend towards increased metabolism could be observed for adults. Negative effects on physiology and rates of shell deposition were demonstrated (Beniash et al., 2010). Exposure to elevated *p*CO2 does influence energy budgets. As pH decreases, energy is reallocated within the organism. If more energy is allocated towards coping with i.e. cellular homeostasis and stress responses, this can lead to a reduced scope for growth (Stumpp et al., 2012).

Even though an increase in metabolic rate was shown in the two studies mentioned, a reduction is probable for pCO_2 higher than 3,500 µatm. Thomsen and Melzner (2010) suggest a trend towards reduction of metabolic rate beyond a certain pCO_2. The change in oxygen consumption could be plotted in a parabolic curve, suggesting an increase of oxygen consumption for lower pCO_2 and indicating a reduction in oxygen consumption with increased pCO_2. It can be argued that a significant reduction might have been found if the same levels of pCO_2 (5,000 µatm) as in Michaelidis et al. (2005) were applied.

Another indicator for difficulties in coping with high CO_2 values could be the dry-weight shell-length relationship. If energy demand exceeds energy supply even though metabolism is decreased, energy stores might be depleted. However, during exposure to elevated pCO_2 of about 5,000 µatm (Michaelidis et al., 2005), the dry-weight shell-length relationship was not significantly different for *M. galloprovincialis*, indicating that despite reduced rates of growth, typical body proportions are maintained.

1.4.3. Oxidative stress

Increasing seawater pCO_2 leads to cellular stress, affecting many other cellular processes. One such a category is oxidative stress.

Oxygen and the control of the oxygen level within the cell are essential for life (Jaffe, 1976). Oxygen is not only essential but can be a highly toxic substance (Kohen, 2002). Reactive oxygen species, or oxyradicals, are potentially damaging substances within the cell (Gilbert, 1981). Reactive oxygen species are formed by partial reduction of molecular oxygen and secondary reactions with free radicals (Byczkowski and Gessner, 1988). Generation of these species is a general phenomenon in the marine environment (Shick and Dykens, 1985; Di Giulio et al, 1989; Winston and Di Giulio, 1991).

One of the toxic effects of oxyradicals is an increase in lipid peroxidation when abnormally high levels of free radicals occur and in addition antioxidant defense mechanisms are insufficient (Maritim, 2003). Lipids are the most vulnerable class of molecules towards oxidative stress and lipidic membranes are seen as being most sensitive towards oxidative damage (Kohen, 2002, Del Rio, 2005). A study of

Tomanek et al (2011) showed that categories associated with oxidative stress, such as antioxidants were upregulated in response to hypercapnia at a level of 3,500 µatm.

Reactive oxygen species are usually analyzed by measurements of secondary products because they are short-lived. Secondary products which can be measured include lipid peroxidation (Kohen, 2002), as reactive oxygen species degrade polyunsaturated lipids. The most studied product formed, and a highly important marker of lipid peroxidation, is Malondialdehyde. MDA is highly toxic and potentially mutagenic; it can destroy mechanisms involved in cell functionality (Del Rio, 2005).

1.4.4. Calcification vs. dissolution

The simplified reaction for the precipitation of calcium carbonate is the same for all specific mechanisms of biomineralisation (Equ.1.3).

$$Ca^{2+} + CO_3^{2-} = CaCO_3 \qquad \text{(Equ. 1.3)}$$

External shell corrosion in calcifying organisms is a direct effect of increasing pCO_2 and the resulting lowered carbonate concentration in the ocean. To protect their shell from surrounding water, bivalves produce an external organic layer called periostracum (Harper, 1997). The periostracum is a proteinaceous, sclerotized layer (Waite, 1983). A thick periostracum protects the shell against environmental influences and against dissolution. The periostracum consists of three different periostracal zones; forming, free and outer periostracum. These are responsible for shell secretion by functioning as a substratum for shell deposition and for shell protection (Saleuddin and Petit, 1983). Dissolution of internal carbonates, which are in contact with the calcifying tissues, can be counteracted by increased calcification (Melzner et al. 2011). Different studies have investigated the effects of acidification on the calcification rates of marine organisms (Gazeau et al., 2007, Ries et al., 2009). Feely et al. and Orr et al. (2004; 2005) suggested that many calcareous organisms may be unable to construct carbonate skeletons as oceans acidify. In short term acute exposure experiments (2-3 hours), Gazeau et al. (2007) showed a linear decrease in net calcification rates in response to seawater pCO_2 (at 1,000 µatm) and

a net shell dissolution for the highest treatments in the bivalves *Mytilus edulis* and *Crassostrea gigas*. In a study by Ries et al. (2009) a decrease in net calcification with increasing pCO_2 was measured in 10 out of 18 species with shell dissolution visible at their highest treatment (3,000 µatm).

In contrast, Thomsen et al. (2010) showed an increase in shell mass during an eight weeks trial for *M. edulis* at even higher pCO_2 (4,050 µatm). Control rates of shell growth were maintained at 1,400 µatm. The amount of increase in shell mass and extension rate were significantly reduced in the highest treatment, suggesting slower shell growth. The reason for the difference to Gazeau et al. (2007) could be acclimation to higher pCO_2 in the long-term experiment. Additionally food availability might play a role for the difference to Gazeau et al. (2007) and Ries et al. (2009).

Melzner et al. (2011) studied inner shell dissolution in the blue mussel *M. edulis*. The external shell might be directly influenced by undersaturated conditions in the ambient water column. However, at the inner site low [CO_3^{2-}] have been measured for the extrapallial fluid which indicate that calcification proceeds in a low [CO_3^{2-}] compartment also under control conditions (Thomsen et al. 2010, Heinemann et al. 2012). Melzner et al. (2011) found increasing internal shell dissolution with increasing pCO_2. At the same time, the extent of the affected dissolution area also depended on the food availability, which emphasizes the importance of physiological control of dissolution and calcification in blue mussels (Melzner et al. 2011).

The effect of elevated pCO_2 on different size classes of bivalves was tested by Green et al. (2004) and Waldbusser et al. (2010). Both found smaller individuals to be more affected. Smaller individuals showed large signs of external dissolution already after two weeks. Mortality rates were lower in larger clams and there was only minimal evidence of dissolution on larger shells. Decreased rates of calcification were shown for all size classes, but size significantly affected calcification rates allowing larger individuals to counteract dissolution pressure and deposit new shell material under corrosive conditions.

Further, shell mineralogy might be an important feature which defines the dissolution sensitivity, as the species using aragonite, the more soluble form of calcium carbonate, would be more susceptible to an increase in pCO_2 (Morse et al., 2007). Generally, significantly different responses to acidification were suggested in a meta-analysis by Kroeker et al. (2011) depending on the mineral form of calcium carbonate

used. A comparison between the bivalves *M. edulis* and *Crassostrea gigas* showed a higher sensitivity for *M. edulis* which was supposedly due to difference in shell mineralogy (Gazeau et al. 2007): the *M. edulis* shell can contain up to 83% aragonite (Hubbard et al.,1981) while the shell of *C. gigas* is mainly composed of calcite (Stenzel, 1963).

1.4.5. Studied species

In this study the effects of elevated pCO_2/lowered pH on three bivalve species from the western Baltic Sea were investigated. All three species, the cockle *Cerastoderma edule*, the soft-shell clam *Mya arenaria* and the Baltic tellin *Macoma balthica*, are infaunal bivalves that dominate sandy benthic communities in the Northeastern Atlantic (Taylor et al., 1973). In general, bivalves are important ecosystem engineers and major components of the biomass in coastal waters (Beukema et al., 2010). Bivalves are suspension-feeding organisms which filter large amounts of water by producing a current with their cilia in order to remove food particles from the water column (Riisgård et al., 2011). The filtration rate is closely correlated to the movement of the water pumping cilia on the gill filaments. The beat frequency of these cilia is directly correlated with the viscosity of seawater which decreases with increasing water temperature (Riisgård and Larsen, 2007).
C. edule and *M. balthica* live burrowed close to the surface of the sediment (Willmann, 1989; Ziegelmeier, 1957, Koie et al., 2001), while *M. arenaria* dwells down to 50 cm sediment depth (Baker and Mann, 1991). *C. edule* can occur at densities up to 60,000 ind m^{-2} in the field (Jensen, 1992). Abundance varies with sediment type and area. Möller and Rosenberg (1983) found abundances of *M. arenaria* of 13,000 to 458,000 ind m^{-2} in sandy areas of Western Sweden and 2,000 to 4,000 ind m^{-2} in soft bottom areas. Abundances of *C. edule* were lower with 5,000 to 59,000 ind m^{-2} and 600 to 1,400 ind m^{-2}. *M. balthica* is present nearly everywhere on tidal flats, it occurs in much lower densities compared to *C. edule* or *M. arenaria* with 30 ind m^{-2} in the Dutch Wadden Sea (Beukema, 1980). Generally all three species are widespread among tidal flats and shallow marine areas and count as an important link between primary producers and consumers.

The shell of all three species consists of aragonite (Taylor et al., 1973; Glover and Kidwell, 1993). The thickness of the periostracum varies with species. In fully grown specimen, *C. edule* (2 µm) and *M. balthica* (5 µm) have a rather thin periostracum, while the periostracum of *M. arenaria* is thicker (20 µm) (Harper, 1997).

Even though these three species have similar lifestyles, their responses to environmental stressors differ. Several studies revealed differences in the resistance towards hypoxic stress. According to Willmann (1989), *C. edule* can survive a couple of days (i.e. 2.9 days at 18 °C as LT_{50} in de Zwaan et al., 2002) without oxygen and, as an intertidal organism, can survive very low oxygen concentrations on a diurnal basis. When comparing *C. edule* with *M. arenaria* and *M. balthica* in physiological experiments without sediment; *C.edule* shows a lower tolerance towards hypoxia than the other two species (Theede, 1973; Dries and Theede, 1974). LT_{50} values in response to oxygen deficiency demonstrated by Dries and Theede (1974) were ca. 8 days for *C. edule*, 21 days for *M. arenaria* and 25 days for *M. balthica*, measured at 10 °C.In an experiment with sediment Henriksson (1969) showed long survival for *M. balthica*. A different *Mya* species (*M. truncata*) was found to be more resistant against hypoxia by Arntz (1977): old individuals of *M. truncata* survived the typical prolonged hypoxia during late summer (Melzner et al., 2012), while most other occurring bivalve spezies died. Even though a different *Mya* species was used in this experiment, a higher tolerance could be expected for *M. arenaria* than for the other two species. Under natural conditions, hypoxia is always correlated to high pCO_2. Therefore, a strong hypoxia tolerance may also imply a certain resistance to elevated pCO_2, which is tested in this study.

The tested community does not exist in the water depth of the Sleipner field, the most important present day CCS project. However, they are typical boreal species which occur in the shallow parts of the North Sea. Therefore, CO_2 leakages from CCS projects planned in shallower areas, for example the Rotterdam project (Hellebrekers et al., 2011) may interfere with these species in their natural distribution. Further, leakages may not occur at the injection point, but may affect shallower areas. The community tested may thus be subjected to possible leakage in future and was therefore used in the framework of this thesis.

1.4.6. Behavior

Lowered calcification rates or lowered metabolism might not be the only change for clams under elevated pCO_2 levels. Another influential parameter is the behavior of clams. Behavior of animals can be influenced by a multiple of factors. Ecological effects and the behavioral responses of benthic macrofauna towards hypoxia were summarized by e.g. Diaz and Rosenberg (1995) and Jörgensen (1980). Different behavioral responses were found. With continuous decline of oxygen concentrations, infauna organisms tended to move closer towards the surface or even onto the surface of the sediment (Jörgensen, 1980; Rosenberg et al., 1991; Diaz and Rosenberg, 1995). Similar responses were also found in a number of other studies (Renaud, 1986; Rahel and Kolar, 1990). In addition *M. arenaria* and *C. edule* stretch their siphons into the water column when exposed to hypoxic conditions, supposedly as more oxygen might be found outside the benthic boundary layer (Jörgensen, 1980). Individuals lying on the surface would not only be stressed by environmental conditions but would additionally be easy prey for all predators being able to enter the hypoxic water column (Diaz and Rosenberg, 1995). No study so far has tested behavioral responses to hypercapnia.

1.5. Objectives and Hypothesis

In order to develop monitoring techniques to identify leakage sites in the field, sensitivity thresholds must be determined. In the case of the present thesis, leakage events were simulated to test various levels of acidification. To test whether organisms are able to acclimate to and to compensate negative effects of severe seawater acidification, short and longer time exposure needed to be tested. Based on existing studies, different hypotheses were formulated:

Hypothesis 1: Mortality of clams will increase with increasing pCO_2 and exposure time.

Hypothesis 2: Mortality will increase and survival time will be shorter for smaller size classes.

Hypothesis 3: Net dissolution of clam shells will occur at higher pCO_2 levels.

Green et al. (2004) and Waldbusser et al. (2010) showed an influence of high pCO_2 on mortality, size and shell dissolution in infauna bivalves. An influence on those parameters is also expected in this study.

Hypothesis 4: Shell free dry mass to shell-length relationship and filtration rate will decrease with increasing pCO_2 in higher treatment levels.

Dry-weight to shell-length relationships are expected to decrease due to metabolic depression at very high seawater pCO_2 and metabolization of body tissues.

Hypothesis 5: MDA content is expected to increase in high pCO_2 treatments.

As oxidative stress is upregulated with elevated CO_2, so would levels of MDA (Tomanek et al., 2011). Assuming that metabolism follows a trajectory as found in (Thomsen and Melzner, 2010) for blue mussels, MDA content might initially increase, to then decrease at highest pCO_2 levels.

Hypothesis 6: Accumulation of C. edule at the sediment surface will increase with increased pCO_2.

This hypothesis is supported by a range of hypoxia studies, e.g. Rosenberg et al. (1991). As hypoxia is always coupled to hypercapnia, similar responses could be expected at very high pCO_2.

Hypothesis 7: Resistance of *M. arenaria* will be highest of all three bivalves; resistance of *C. edule* will be lowest.

As a study of Theede (1973) showed a lower tolerance of *C. edule* towards hypoxia than in the other two species, a similar result is expected when exposing the three species to hypercapnia. *M. arenaria* is expected to be more resistant as a thicker periostracum leads to better protection of the shell against dissolution pressure (Harper, 1997).

2. Materials and Methods

2.1. Structure of results and discussion

Generally if not mentioned otherwise, the results and discussion sections exclusively cover *C. edule*. All results concerning *M. arenaria* and *M. balthica* are treated under a separate heading.

2.2. Experimental setup

For the experiment, the following flow - through experimental set up was used (Fig.2.1, Fig.2.2). The design of the experiment included five treatment levels and a control with six replicates each.

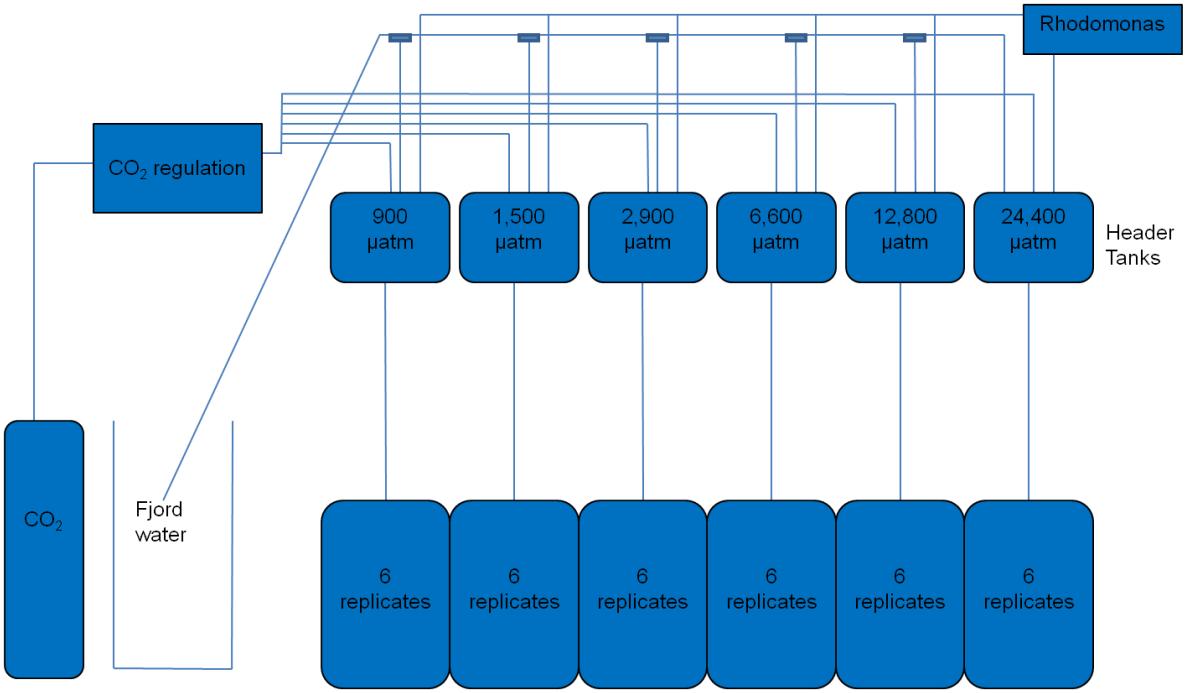


Fig.2.1: Experimental set up; six header tanks with six different CO_2 levels generated through pH controlled CO_2 addition (IKS Aquastar); *Rhodomonas sp.* supplied to six header tanks; six replicates for each treatment

Six header tanks were used, which were supplied with filtered sea water from Kiel Fjord. In order to reach a desired pH, pure CO_2 was automatically added to the header tanks. The amount of CO_2 added was controlled through a computer (IKS Aquastar, iks Computersysteme GmbH, Karlsbad, Germany) through constant

measurement of pH within each header tank. If pH exceeded the target pH by more than 0.05 units, CO_2 was added into the specific header tank until a predefined pH, corresponding to a specific target pCO_2, was reached. However, pH levels could not be raised above the ambient values using this system (Kiel Ford water is naturally acidified). Therefore, pH level of the control was thus equal to the pH of the incoming water of the Fjord. Calculated pH (NBS scale) levels ranged from 6.4 to 8. The corresponding pCO_2 levels were 900 (control), 1,500 (treatment 1), 2,900 (treatment 2), 6,600 (treatment 3), 12,800 (treatment 4) and 24,000 µatm (treatment 5). Those levels were calculated based on pH (mol kgH_2O^{-1}, NBS scale), temperature, salinity, and first and second dissociation constants of carbonic acid in seawater (according to Roy et al., 1993) using the programme CO2SYS (Lewis and Wallace, 1998). For temperature (10 °C) and salinity (20 psu) approximate values at the start of the experiment were taken, alkalinity values were taken as average Baltic Sea alkalinity (2,000 µmol $kgSW^{-1}$) at a salinity of 20 psu (Beldowski et al., 2010).

In addition, an algae storage tank was connected to each header tank assuring a constant algae supply to all aquaria. The algae flow rate into the header tanks was controlled through a peristaltic pump (MCP, ISMATEC, IDEX Health & Science GmbH, Wertheim-Mondfeld, Germany). The algae concentration in the header tanks was thus maintained at circa 4,000 cells ml^{-1}. Live *Rhodomonas* sp. was used as food algae during the experiment. Each header tank supplied six replicate experimental units (EU) of a size of 11.5 l. The EUs were distributed randomly within the room to avoid an effect of slight gradients in temperature and light intensity. Round plastic buckets were used as EUs to ensure a constant circulation within the replicates. A flow rate of 100 ml min^{-1} was constantly channelled towards each EU from the respective header tanks via gravity feed. Throughout the experiment pH, salinity, temperature, flow rate and mortality was controlled each day. A pH meter equipped with a pH electrode (SenTix 81 Plus, WTW Wissenschaftlich Technische Werkstätten GmbH, Weilheim, Germany) was used to measure temperature and pH; a salinometer (Cond315i instrument, WTW Wissenschaftlich Technische Werkstätten GmbH, Weilheim, Germany) was used to measure salinity. EUs were checked daily for bivalve mortality. For each dead bivalve found during the experiment the dissolution rate of the shell (holes, no holes present) was noted. Carbonate chemistry and algae concentration in the EUs was measured weekly. C_T was measured using

an Automated Infra Red Inorganic Carbon Analyzer (AIRICA, Marianda, Kiel, Germany). pCO_2 and calcium carbonate saturation were then calculated according to the *Guide to best practices for Ocean CO_2 measurements* (Dickson et al., 2007). For calculations the programme CO2SYS (Lewis and Wallace, 1998) was used with measured pH (NBS-scale), salinity, C_T (µmol $kgSW^{-1}$), and temperature.

The experiment took place during 17th of December 2011 to 06th of March 2012, the salinity ranged between 14.6 and 20.5 psu. Temperatures ranged between 8.9°C in December and 4.3°C towards the end of the experiment. The temperature and salinity fluctuations varied with natural occurring changes during the experimental period. Light conditions were similar for all basins. The light level ranged from 5.53 to 7 µmol $s^{-1}m^{-2}$, with light hours being from 8:00 to 17:00.

Each EU contained a constant number of experimental animals from three bivalve species: five *M. arenaria* (size classes: 0.5-1 cm: two animals; 1-1.5 cm: two animals; 2-2.5 cm: one animal); one *M. balthica* and 40 *C. edule* (size classes: 0-0.5 cm: three animals; 0.5-1 cm: 18 animals; 1-1.5 cm: 11 animals; 1.5-2 cm: seven animals; 2-2.5 cm: 1 animal). Bivalves were sampled in Kiel Fjord at Falckenstein. Sampling took place with a Van Veen grab in 1-2 m depth on 21st of November 2011. Density of the three bivalve species was estimated from one Van Veen grab and used to stock the EUs at field specific species proportions. Bivalves were transferred into holding basins at a constant temperature (9 °C) before being placed in the experimental setup. Each basin contained sediment (ca. 20 cm deep) and a free water column (ca. 10 cm deep). The lower 10 cm of the sediment consisted of sieved sand taken from a local beach; the upper 10 cm consisted of surface sediment (top 5-10 cm) from the station at which the experimental animals were sampled to resemble the natural environment. The sandy environment thus contained a natural microbial and meiofauna community, at similar densities as in the field. Microbial and meiofauna communities were thus randomly distributed within the replicates through random distribution of sediment. Bivalve shell lengths were measured and the same size class distribution for each species was placed in each replicate / EU.

Behaviour was noted every second day starting in the third week, by counting the number of bivalves on the sediment. Additionally, pictures were taken every second day to assess opening status of the exhalant / inhalant siphos (open vs. closed) in each treatment.

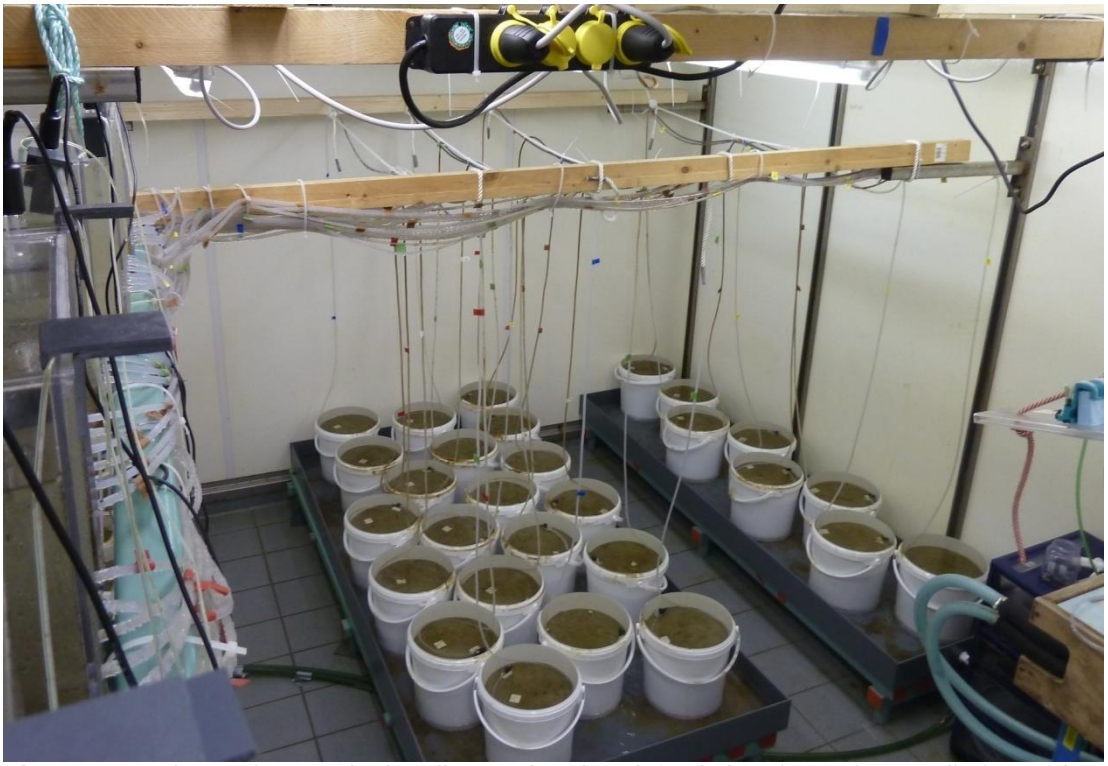


Fig.2.2: Experimental set-up in the climate chamber; left: six header tanks supplied experimental units (bottom) at a rate of 100 ml min^{-1}. Six replicates for each header tank; replicates were distributed randomly within the room.

2.3. Sampling

Sampling took place after six weeks and at termination of the experiment (12 weeks). Samples were taken for determination of bacterial community composition, meiofauna community composition and chlorophyll determination (not part of this thesis). Bacterial community samples were taken with a corer of 2.5 cm diameter and 1 cm depth, transferred into Eppendorf tubes and kept frozen at -20°C. Two samples were taken for each replicate. One sample for meiofauna composition was taken for each replicate the same way as for bacteria and stored in 4% buffered formalin. In addition one sample of each replicate was taken in the same manner and frozen for analysis of chlorophyll concentration. During the first sampling, all bivalves located in one quadrant of the circular EUs were sampled. Thus, the rest of the sediment surface was left undisturbed. Bivalves removed from the experiment were frozen and stored at -20 °C until the measurements of shell width and shell free dry mass. For measurement of shell mass and shell free dry mass, bivalves were cooked in a microwave at maximum heat (700 W) for one minute and the shell was separated

from the meat. Subsequent, meat and shell were dried at -80 °C for 20 hours in order to determine somatic / shell dry mass.

Ceramic settlement plates of a size of 1 cm² were added to each replicate for the last six weeks for analysis of settlement of benthic microalgae.

After twelve weeks, algae plates were removed for analysis and samples were taken again for bacterial community analysis (3 samples); meiofaunal community analysis (2 samples), chlorophyll analysis (1 sample). Bivalves were frozen separately. Two *C. edule* were frozen at -80 °C for genetic analysis and four *C. edule* for oxidative stress analysis. All other bivalves left were frozen at -20°C for measurements of shell width and shell free dry mass. Six *C. edule* were maintained under experimental conditions (control, treatments 2, 3, 4) for determination of filtration rate following the termination of the main experiment.

All bivalves were treated and measured the same way as after the first sampling. Images of shells were taken under the stereomicroscope to assign dissolution categories to different shells. In addition, SEM analysis were carried out using *C. edule* shells to gain a closer insight into the degree of shell dissolution. For analysis of shell integrity, images of five random cockles of each treatment were taken under the stereomicroscope and assigned to different categories. Four categories were chosen: 0 - no dissolution; 1 - slight dissolution; 2 - strong dissolution and 3 - very strong dissolution and holes present. Images of each cockle were then sorted independently into different categories by five different investigators. Averages of each treatment for each category were plotted for the analysis.

2.4. Energy budget

Energy content for the somatic dry mass of *C. edule* was calculated using the value 18.85 J mg^{-1} Brey et al. (1988). Oxygen consumption was estimated using the formula by Newell and Bayne (1980): Equ.2.1, W [g] being dry tissue weight.

$$VO_2 = aW^b \qquad \text{(Equ.2.1)}$$

Oxygen consumption rates of *C. edule* were calculated for two size classes of 0.1057 g dry weight and 0.0057 g dry weight for corresponding temperatures (~8 °C) and a- (0.583) and b-values (0.852) for animals sampled in February (Newell and

Bayne, 1980). The oxygen consumption rates were converted into energy turnover using an oxycaloric equivalent of 0.44 J µmol^{-1} O_2 (Lauff and Wood, 1996). Using these values, hypothetical (maximum) survival times were calculated assuming total depletion of somatic body mass.

2.5. Filtration rate

Filtration rates (control, treatment 2, 3 and 4) were measured for *C. edule* directly after the experiment. Filtration rate measurements were carried out at the same pH as the experiment.

The water in the measurement aquaria was filtered 0.2 µm prior to measurements, each aquarium contained 1.5 l. A current within each aquarium was created through a bubble stone to ensure an equal distribution of algae within each aquarium and avoid sinking of the algae. For each measurement, three cockles were placed in the measurement aquaria. The cockles were allowed to acclimate to the experimental conditions for thirty minutes before an algae solution was added. The desired algae concentration was calculated to be approximately 5,500 cell ml^{-1}. 10 ml samples were taken out of each aquarium every 5 or 10 minutes and measured for algae concentration using a coulter counter (Z2 Coulter® Particle count and size analyzer, Beckmann Coulter^{TM}) starting after five minutes after addition of algae. The first five runs were measured for 30 minutes with a sample taken every five minutes. All other runs were measured for one hour with a sample taken every ten minutes.

Clearance rate was then calculated after Riisgård (2001) (Equ.2.2) and related to shell width.

$$CR = \frac{V}{nt} \times ln \frac{C_0}{C_t} \qquad \text{(Equ.2.2)}$$

CR = clearance rate [ml/ min]

V = used water volume [ml]

n = amount of bivalves in the aquarium

t = time [min]

C = the concentration at start time [cell ml^{-1}]

C_t = the concentration at time t.

For the measurement of Malondialdehyde (MDA) content, the frozen bivalve tissues were ground in liquid nitrogen using mortar and pestle. The four bivalves taken from each replicate were ground separately. From each individual, 50 mg were taken to mix all four bivalves of each replicate in one pool. This was due to the requirement for sufficient amounts of bivalve tissue for the measurements. Each replicate pool was then measured separately. All samples were constantly kept frozen at -80 °C or in liquid nitrogen. MDA concentration was determined following the protocol of Mihara and Uchiyama (1978). Tissue was homogenized with phosphoric acid (0.2%) in relation 1:5 and the same amount of phosphoric acid (2%) was added. One blank (homogenate and 3mM hydrogen chloride) and two samples (homogenate and TBA solution) were incubated at 100 °C for one hour for each treatment. 0.5 ml of butanol was then added. After several vortexing procedures all samples and blanks were measured in a plate reader (Plate Chameleon, Hidex, Turku, Finland) at 532 and 600 nm. A difference between the two extinctions was calculated to then assess MDA concentration with a standard curve (buffer solution containing 1.01 mM MDA in 1.1 % H_3PO_4). Tissue concentration of MDA was calculated followed the Equation Equ.2.3.

$$C_{tiss} = \frac{C_{MDA} \times V_{But} \times V_{Extr}}{V_{aliq} \times W} \qquad \text{(Equ.2.3)}$$

C_{MDA}: MDA concentration, calculated after standard curve
V_{But}: Volume of butanol [ml]
V_{Extr}: Extraction volume [ml]
V_{aliq}: Volume of Homogenate [ml]
W: weight of tissue [g]

2.7. Statistical analysis

For most of the statistical analysis the program R was used.
Mortality of different size classes within each treatment was tested with a Kruskal Wallis test as normal distribution and homogeneity of variances could not be achieved. Without normal distribution a nonparametric test had to be used. Kruskal mc was used as a post hoc test. Results for a Tukey HSD test are also given. The

Tukey HSD test cannot be relied upon, as it is a parametric test. The p-value was lowered to 0.01 and could then compare results of both tests.

Mortality overall size classes between the treatments and percentage of dissolute shells were tested the same way.

The influence of pCO_2 on shell free dry mass, filtration rate and Malondialdehyde (MDA) were tested with an ANOVA and a Tukey HSD post hoc test. Shell free dry masses were transformed (box-cox transformation) but showed no normal distribution. However, the histogram (Fig.2.3) showed a near normal distribution of values, thus the parametric test was used nevertheless. Homogeneity of variances was achieved after box-cox transformation of values.

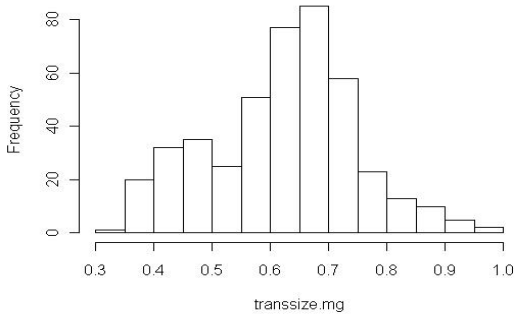


Fig.2.3: Histogram of transformed values of size/shell free dry mass

MDA values were normal distributed after box-cox transformation. As no homogeneity of variances was achieved the p-level was lowered to 0.01 for the analysis.

Behaviour was tested with PERMANOVA. As the same individuals were counted repeatedly, a repeated measures analysis would have to be used if achieving normal distribution. As normal distribution could not be achieved a non parametric alternative to repeated measures ANOVA had to be used. PERMANOVA does not assume normal distribution and could be used in this case.

For all graphs plotted in the results, standard deviation (SD) is given.

3. Results

3.1. Experimental Parameters

Experimental parameters were observed continuously during the experiment. Concentration of food algae (*Rhodomonas sp.*) was constantly at a level between 3,000 and 4,500 cell/ml in the header tanks (Fig.3.1). pH levels were relatively stable (Fig.3.2). Fluctuations in pH were observed during the first 15 days in the treatments 1, 2 and 3. Those fluctuations were due to electrical problems within the pH control system, which did not occur during a prior test run. The control and the two lowest pH treatments were stable throughout the experiment.

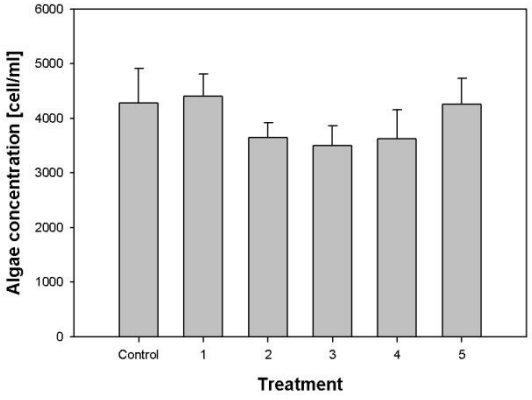


Fig.3.1: Food supply during the experiment; Concentration of *Rhodomonas sp.* algae measured in the header tank and counted with a coulter counter

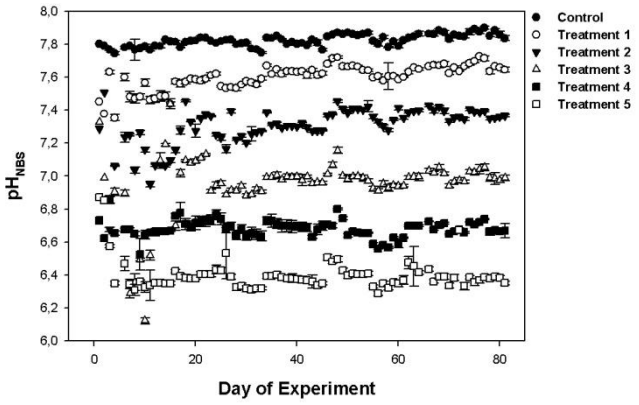


Fig.3.2: pH_{NBS} measured during the experiment, fluctuations of pH treatment 1, 2 and 3 during the first 10 days were due to electrical problems with the pH control system

3.2. Mortality

Mortality was observed during the entire duration of the experiment (Fig.3.3). Mortality increased with pCO_2 and duration of the experiment. Significant differences in mortality were found for all time periods (Kruskal Wallis, $p < 0.01$ for week 0-6; 6-12 and 0-12).

For the week 0-6 and 0-12 interval, the Kruskal mc test ($p < 0.05$) indicated significant differences in mortality between control, treatment 1 and 2 in comparison to treatment 5. Difference in mortality was also found for treatment 1 in comparison to treatment 4 (Kruskal mc). For the second experimental period, week 6-12,

differences in mortality were found between the control, treatment 1 and 2 to treatment 5 (Kruskal mc).

Mortality is plotted in Fig.3.3, showing a strong difference for treatment 5 in comparison to all other treatments. Using a parametric test to discriminate between mortality rates (a Tukey post-hoc test) and lowering the p-value to 0.01, yielded slightly different results than the non-parametric test (Kruskal mc) (Tab.3.1). Mortality in treatment 5 was significantly different to all other treatments and the control for all time periods (Tukey HSD, $p < 0.01$). Mortality in treatment 4 differed significantly to the control, treatment 1 and 2 for week 0-6 and 0-12 (Tukey HSD, $p < 0.01$), while mortality in treatment 4 was not significantly different to any other treatment for week 6-12. Significant differences in mortality were also shown for treatment 3 versus treatment 1 for week 0-12 (Tukey HSD, $p < 0.01$). In treatment 5, 50% of *C. edule* survived for 68 days.

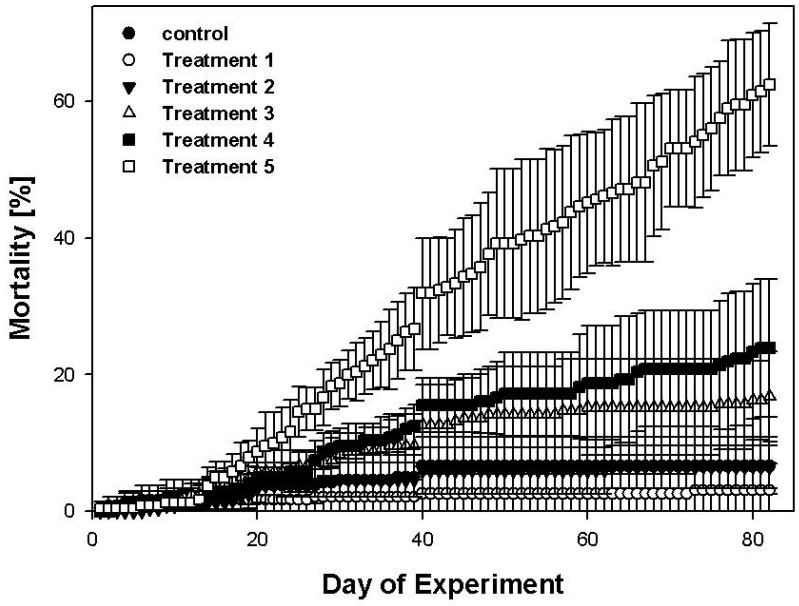


Fig.3.3: Cumulative mortality plotted for the duration of the experiment. The first sampling after 40 days of the experiment leads to the gap; 50% mortality in treatment 5 on day 68

Tab.3.1: Results of a Kruskal Wallis test, a Tukey HSD and a Kruskal mc post-hoc test for mortality within the treatments over three different time periods. This table contains pair wise test results (i.e. T5-T1 is the difference between treatment 5 and treatment 1). P-values for the Tukey test, results of Kruskal mc in bold letters

Week: 0-6: Treatment x-treatment x	**Week: 6-12: Treatment x-treatment x**	**Week: 0-12: Treatment x-treatment x**
χ^2: 27.037; p: 0.0001	χ^2: 24.834; p: 0.0002	χ^2: 28.813; p: 0.0000
T4-control 0.0079	T4-control 0.0408	T3-control 0.0478
T5-control 0.0000	**T5-control 0.0000**	T4-control 0.0001
T3-T1 0.0179	**T5-T1 0.0000**	**T5-control 0.0000**
T4-T1 0.0002	**T5-T2 0.0000**	T3-T1 0.0046
T5-T1 0.0000	T5-T3 0.0000	**T4-T1 0.0000**
T4-T2 0.0051	T5-T4 0.0000	**T5-T1 0.0000**
T5-T2 0.0000		T4-T2 0.0001
T5-T3 0.0000		**T5-T2 0.0000**
T5-T4 0.0000		T5-T3 0.0000
		T5-T4 0.0000

Size class dependent mortality was analyzed for the same time periods as total mortality. Smaller individuals were more sensitive than larger individuals. The control (Fig.3.4) and treatment 1 (Fig.3.5) showed no significant differences in mortality between size classes. Mortality for the smallest size class (0-0.5 cm) in treatment 2 was significantly higher than mortality for the three largest size classes (1-1.5 cm, 1.5-2 cm, 2-2.5 cm) when tested with a Tukey HSD ($p < 0.01$). Mortality in treatment 2 was not significantly different between size classes when tested with Kruskal mc (Tab.3.2). Mortality in treatment 3 was significantly higher, when tested with a Tukey HSD, for the smallest size class in comparison to all other size classes except the largest (2-2.5 cm) for week 0-12 and significantly higher in comparison to all size classes for week 0-6 (Tukey HSD, $p < 0.01$). Mortality in the smallest size class in treatment 3 was significantly higher than mortality in the largest size class for week 0-6 and significantly higher than mortality of cockles of a size 1-1.5 cm for week 0-12 when tested with a Kruskal mc. Mortality of the smallest cockles (0-0.5 cm) in treatment 4 was significantly higher than that of the three largest size classes (1-1.5 cm, 1.5-2 cm, 2-2.5 cm) for week 0-12 and 0-6 when testing the Kruskal mc post-hoc test. When testing the Tukey HSD, mortality of the smallest size class in treatment 4 was significantly higher than that of all other size classes for week 0-6 and 0-12. In addition mortality of the smallest size class was significantly higher in comparison to the two largest size classes (1.5-2 and 2-2.5 cm) for week 0-12 (Tukey HSD, $p < 0.01$).

Mortality in treatment 5 was significantly higher in the two smallest size classes (0-0.5 and 0.5-1 cm) in comparison to the other size classes in week 0-6 (Tukey HSD, $p < 0.01$). When testing the Kruskal mc, mortality was significantly higher in the smallest size class in comparison to cockles sized 1-1.5 cm for week 0-12 and in comparison to the three largest size classes for week 0-6. The result of a Kruskal mc for treatment 5 for week 0-6 was also found when testing the Tukey HSD on week 0-12. In addition mortality in treatment 5 of cockles sized 0.5-1 cm was significantly higher than mortality in all larger size classes when testing the Tukey HSD ($p < 0.01$). A Tukey HSD and a Kruskal mc do have different results but results indicate the same tendency of smaller cockles being more susceptible towards elevated pCO_2

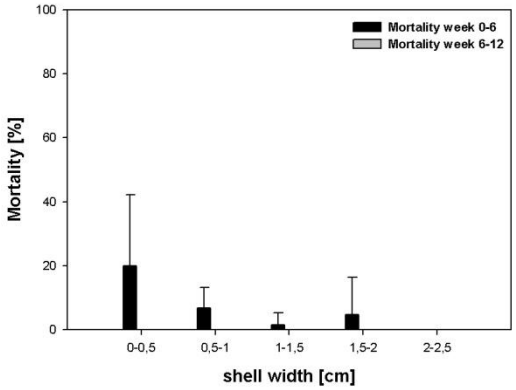


Fig.3.4: Control; Mortality of different size classes during the first and second half of the experiment

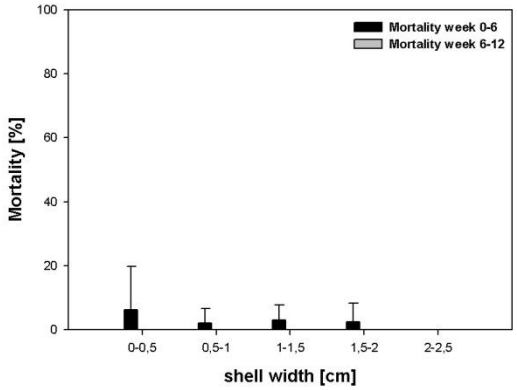


Fig.3.5: Treatment 1; Mortality of different size classes during the first and second half of the experiment

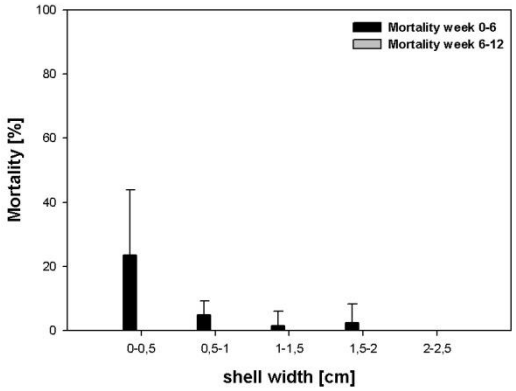


Fig.3.6: Treatment 2; Mortality of different size classes during the first and second half of the experiment

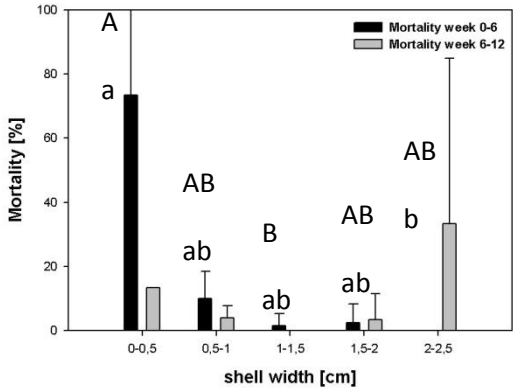


Fig.3.7: Treatment 3; Mortality of different size classes during the first and second half of the experiment; capital letters indicate significant differences in mortality for 0-12 weeks, lower case letters indicating significant difference in mortality for 0-6 weeks (Kruskal mc test)

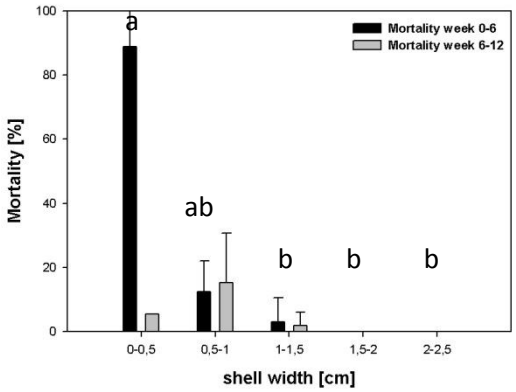


Fig.3.8: Treatment 4; Mortality of different size classes during the first and second half of the experiment; lower case letters indicating significance difference in mortality for 0-6 and 0-12 weeks (Kruskal mc test)

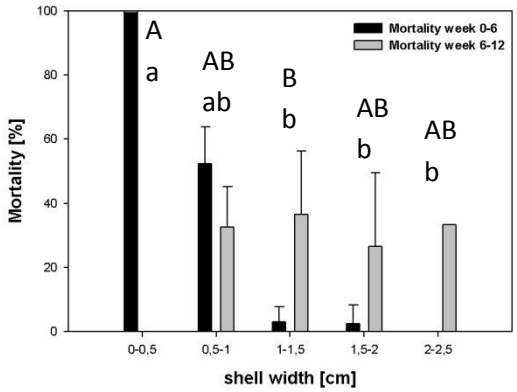


Fig.3.9: Treatment 5; Mortality of different size classes during the first and second half of the experiment; capital letters indicating significant difference in mortality for 6-12 weeks, lower case letters indicating significant difference in mortality for 0-6 and 0-12 weeks (Kruskal mc test)

Tab.3.2: Results of a Kruskal Wallis test, a Tukey and a Kruskal mc post-hoc test for mortality of differently sized cockles; all size classes tested over three time periods: week 0-6; week 6-12 and week 0-12; number indicating different size classes: 1 = 0-0.5 cm; 2 = 0.5-1 cm; 3 = 1-1.5 cm; 4 = 1.5-2 cm; 5 = 2-2.5 cm. This table contains significant differences always between two size classes (i.e. 2-1 is the difference between size class 2 and 1). p-value for the Tukey test; results of Kruskal mc in bold letters

Treatment 2, week 0-6 χ^2: 9.992; p: 0.0406	**Treatment 3, week 0-6** χ^2: 17.018; p: 0.0019	**Treatment 4, week 0-6** χ^2: 22.216; p: 0.0002	**Treatment 5, week 0-6 and 0-12** χ^2: 24.833 and 19.181; p: 0.0001 and 0.0007
2-1 0.017	2-1 0.0000	2-1 0.0000	2-1 0.0000
3-1 0.0047	3-1 0.0000	**3-1 0.0000**	**3-1 0.0000**
4-1 0.0059	4-1 0.0000	**4-1 0.0000**	**4-1 0.0000**
5-1 0.0035	**5-1 0.0000**	**5-1 0.0000**	**5-1 0.0000**
			3-2 0.0000
			4-2 0.0000
			5-2 0.0000
Treatment 2; week 0-12 χ^2: 10.095; p: 0.0389	**Treatment 3; week 0-12** χ^2: 11.266; p: 0.0237	**Treatment 4; week 0-12** χ^2: 22.858; p: 0.0001	**Treatment 5; week 6-12** χ^2: 10.594; p: 0.0315
2-1 0.0206	2-1 0.0072	2-1 0.0000	**3-1 0.0005**
3-1 0.005	**3-1 0.0014**	**3-1 0.0000**	4-1 0.0001
4-1 0.0062	4-1 0.0024	**4-1 0.0000**	5-1 0.0000
5-1 0.0038		**5-1 0.0000**	3-2 0.0999
		3-2 0.0460	4-2 0.0025
		4-2 0.0093	5-2 0.0006
		5-2 0.0093	

3.3. Somatic growth

As individual animals were not marked prior to the experiment and owing to mortality in all treatments, growth rates could not be analyzed. Fig.3.10 and Fig.3.11 indicate

growth in shell width. In Fig.3.10 average shell width (and SD) at start and end points are plotted. A small increase in shell width seems to occur in the control and higher pH treatments. Average shell width increases more for treatments with lower pH, which is due to the very high mortality of smaller *C. edule*. This can also be seen in Fig.3.11, which demonstrates changes in size composition of experimental populations towards larger amounts of larger sized clams.

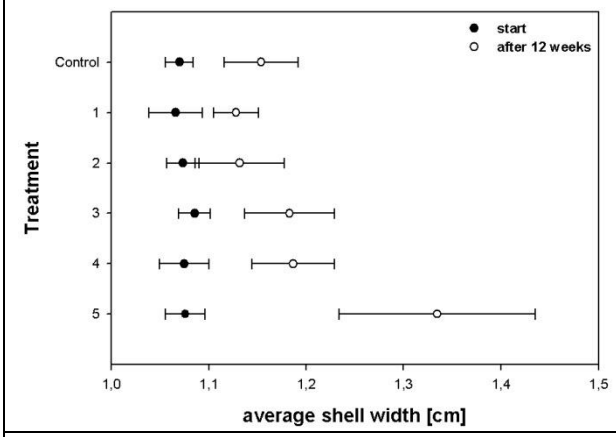


Fig.3.10: Average size before and after the experiment; high mortality in smaller size classes resulting in an increase in average shell width

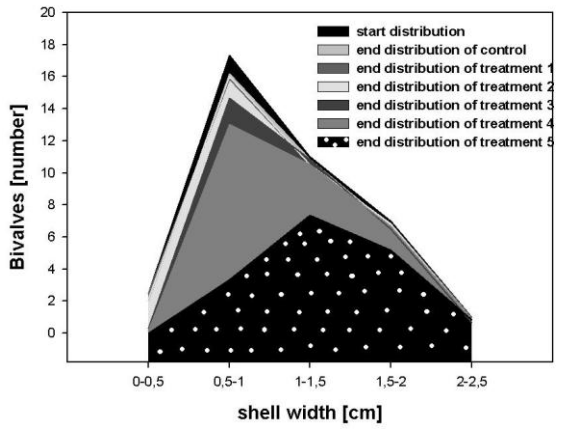


Fig.3.11: Average of n per size class before and after the experiment

3.4. Shell integrity

Dead clams were examined for signs of shell dissolution (Fig.3.12). The comparison of intact shells against shells with holes showed differences between treatments for all time periods tested (Kruskal Wallis, $p < 0.01$). Differences in rate of dissolution were found for treatment 4 and 5 in comparison to the control and treatment 1 for week 0-6 and 0-12 when testing the Kruskal mc test. When testing the Tukey HSD more pair wise tests were significant (Tab.3.3). The amount of shells with holes was significantly higher in treatment 4 and 5 in comparison to the control and treatment 1 and 2 for all time periods (Tukey HSD, $p < 0.01$). In addition the amount of shells with holes was significantly higher in treatment 3 in comparison to the control and treatment 1 (Tukey HSD, $p < 0.01$). Dissolution was demonstrated to increase with increasing pCO_2, the amount of dead shells with holes already increased in treatment 3.

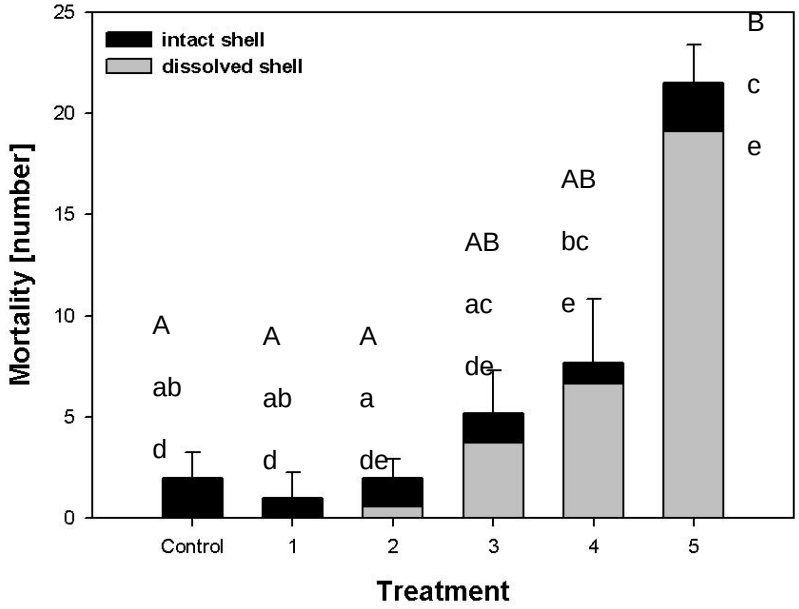


Fig.3.12: Bars showing *C. edule* mortality over the entire experimental duration (mean +- SD); color coding showing dissolved vs. intact shell; lower case letters (abc) indicate significant differences in mortality for 0-6 and 6-12 weeks, capital letters indicate significant differences in mortality for 6-12 weeks, lower case letters (de) indicate significant difference in dissolution of shells for 0-6 and 0-12 weeks

Tab.3.3: Results of a Kruskal Wallis test, a Tukey and a Kruskal mc post-hoc test test for shell dissolution of cockles that died in the course of the experiments over three different time periods. This table contains significant differences always between two treatments (i.e. T5-T1 is the difference between treatment 5 and treatment 1). P-values for the Tukey test, results of Kruskal mc in bold letters

Week: 0-6: Treatment x-treatment x	**Week: 6-12: Treatment x-treatment x**	**Week: 0-12: Treatment x-treatment x**
χ^2: 25.747; p: 0.0001	χ^2: 23.235; p: 0.0003	χ^2: 26.753; p: 0.0001
T4-control 0.0001	T4-control 0.0007	T3-control 0.0023
T5-control 0.0000	T5-control 0.0001	**T4-control 0.0000**
T4-T1 0.0001	T4-T1 0.0007	**T5-control 0.0000**
T5-T1 0.0000	T5-T1 0.0001	T3-T1 0.0023
T4-T2 0.0101	T4-T2 0.0007	**T4-T1 0.0000**
T5-T2 0.0002	T5-T2 0.0001	**T5-T1 0.0000**
T5-T3 0.0034	T4-T3 0.0416	T4-T2 0.0029
	T5-T3 0.0140	T5-T2 0.0003

After the experiment, images of clams were taken and shells were sorted into different categories, depending on the degree of dissolution. Cockles were assigned to four different categories (Fig.3.13): 0 - no dissolution; 1 - slight dissolution; 2 - strong dissolution and 3 - very strong dissolution and holes present. For the control and treatment 1 and 2, most cockles were classified into the first two categories. For treatment 3, most cockles were fitted into category 2. In treatment 4 and 5, mostly

clams were classified into category 2 and 3. With increased pCO_2 more shells with holes or strong signs of dissolution were found.

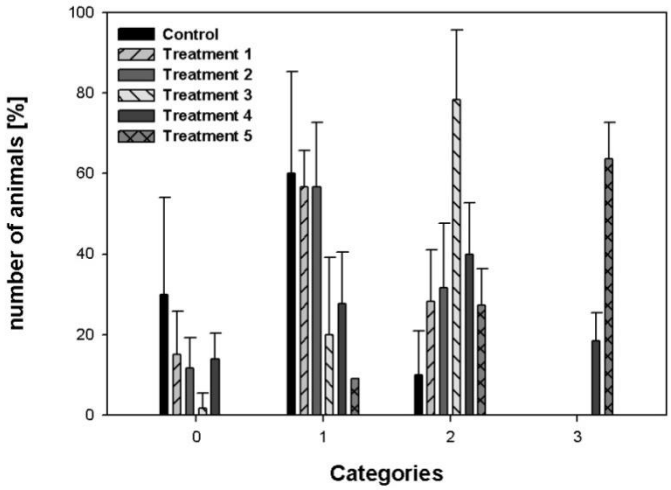


Fig.3.13: five *C. edule* of each treatment were fitted into categories according to shell dissolution index; 0 - no dissolution; 1 - slight dissolution; 2 - strong dissolution and 3 - very strong dissolution and holes present.

In addition to stereo microscopic images, SEM analysis was carried out for the control, treatment 1, 2 and 3 shells. SEM analysis supports the finding of increased dissolution with higher pCO_2. The control was not characterized by shell dissolution on the outside of the shell (Image 1 and 2) and no dissolution signs were visible under the stereomicroscope (Image 4). The inside of the shell was also not corroded (Image 3). Shells from treatment 1 were characterized by external and internal dissolution (Image 7). Dissolution on the outside of the shell (Image 5 and 6) was clearly visible on SEM images. However, these subtle signs of corrosion could not be resolved with the stereomicroscope (Image 8). Dissolution on the outside of the shell can clearly been seen in shells from treatment 2 and 3 on stereomicroscopic images (Image 10 and 14), with the shell from treatment 3 showing stronger signs of dissolution. SEM analysis demonstrated signs of dissolution for both treatments on the outside (Image 9, 11 and 12) and dissolution on the inside of the shell for treatment 3 (Image 13). SEM images were not obtained for treatment 4 and 5, as dissolution was obvious in the stereomicroscopic images already (Image 15 and 16).

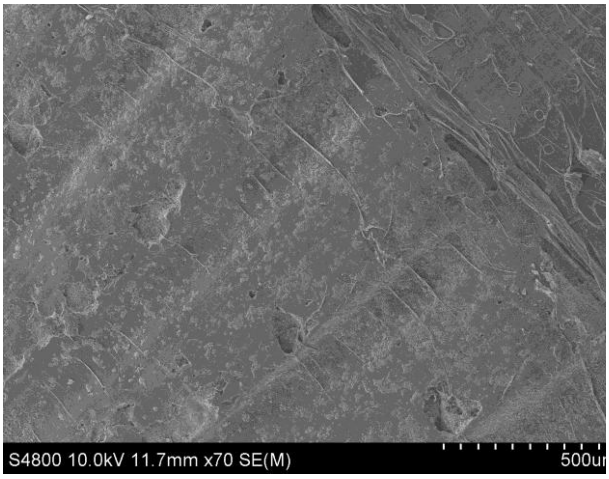


Image 1: SEM; shell dissolution of the outside of the shell for the control. Scale bar 500 µm

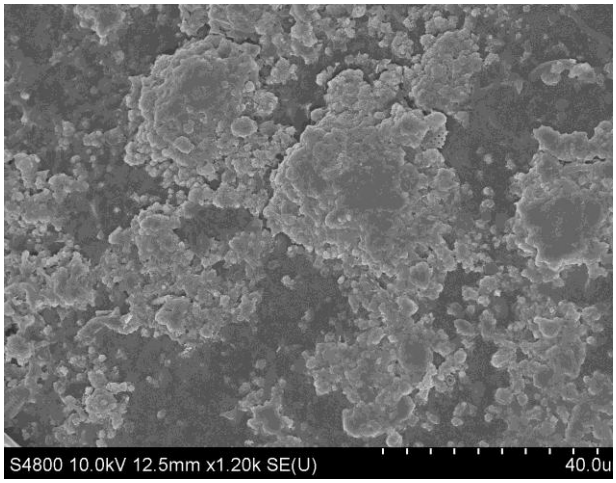


Image 2: SEM; the outside of the shell for the control. Scale bar 40 µm

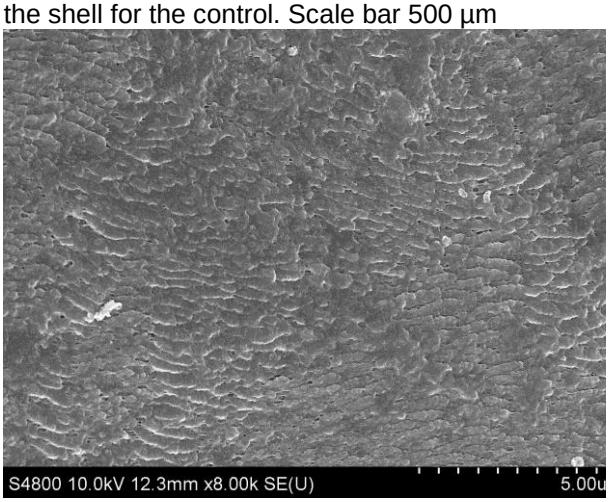


Image 3: SEM; the inside of the shell for the control. Scale bar 5 µm

Image 4: Control, intact shell, no dissolution signs. Scale bar 5 mm

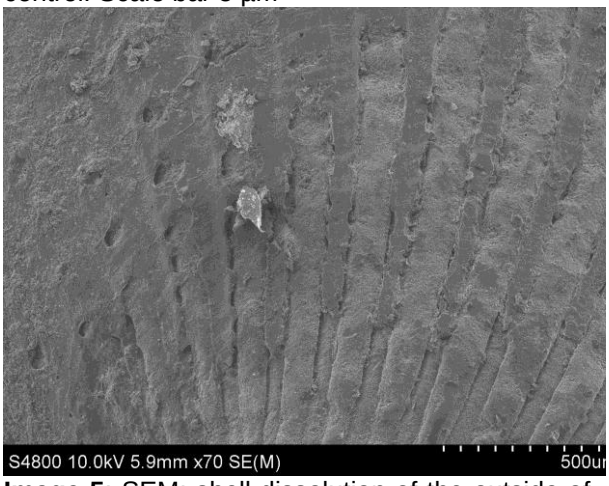


Image 5: SEM; shell dissolution of the outside of the shell for treatment 1. Scale bar 500 µm

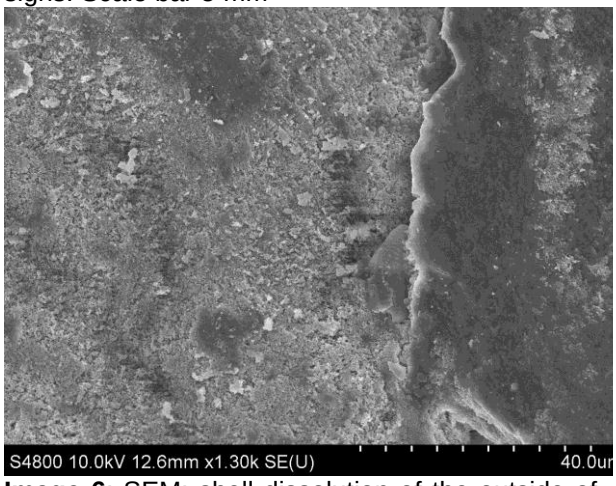


Image 6: SEM; shell dissolution of the outside of the shell for treatment 1. Scale bar 40 µm

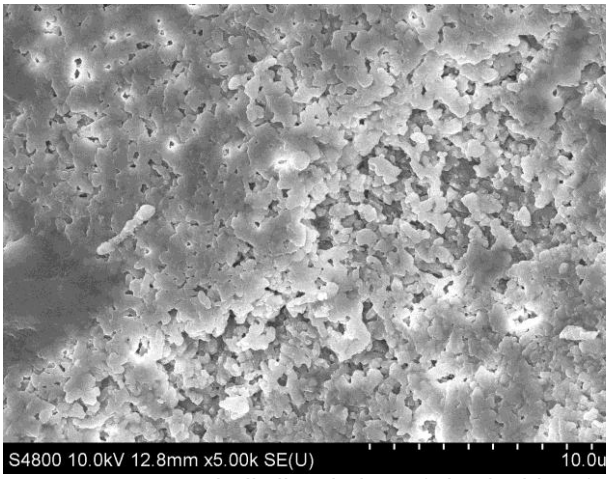


Image 7: SEM; shell dissolution of the inside of the shell for treatment 1. Scale bar 10 µm

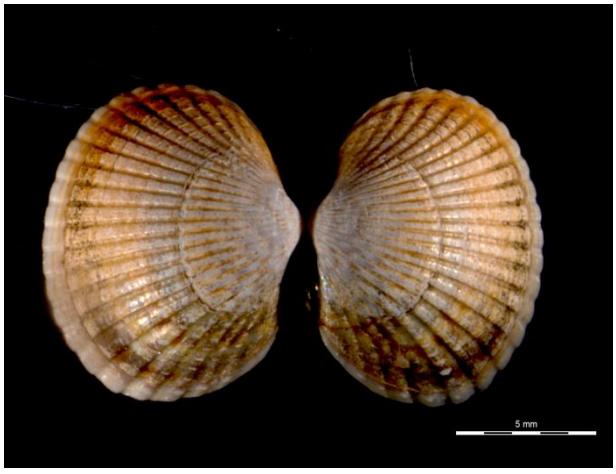


Image 8: Treatment 1, intact shell, light dissolution signs. Scale bar 5 mm

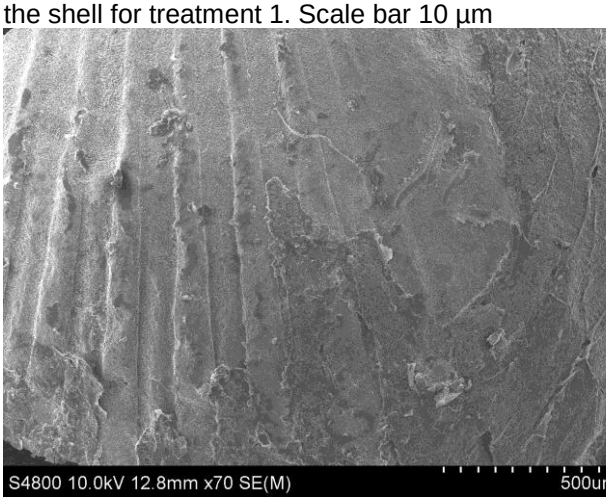


Image 9: SEM; shell dissolution of the outside of the shell for treatment 2. Scale bar 500 µm

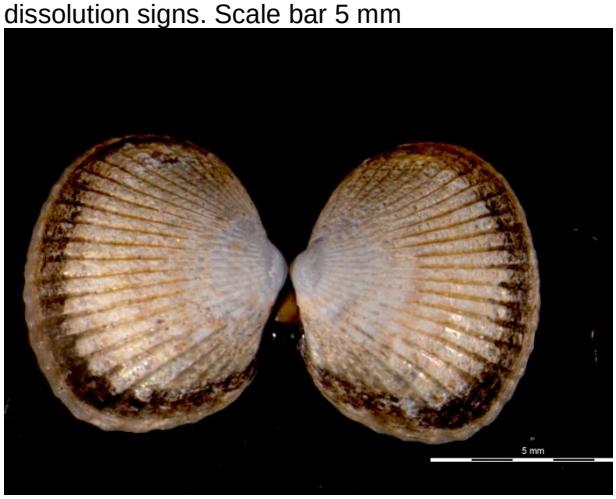


Image 10: Treatment 2, dissolution signs Scale bar 5 mm

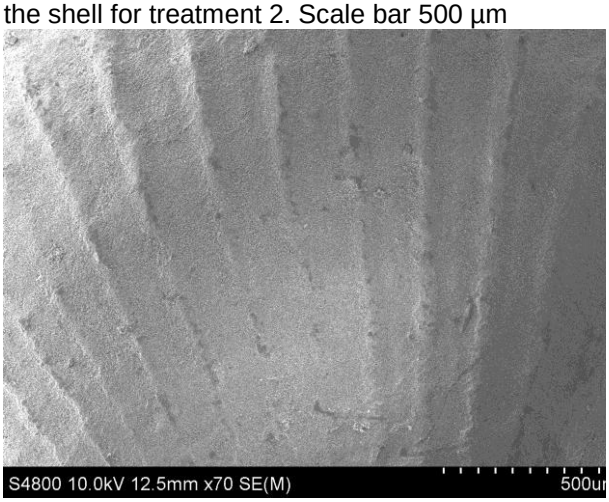


Image 11: SEM; shell dissolution of the outside of the shell for treatment 3. Scale bar 500 µm

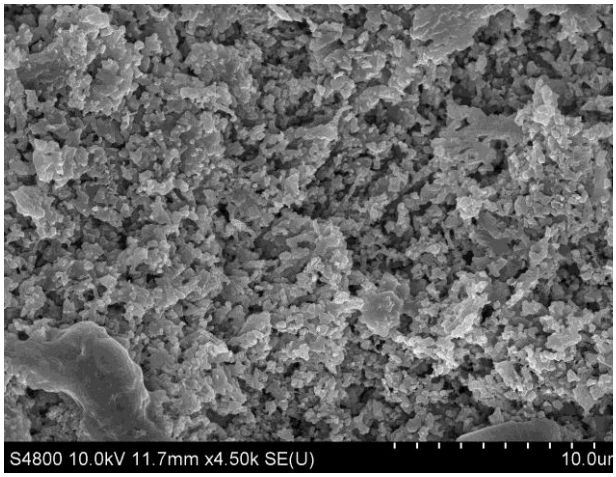


Image 12: SEM; shell dissolution of the outside of the shell for treatment 3. Scale bar 10 µm

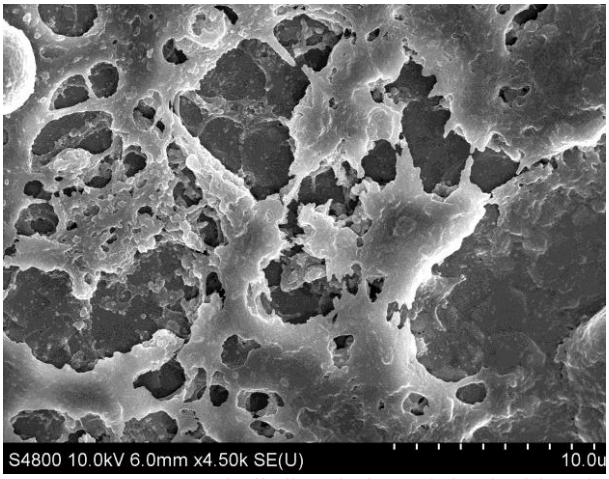


Image 13: SEM; shell dissolution of the inside of the shell for the treatment 3. Scale bar 10 µm

Image 14: Treatment 3, strong dissolution signs. Scale bar 5 mm

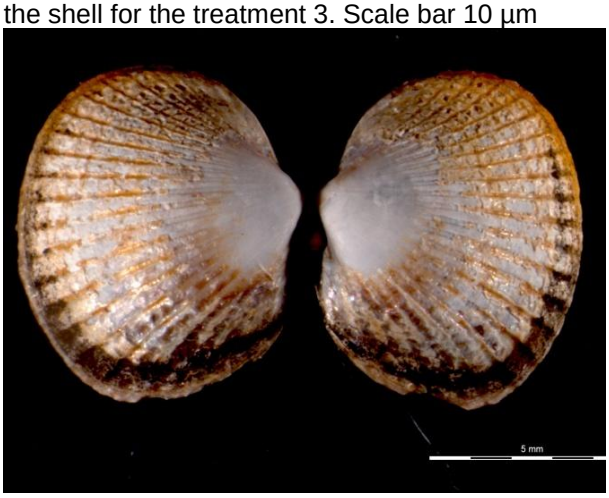


Image 15: Treatment 4, strong dissolution signs. Scale bar 5 mm

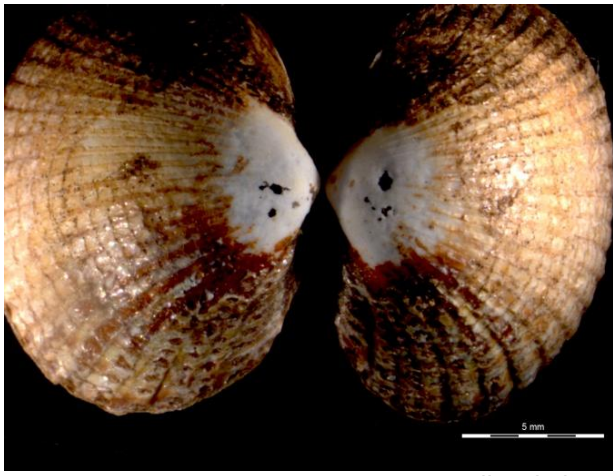


Image 16: Treatment 5, very strong dissolution signs, holes. Scale bar 5 mm

3.5. Shell free dry mass

The relationship between log shell width and log shell free dry mass was tested for all treatments for *C. edule* using an ANOVA and a Tukey HSD post-hoc test. Significant differences were shown for treatment 5 against the control, treatment 2, 3 and 4 Tukey HSD, $p < 0.05$). A significant difference was also found for treatment 4 to treatment 1. In all cases with significant differences between treatments, shell free dry mass at a particular shell width was lower for high pCO_2 treatments.

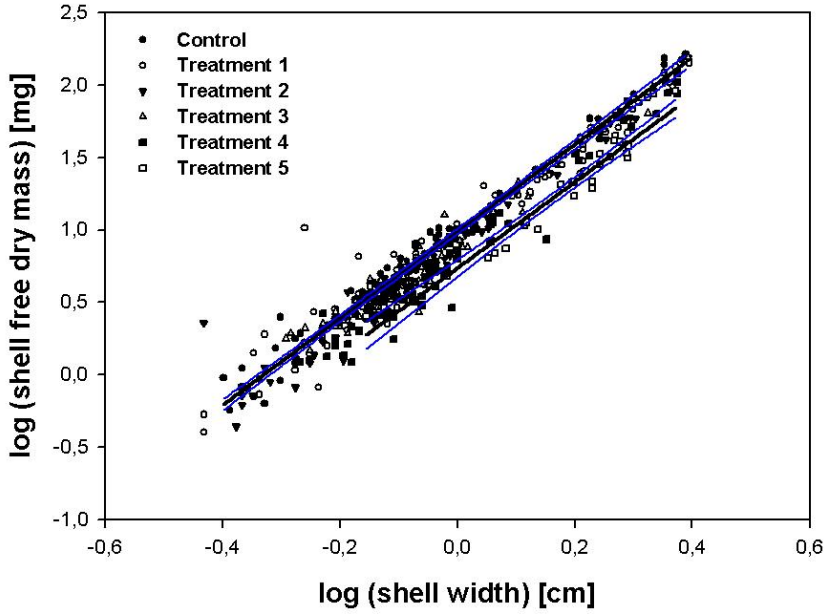


Fig.3.14: Shell free dry mass versus shell width for control and T5; with 95% confidence interval; all measured values were plotted with log transformation. Significant difference for treatment 5 vs. control

3.6. Filtration rate

Filtration rate was found to be not significantly different between *C. edule* from the tested treatments (ANOVA, $p > 0.05$). Large variability in filtration rate in all groups tested obscured potential differences. A slight insignificant increase in filtration rate for treatment 2 and a decrease for treatment 3 and 4 is visible in Fig. 3.15.

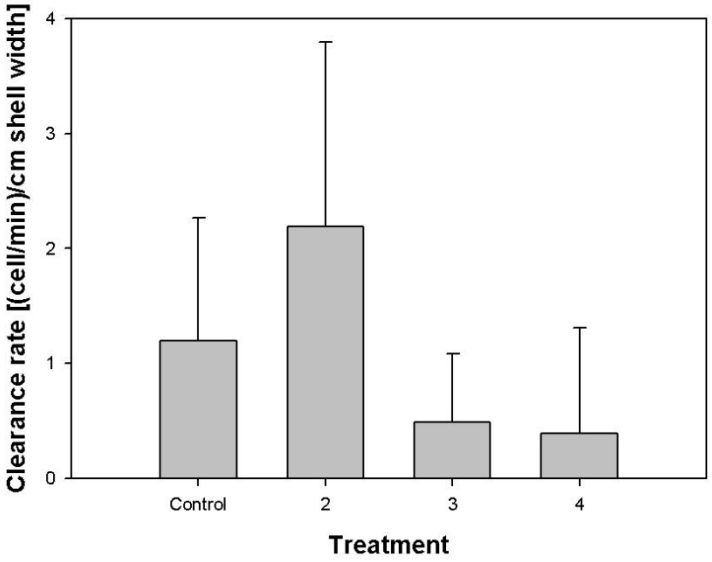
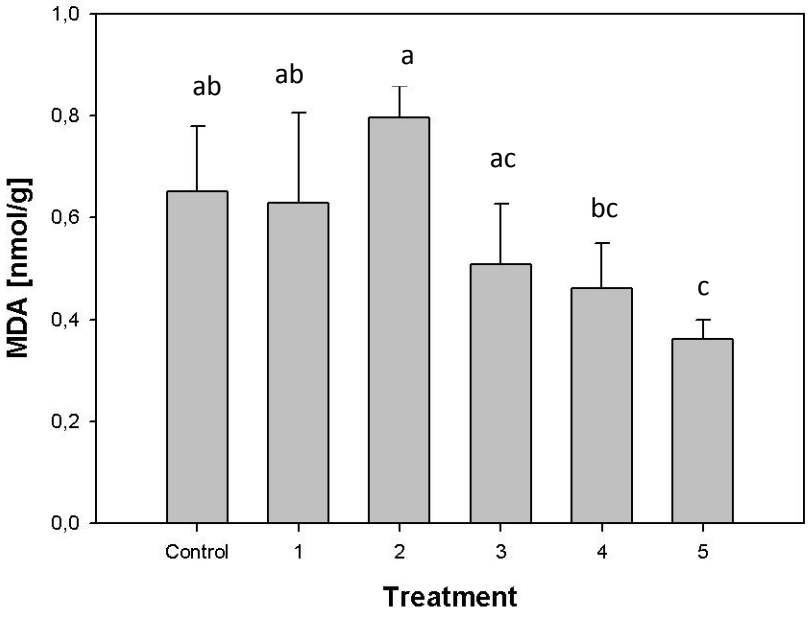


Fig.3.15: Average clearance rate [cell/min] for treatment 2, 3 and 4 in relation to shell width [cm], no significant difference

3.7. Oxidative stress

MDA content was found to be significantly affected by pCO_2 (ANOVA, F 8.83, $p < 0.01$). The significance level was lowered to 0.01 as no homogeneity of variances could be achieved with various transformations. MDA content in treatment 5 was significantly different to that of the control, as well as MDA content of treatment 1 and treatment 2 (Fig 3.16) (Tukey HSD, $p < 0.01$). MDA content in treatment 2 was in addition significantly higher than MDA content in treatment 4 (Tukey HSD, $p < 0.01$). The differences found with the statistical analysis are supported by the graph as standard variations are not overlapping. Fig.3.16 depicts a slight increase in MDA content for treatment 2 and a decrease for treatment 3, 4 and 5.

MDA content and filtration rate were correlated in a significant, positive linear regression ($F=5.64$, $p < 0.05$). However, an R^2-value of 0.29 indicates that only 29% of the observed variance could be explained by the regression (Fig.3.17).



T5-control: 0.0035764
T5-T1: 0.0086862
T4-T2: 0.0080446
T5-T2: 0.0001671

Fig.3.16: Average of MDA content [nmol/g] for the different treatments; lower case letters indicating significance in MDA content; p-values for the Tukey post-hoc test for all significant levels

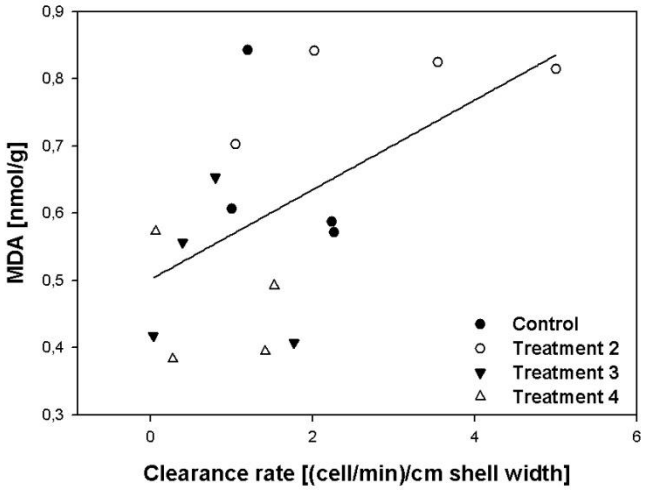


Fig.3.17: MDA content vs. clearance rate. F: 5.6368; $p < 0.05$; R^2 0.29

3.8. Energy budget sensitivity analysis

Energy content for *C. edule* was calculated using a conversion factor of 18.85 J/mg (Brey et al., 1988). This calculation revealed an energy content of 2026.375 J for a cockle with a shell width of 2.08 cm and a weight of 0.1075 g. A cockle with a shell width of 0.74 cm and a weight of 0.0057 g was found to have an energy content of 107.445 J.

Oxygen consumption was calculated using the formula and parameters determined by Newell and Bayne (1980). Using the values given in the paper, examples were calculated using February (~8°C) values, as this temperature value was close to measured temperatures in this experiment. Assuming normoxic metabolic rates (no metabolic reduction), a 2.08 cm cockle would consume 1.6 J/h in February. Hypothetical survival rates in dependency of the available energy under the assumption of neutral energy uptake (no algae consumption) was 52 days for this size, assuming total depletion of the energy reserves (i.e. the entire somatic body mass). A cockle with a shell width of 0.74 cm needs less energy but also has less tissue available that it can metabolize. In February, the smaller cockle would consume 0.132 J/h limiting maximum survival to 34 days. February surviving times with above mentioned calculations were plotted for three different sizes: 2.08 cm; 1.38 cm and 0.74 cm (Fig.3.18). The graph shows different survival times and different slopes of energy depletion.

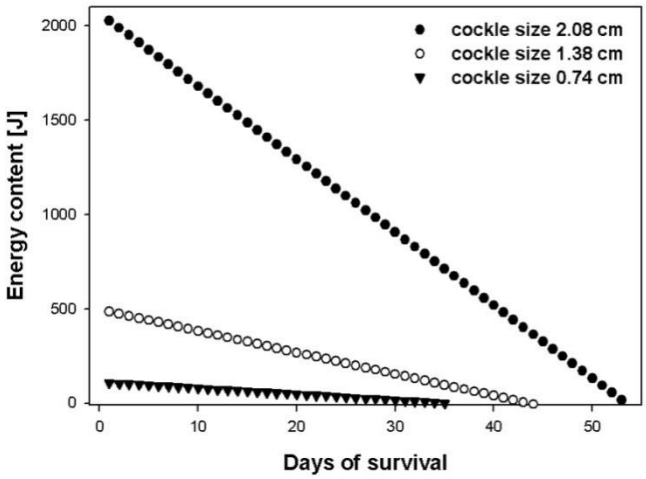


Fig.3.18: Theoretical survival time in days of different sized cockles assuming 0 uptake of food algae: 2.08 cm; 1.38 cm and 0.74 cm; calculations after O_2 consumption calculations after Newell and Bayne (1980) and energy content after Brey et al. (1988)

3.9. Behavior

Different types of behaviour could be observed: (1) cockles burrowed with open siphos, (2) cockles burrowed with closed siphos (not visible) and (3) cockles lying on the sediment surface. Up to treatment 2, clams were mostly displaying type 1 behavior (Fig.3.19).

With decreasing pH, clams were less visible (Fig.3.20). Their siphos could not be differentiated within the sediment for most of the time as they were closed. In the highest CO_2 treatments, cockles migrated towards the surface, displaying type 3 behavior (Fig.3.21, Fig.3.22). In treatment 5, an increasing number was observed to be located on the surface (Fig.3.22). In treatment 4, some clams were observed on the surface (Fig.3.21), however, always less than in treatment 5. Cockles on the surface of the sediment were counted regularly.

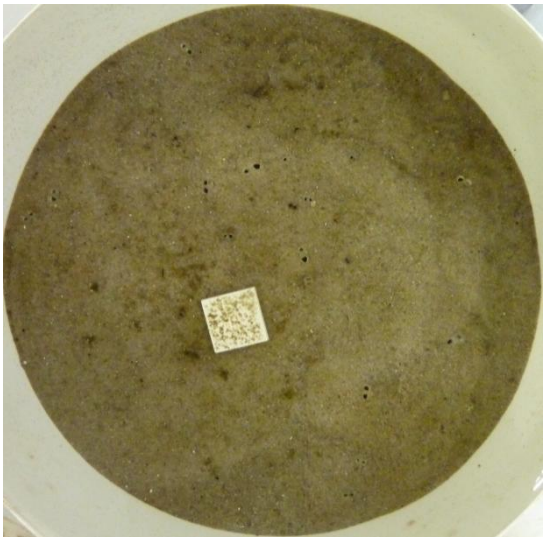


Fig.3.19: Control; sediment surface with siphos opened and visible

Fig.3.20: Treatment 3; observed behavior during the experiment

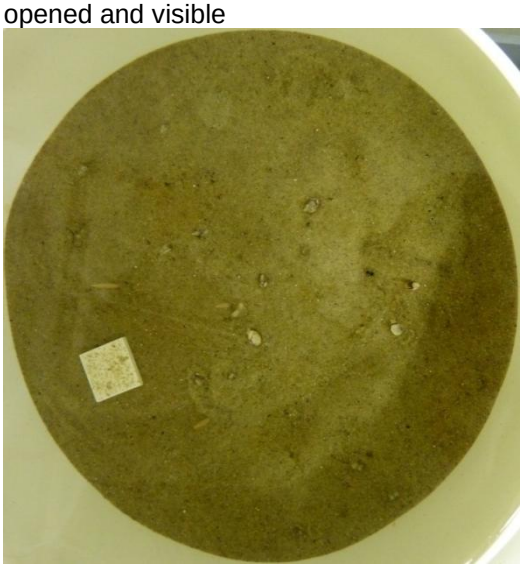


Fig.3.21: Treatment 4; observed behavior during the experiment

Fig.3.22: Treatment 5; observed behavior during the experiment

Over the duration of the experiment, *M. arenaria* and *M. balthica* were seldomly observed on the surface and were not included in the following analysis.

Fig.3.23 shows the average percentage of cockles located on the sediment surface for each treatment from week 0-12. Fig.3.24 shows the average percentage of cockles on the sediment surface for each treatment for each day counted over the time period of the experiment. Fig.3.24 shows an increasing amount of *C. edule* on the surface with ongoing experimental duration. PERMANOVA results (F 2.80, $p < 0.01$) showed that significantly more cockles were located on the sediment in treatment 5 than in all other treatments (PERMANOVA, $p < 0.01$). In treatment 5,

50% of clams were located on the surface of the sediment on day 50 (LD 50 on day 50.3; Fig.3.23).

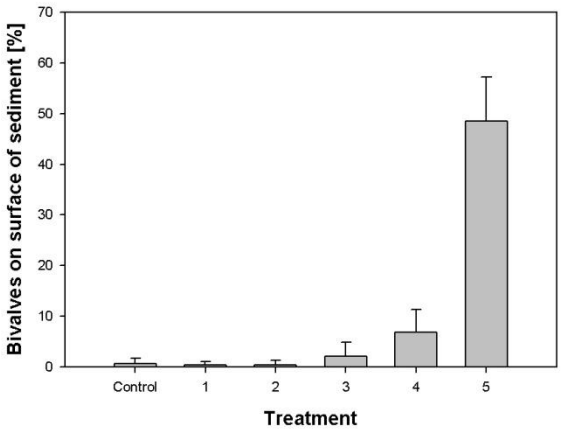


Fig.3.23: Average of non-buried *C. edule* over the complete experimental phase in % of total *C. edule*

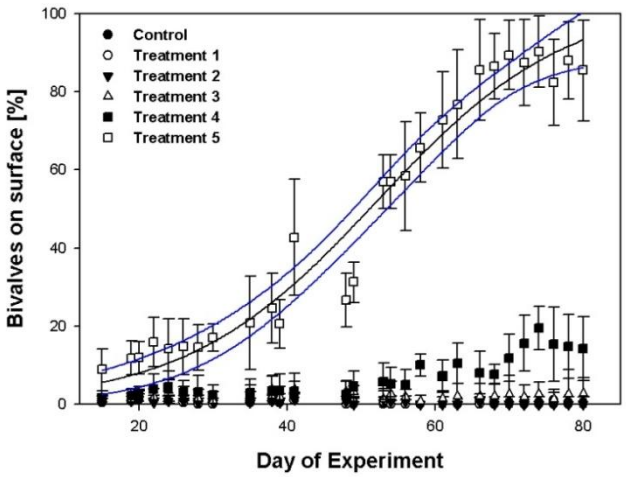


Fig.3.24: Average of non-buried *C. edule* over the complete experimental phase in % of total *C. edule*, LD 50 on day 51.3, curve fitted for treatment 5 including 95% confidence interval.

3.10. *M. arenaria* and *M. balthica*

M. arenaria and *M. balthica* were burrowed during the whole experimental duration in all treatments. As there were only a few individuals, a statistical analysis for shell free dry mass or MDA was not completed. Mortality and behavior were observed during the experiment. Both species were observed with one individual on the surface in treatment 5. No mortality was observed during the entire experiment.

4. Discussion

4.1. Method discussion

Food conditions and water flow through the experimental units were stable during the experiment. In addition to food algae concentration measurements in the header tanks, algae concentrations were also measured in the experimental units. This assured comparable food supply during the experiment. Experimental conditions (pH, flow, T and salinity) were stable for most of the experimental duration.
pCO_2 / pH conditions varied during the first 15 days in treatment 1, 2 and 3 (Fig.3.2). pH was stable for half a day, always following readjustment of the pH control system and fluctuated in these three treatments between 6.3 and 7.5 during the rest of the day. Measurements of pH were always noted prior to system adjustment. The initial variability was due to defective contacts within the system. This did not occur in the test phase prior to starting the experiment. Fluctuations did not have a measurable influence on the results. Even though fluctuations were quite high, the mortality rate in treatment 1, 2 and 3 was not significantly higher than that of the control.

4.2. Mortality

Hypothesis 1: Mortality of clams will increase with increasing pCO_2 and exposure time.

Hypothesis 2: Mortality will increase and survival time will be shorter for smaller size classes.

Hypothesis 1 was confirmed as *C. edule* mortality increased with increasing pCO_2. Regardless which statistical method used, a significant influence of the highest applied pCO_2 on mortality was demonstrated. For the analysis, a Kruskal mc post hoc test and a Tukey post hoc test were used as data were not normal distributed. Cockles kept at 24,000 µatm were characterized by continuous and high mortality after an initial period of one to two weeks. Fig.3.3 shows a strong mortality for 12,800 µatm and 6,600 µatm. Depending on the method used, this observation is

supported by the applied statistical procedures. Cockles of the size range used in this experiment survived maximum levels of expected ocean acidification (< 6,000 µatm). Very high pCO_2 > 6,600 µatm, which might occur during leakage events, led to increased mortality for this species. The results of this study support findings of earlier studies, even though different species were used. Green et al. (2004) applied 5,000 µatm during a two weeks study on *M. mercenaria* and found significantly higher mortality, while lower levels of 3,800 µatm showed no difference in mortality in the bivalve *M. galloprovincialis* during an 11 week trial (Range et al., 2012). A study by Berge et al. (2006) observed mortality for *M. edulis* at a level of 14,000 µatm. A threshold for mortality can be defined to be around 5,000 µatm. However, this accounts solely for *C. edule* in this present study, as both other species tested in this study showed no mortality at any level applied.

Hypothesis 2 was confirmed as mortality differed over different size ranges. As already found by previous studies (Waldbusser 2010, Green et al., 2004) smaller bivalve individuals were more susceptible towards higher pCO_2. Waldbusser et al. (2010) showed significant effects of size on calcification rates for *Mercenaria spp.* and Green et al. (2004) showed additionally differences in mortality. Even though levels at which effects are visible differ for these two studies, the trend of smaller individuals being more sensitive towards acidification is consistent in both studies and agrees with results of this study. Additionally, a study of Beniash et al. (2010) showed effects on mortality, growth, net calcification and metabolism for juvenile *C. virginica* already at 3,500 µatm.

Significantly higher mortality in this study was observed in the highest experimental treatment for all size classes. While larger cockles could still be observed at the termination of the experiment (29% mortality in treatment 5), smaller size classes suffered from high mortality (92% mortality in treatment 5). In all treatments with significantly higher mortality, a difference in mortality between size classes was observed (Fig.3.7-9/ Tab.3.2). Smaller cockles were affected earlier, as well as at lower levels of acidification. While the overall mortality as an average over all size classes was significantly increased at 6,600 µatm, the smallest size class was already susceptible to 2,900 µatm. Depending on levels of pCO_2 and duration of CO_2 leakage from sub-seabed storage sites, leakage would have a strong influence on mortality of *C. edule* occurring in the potential leakage site. The present results

suggest an influence of lower pCO_2 around 3,000 µatm on *C. edule*. These are levels that are already occasionally observed in the Baltic today and that might be more common due to future ocean acidification in the area (Melzner et al., 2012).

4.3. Shell integrity

Hypothesis 3: Net dissolution of clam shells will occur at higher pCO_2 levels.

An often studied response to experimental seawater acidification is the calcification rate, as calcifiers have been hypothesized to be most strongly influenced by acidification (Widdicombe et al., 2009, Hall-Spencer et al., 2008).
In this study shells were screened for signs of dissolution following termination of the experiment and classified into different categories. Shells of deceased animals were observed post-mortem during the experiment for presence or absence of holes. Shells of dead cockles from treatment 4 and 5 had significantly more holes than those from the control and treatment 1 and 2. When sorting cockles into categories, increased corrosion with increasing pCO_2 was already visible for the 2,900 µatm treatment, when studied with the stereomicroscope. Using SEM analysis, corrosion was even visible for the 1,500 µatm treatment.
Signs of dissolution already occur in lower pCO_2 treatments. Cockles kept under extremely high pCO_2 (> 12,800 µatm) showed not only signs of dissolution, but were characterized by holes in the shell, confirming hypothesis 3. At 6,600 µatm, shells with holes were observed, however only very few individuals and exclusively in smaller size classes (< 0.7 cm). The degree of shell dissolution strongly increased with the level of acidification. Shell corrosion at a water pCO_2 of 1,500 µatm was not lethal in this treatment as it did not correlate with increased mortality. Strong dissolution at higher treatment levels could be due to movement to the sediment surface, see section 4.8.
This work supports all studies observing increased shell dissolution in bivalves with increasing pCO_2 (Ries et al., 2009; Gazeau et al., 2007, Green et al., 2004).
Short term studies such as the one by Gazeau et al. (2007) on the blue mussel *M. edulis* typically witnessed strong influences of pCO_2 on calcification. Ries et al. (2009) showed a decrease in net calcification for different calcifiying species at a

level of 3,000 µatm in a 60 day experiment. Both, Ries et al. (2009) and Gazeau et al. (2007) witnessed dissolution in their highest treatments (ca. 2,000 and 3,000 µatm). Green et al. (2004) observed reduced calcification in their highest treatment (5,000 µatm) in a two weeks experiment. In contrast, Thomsen et al. (2010) demonstrated less dramatic effects and even an increase in shell mass at 4,050 µatm for *M. edulis*. Range et al. (2012) found a decrease in calcification rate for *M. galloprovincialis* only at 3,800 µatm. A threshold for calcification is thus difficult to define.

4.4. Shell free dry mass and filtration rate

Hypothesis 4: Shell free dry mass to shell-length relationship and filtration rate will decrease with increasing pCO_2 in higher treatment levels.

Shell free dry mass was found to be significantly lower for treatment 5, supporting hypothesis 4. This weight loss could be related to spawning activities. However as *C. edule* is spawning between March and August in the Baltic Sea (Jaekel, 1952), spawning does not take place during winter; thus the season during which the experiment was conducted.

Reduction in shell free dry mass demonstrated that *C. edule* maintenance costs exceeded energy uptake during the experimental incubation. Hence, growth rates were negative during the experimental period. We cannot conclude from these results, whether metabolism was down- or upregulated. Metabolism could be downregulated as a result of increased pCO_2 in order to conserve energy, as observed in Michaelidis et al. (2005) in a 3 months study on *M. galloprovincialis* at 5,000 µatm. On the other hand, metabolism was shown to be upregulated in juvenile *C. virginica* during a 30 weeks trial at 3,500 µatm (Beniash et al. 2010), possibly to counteract higher energy demands for maintenance (Stumpp et al., 2012).

The filtration rate was expected to decrease with increasing pCO_2 (hypothesis 4) as energy demand and energy supply were expected to be down regulated for higher treatment levels. This expectation was in agreement with observations within the treatments. The siphos in treatment 5 (Fig.3.22) were mainly observed to be close when cockles were burrowed, suggesting that no or very little filtration took place.

Filtration rate correlates with metabolism: Riisgård (1981) observed higher O_2 consumption and a higher filtration rate under higher food concentration. Filtration rate could not be tested for treatment 5 because of very high mortality resulting in too few individuals being alive at the end of the experiment. Filtration rate was tested for *C. edule* for the control, treatment 2, 3 and 4. Variations within filtration rates were too high to evidence any significant differences; hypothesis 4 could thus not be confirmed for filtration rates. High variations could possibly be due to a too short acclimation period or to the lack of sediment. However, a trend towards increasing filtration rate until a threshold and decreasing filtration rate for the highest pCO_2 treatments was observed. This trend agrees with findings of Thomsen and Melzner (2010) who found oxygen consumption rates resembling a parabolic curve as they increase with increasing pCO_2 and decrease in higher levels; however filtration rates were not measured in their study.

4.5. Oxidative stress

Hypothesis 5: MDA content is expected to increase in high pCO_2 treatments.

MDA (Malondialdehyde) concentrations were measured as an indicator for oxidative stress (Kohen, 2002). Expectations for MDA concentrations were based on expectations for changes in metabolic rate with changing pCO_2. MDA concentration correlates with oxidative stress (Rio, 2005). The production of reactive oxygen species increases with increased metabolic rates of organisms (Finkel and Holbrook, 2000), thus MDA concentration should correlate with metabolic rate.

Metabolic rate increased for intermediate pCO_2 levels (i.e. 3,500 µatm, Beniash et al., 2010) and Tomanek et al. (2011) observed an increase in oxidative stress with increasing pCO_2 in the oyster *C. virginica*. MDA concentrations were expected to increase with increasing pCO_2. The highest treatment level in both studies was around 3,500 µatm. Metabolic rate was observed to decrease with very high pCO_2 (Michaelidis et al., 2005; Pörtner et al., 2005, Thomsen and Melzner, 2010), oxidative stress and MDA content might thus be reduced at higher pCO_2. In the present study, significantly higher values to the control could not be directly observed. A significant difference between treatment 2 (2,900 µatm) and treatment 4 (12,800 µatm) was

found, thus an increase in MDA values can be argued for as treatment 2 showed a slight increase and treatment 4 showed a slight decrease in MDA values (Fig.3.16). A significantly lower value was observed for the highest treatment, thus MDA values decrease with increasing pCO_2 potentially coupled to reductions in metabolic rate. The pattern in MDA concentration resembled the parabolic response in metabolic rate in the mussel *M. edulis* observed by Thomsen and Melzner (2010).

Results of MDA content, filtration rate and changes in shell free dry mass together indicate that the energy budget of cockles was severely impacted by hypercapnic stress. Even though filtration rate was not significantly different for any treatment, filtration rate significantly correlated positively with MDA (Fig.3.17, $p < 0.03$) with an R^2 of 0.29. Increased filtration would correlate with increased metabolic rates and increased energy uptake. Increase in metabolic rate with an increase in pCO_2 could be due to increased energy demand as more energy is needed for maintenance (Stumpp et al., 2012).

4.6. Energy budget sensitivity analysis

With respect to the determined MDA concentrations there is a significant reduction in [MDA] above 12,800 µatm and a trend towards a reduction above 6,600 µatm, agreeing with a trend towards a reduction in filtration rate and the reduction in shell free dry mass in the highest treatment. Reduced filtration rates would lead to less energy supply to the organism, which would explain the reduced shell free dry mass in the highest treatment. This leads to the suggestion of a critical CO_2 threshold.
Agreeing with results of this study, Michaelidis et al. (2005) showed a reduction of metabolism for 5,000 µatm. A reduction of metabolism is a general response to environmental stress as it was also shown for hypoxic conditions (Diaz and Rosenberg, 1995; Boutilier, 2001; Guppy et al., 2005; Pörtner et al., 2005).
Even though metabolic rates were not measured it becomes clear from the present results that calcification and dissolution are only one problem occurring with increased pCO_2. Leakage scenarios as simulated here can lead to disturbances in energy budgets. Organisms being exposed to slightly higher pCO_2 (levels assumed to occur with future acidification, e.g. pCO_2 1,000 - 4,000 µatm) seem to increase

metabolism, while organisms exposed to higher levels of elevated CO_2 seem to decrease metabolism.

Difference in mortality for different sizes was possibly partly related to energy content in relation to mass specific metabolic rates. Energy utilization values were calculated for ~8°C using the conversion factors of Newell and Bayne (1980). Energy utilization calculated for *C. edule* (Fig.3.18) can only be regarded as a guideline. If only utilizing the available energy and assuming no food uptake, calculated survival times fit more or less to survival times in the 24,400 µatm treatment. E.g., a *C. edule* of 0.74 cm shell length would survive for 34 days; a cockle with 2.08 cm shell length for 52 days. An average, normoxic metabolic rate was assumed for the calculated survival times. Findings of the present and other studies (Green et al., 2004; Waldbusser et al., 2010) coincide, as all reveal a higher/ longer survival rate for larger bivalves.

Intracellular pH is usually regulated by organisms (Michaelidis et al., 2005) and more energy might be needed to regulate intracellular pH. Increased metabolism could indicate increased demands for maintenance. Under very highly elevated pCO_2, metabolism is suggested to be downregulated (Michaelidis et al., 2005), thus the energy demand and uptake would be lower, resulting in possibly longer survival times. Survival times during exposure to unfavorable environmental conditions (e.g. anoxia) are typically increased by bivalves by severely constraining metabolic demand and supply (e.g. Boutilier 2001, Guppy et al 2005).

4.7. Behavior

Hypothesis 6: Accumulation of C. edule at the sediment surface will increase with increased pCO_2.

Behavioral responses of *C. edule* on hypercapnia were the same as responses observed during exposure to hypoxia (Diaz and Rosenberg, 1995; Rosenberg et al., 1991), confirming hypothesis 6. With increasing pCO_2 more cockles came to the surface. Seemingly, cockles move to the surface when already in a bad condition and prior to death. A strong behavioral response could be observed in the highest treatment but not for any of the other treatments. The fraction of *C. edule* on the surface of the sediment increased with duration of the experiment. In treatment 4,

movement on to the surface could be observed; however cockles on the surface were only few (average 6%). Behavioral responses such as massive accumulation of bivalves on the seafloor, especially during the short-term period (0-6 weeks) could be used as a cheap and efficient monitoring tool for future monitoring of sub-seabed CCS storage sites, e.g. by towing camera systems across large sea floor areas.

4.8. Abiotic conditions in the sediment

Animals in the sediment are used to lower pH and relatively low calcium carbonate saturation states (Ω aragonite, calcite) even under control seawater pCO_2. Saturation conditions in the water column differ from saturation conditions within the sediment pore water due to alkalinity differences. A_T in the sediment is higher than in the seawater column resulting in a higher carbonate concentration and a higher saturation state (Haynert et al., 2012). This results from anaerobic generation of bicarbonate during nitrate and sulfate reduction (Yao and Millero, 1995). Fig.4.1 shows calculated seawater and sediment Ω for aragonite. Because of higher buffering capacities within the sediment, conditions are more stable. Saturation for aragonite was calculated for all pCO_2 levels and undersaturation occurred in all levels. On the other hand sediment Ω for aragonite is higher than seawater Ω (aragonite) at elevated pCO_2 levels (Fig.4.1); therefore infauna species are subjective to smaller changes compared to open water conditions. Under acidified conditions it would be advantageous to be burrowed as pCO_2 is lower and Ω is higher within the sediment and thus conditions in the sediment are be less corrosive than in the water column.

Cockles within the sediment might endure acidified conditions of the water column more easily. Moving onto the sediment surface would increase the rate of shell dissolution.

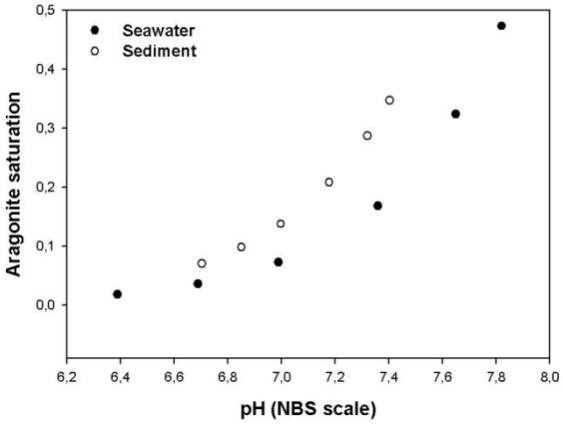


Fig.4.1: Calculated seawater and sediment omega for aragonite. Values were calculated for alkalinity of 1980 µmol/kg for seawater (average value of this experiment); 3700 µmol/kg for sediment at 2cm depth (Haynert, unpublished). Average temperature of 6.2°C and salinity of 17.8g/kg; sediment pH (seawater – sediment) was calculated from seawater pH using linear regression (-0.5116*pH+3.5837) between seawater pH and sediment pH according to Widdicombe et al. (2009).

4.9. M. arenaria and *M. balthica*

Hypothesis 7: Resistance of *M. arenaria* will be highest of all three bivalves; resistance of *C. edule* will be lowest.

All species studied are calcifiers, infaunal clams and their shells consist of aragonite, the more soluble calcium carbonate polymorph. Even though *C. edule* was the only species in this study investigated for shell free dry mass and MDA, it is obvious from the results that *M. balthica* and *M. arenaria* are more resistant towards higher pCO_2 values as suggested in hypothesis 7. Observing mortality and behavior, no difference between those two species could be found. During the experimental duration, no mortality was found and only one individual of each species came to the surface. Their resistance towards elevated pCO_2 was very high and even smaller individuals survived 12 weeks in very low pH. The expectation of *M. arenaria* being more resistant towards higher pCO_2 than *M. balthica* was not confirmed. However, observations for *M. balthica* need to be regarded with care as there was only one individual per replicate.

M. arenaria is a highly resistant species not only against future levels of ocean acidification but also against extremely high CO_2 partial pressures expected to occur at leakage sites. Resistance of this species is probably due to a greater burrowing

depth (< 50 cm; Baker and Mann, 1991) and thus possibly a lower influence of acidified water. In addition, a thicker periostracum (Harper, 1997) leads to a better protection of the shell and higher resistance towards dissolution. These observations are a clear indication against organisms with an aragonitic shell being generally vulnerable towards acidified water. Saturation states for aragonite are lower compared to calcite (Orr et al., 2005), but this can obviously not be the only influence on shell vulnerability to dissolution. Constituent mineralogy might be an important factor in susceptibility of bivalves with differently composed shells (Ries et al., 2009) but there is a high variation between species with the same shell composition. Crystal size and proportion of organic matrix play an additional factor in resistance against environmental stress like hypercapnia (Harper, 2000).

Species	Duration of Experiment	Approx. CO_2 Level	Mortality	Growth	Net Calcification	Metabolism	Oxidative stress	Effect	Reference
3 species, e.g. *M. mercenaria*	20 days	750 µatm		↓				Juv. *M. mercenaria* was not negatively affected by higher CO_2	Talmage and Gobler, 2011
5 species, e.g. *M. edulis*	60 days	1,000 µatm						Ability to maintain levels of calcium carbonate	Findlay et al., 2011
M. edulis; C. gigas	2-3 hours	2,000 µatm			↓			Dissolution > 1,800 µatm	Gazeau et al., 2007
Mercenaria spp.	8 hours	2,000 µatm			↓			significant effects of size on calcification rates	Waldbusser et al., 2010
13 species; e.g. *M. mercenaria; M. arenaria*	60 days	3,000 µatm			↓			Net calcification decreased; net dissolution was observed; Mineralogy important	Ries et al., 2009
Crassostrea virginica	30 weeks (juv.); 2 weeks (adults)	3,500 µatm	↑ (juv.)	↓(juv.)	↓ (juv.)	↑ (juv.)		Decreased mortality, soft-body and shell growth and higher standard metabolic rate for juveniles	Beniash et al., 2010
C. virginica	2 weeks	3,570 µatm					↑	Functional categories such as oxidative stress upregulated	Tomanek et al., 2011
M. galloprovincialis	78 days	3,800 µatm						No difference between clearance and respiration	Fernández-Reiriz et al., 2012
M. galloprovincialis	84 days	3,800 µatm			↓			No difference in growth or mortality	Range et al., 2012
M. edulis	7 weeks	4,000 µatm		↓(>4,000 µatm)	Internal shell corrosion (>2400 µatm)			Low food algae concentration and high pCO_2 values each significantly decrease shell length growth	Melzner et al., 2011
M. edulis	8 weeks	4,050 µatm		↓	↑			Increase in shell mass for all treatments, slowed shell growth for elevated pCO_2	Thomsen et al., 2010
M. mercenaria	2 weeks	5,000 µatm	↑		↓			Significant mortality for each size class; larger bivalves	Green et al., 2004

								less susceptible to dissolution mortality	
M. galloprovincialis	3 month	5,000 µatm		↓	↓	↓		Significantly lower oxygen consumption and shell growth	Michaelidis et al., 2005
M. edulis	44 days	14,000 µatm	↑ (indication)	↓ (at 7.1)				Low food, mortality due to T	Berge et al., 2006
Acesta excavata	96 hours	33,000 µatm			constant	↓ (end ↑ to norm)		Decline in oxygen consumption, return towards control values at the end	Hammer et al., 2011

Tab.4.1: CO_2 levels calculated after pH values and CO2SYS or taken as data from paper

4.10. Responses of bivalves to high environmental pCO_2

The response of bivalves to elevated pCO_2 in experimental studies has been shown to depend on numerous factors. Among the most important factors that have been shown to influence experimental outcomes are (i) pCO_2 treatment level, (ii) feeding conditions, (iii) exposure time, (iv) ontogenetic stage tested, (v) temperature, (vi) habitat (e.g. infauna vs. epifauna). Experimental outcome would also be strongly affected by species tested. Studies being compared in this case all studied effects on bivalves. Accordingly, available studies (Tab.4.1) showed a variety of different results. These varying results of different studies however, show certain trends and approximate thresholds which agree with findings of this study. Obviously not all effects were tested in all studies but a combination leads to possible assumptions about certain thresholds for different parameters as defined above for parameters measured within this study.

The present study investigated the response of three species of infauna bivalves to prolonged exposure to very high seawater pCO_2. There are only a few studies available that have also investigated infauna species mainly regarding *M. mercenaria* (Green et al., 2004; Waldbusser et al., 2010; Ries et al., 2009 and Talmage and Gobler 2011). All three species investigated here are highly important species for the Baltic & North Sea ecosystem (Taylor et al., 1973; Möller and Rosenberg, 1983; Beukema, 1979). As infauna species are trapped in potential leakage areas, while other, mobile species like teleost fish could rapidly migrate from the leakage site, studies on infauna species are highly important.

Generally, studies showed that susceptibility to high pCO_2 depends on size, with smaller individuals being more vulnerable (Waldbusser et al., 2010; Beniash et al., 2010). This study only tested cockles of a size > 0.4 cm, thus there is no result for even smaller organisms. However, studies in juveniles lead to the assumption of larvae being even more sensitive towards increased pCO_2 (Beniash et al., 2010, Talmage and Gobler, 2009, 2011).

Growth could not be assessed in this study; however thresholds can be defined regarding results in other studies. Growth in juveniles was as well already affected at comparatively low levels of acidification (e.g. 750 µatm). Juvenile *M. mercenaria* showed slowed growth in a study by Talmage and Gobler (2011) at a level of

750 µatm while no other measured parameter was affected. The threshold for an effect on growth might argued to be around 3,000 µatm, as slowed growth (i.e. > 10%) was found for M. edulis above 4,000 µatm (Melzner et al., 2011; Thomsen et al., 2010) and also by Michaelidis et al. (2005) for *M. galloprovincialis* at 5,000 µatm. Range et al. (2012) tested a level of 3,800 µatm and found no difference in growth for *M. galloprovincialis* however, this could be due to slow growth in general or high variances.

Metabolism has been shown to be differentially affected depending on the CO_2 exposure level applied. Juvenile *C. virginica* showed an upregulation of metabolism at a level of 3,500 µatm (Beniash et al., 2010) and Tomanek et al. (2011) showed an upregulation of oxidative stress for the same species which could lead to assumptions about upregulated metabolism causing increased oxidative stress. In a study of Michaelidis et al. (2005) metabolism was downregulated at 5,000 µatm in *M. galloprovincialis*. Fernández-Reiriz et al. (2012) showed no significant difference in respiration for *M. galloprovincialis* at 3,800 µatm, however downregulation above this level could be possible. According to above mentioned studies and also agreeing with results of MDA content in this study, metabolism is upregulated until 4,000 µatm and downregulated for even higher pCO_2. This agrees with the parabolic response in oxygen consumption with increasing pCO_2 observed by Thomsen and Melzner (2010) for *M. edulis*.

A threshold for calcification was difficult to define. Rates in the short-term studies of Gazeau et al., (2007) and Ries et al., (2009) need to be treated with care as there was no possibility for acclimation. In addition both studies might have had feeding difficulties which would have high influence on the results as shown by Melzner et al. (2011). Melzner et al. (2011) found a significant influence of food algae concentration on shell length growth.

Internal shell might be more vulnerable as it is in direct contact with low pH in the extrapallial fluid is a result of high body fluid pCO_2. The inside shell surface is directly exposed to the extrapallial fluid and was found to be corroded for pCO_2 of 2,400 µatm (Melzner et al., 2011). The internal shell might be influenced at lower thresholds as it is in contact with a more corrosive fluid. Calcification might be impacted at a different level in this study compared to epifauna studies as Ω for aragonite was shown to be different in sediments (Haynert et al., 2012). A short-term study by

Waldbusser et al. (2010) on *M. mercenaria* found a decrease in calcification rate already at a level of 2,000 µatm, while Green et al. (2004) tested the infauna bivalve *M. mercenaria* and found a decrease in calcification rate at a level of 5,000 µatm. This difference could again be due to feeding conditions. Considering the results of this study, there is already an influence of elevated pCO_2 at 1,500 µatm on corrosion; however no net calcification rates were measured.

Differing from these results is a study on the deep-sea bivalve *Acesta excavate.* Contrary to many other studies on different species, calcification was found to be constant in this species even in the highest treatment. Additionally Hammer et al. (2011) did show a downregulation of metabolism for this species at 33,000 µatm but they showed a backregulation towards control values at the end of their 96 hour study. Even though this is only a short – term study this study shows the importance of testing different species as effects on different species might vary. Of the three species studied in the present experiment, only one was found to be sensitive regarding the measured parameters, while the two other species did not show any effect at least on mortality. Conclusively thresholds can thus be an indication but are not applicable to all species.

5. Conclusion and Outlook

Calcifiers are sensitive to elevated levels of pCO_2, and sensitivity increases with increasing pCO_2. In this study, the effects of different levels of pCO_2 on different bivalve species were tested. *C. edule* was found to be sensitive to most levels tested, however mortality increased significantly at pCO_2 levels of 6,600 µatm. Dissolution of the shell already occurred at lower levels of acidification. In addition to dissolution, oxidative stress and influence of acidificiation on different size classes was tested. Larger individuals were less susceptible towards acidification but with ongoing duration, mortality increased. MDA content for *C. edule* was found to increase until a certain level and decrease for the highest treatments. This suggests a regulation against stress until a certain level and less or no regulation for higher levels. MDA increased to a level of 2,900 µatm, one level beneath 6,600 µatm, at which mortality started to be significant.

C. edule is very sensitive to acidification of the water column but the size range tested can survive stressful conditions up to 2,900 µatm for 12 weeks and thus any level predicted for future ocean acidification. Leakages with a rise of pCO_2 to 6,600 µatm and upwards, would be lethal for *C. edule* in the tested time frame. For both other bivalve species tested, no mortality was found during 12 weeks. Dissolution was only tested for *C. edule*, thus no results are given for the other two species. As *M. arenaria* and *M. balthica* showed no mortality, a very high resistance against environmentally stressful conditions at elevated pCO_2 can be argued.

C. edule could potentially be used for initial monitoring of leakages as cockles move to the surface before dying. This is a very obvious sign of acidification. At early stages of acidification small cockles move to the surface as they are more susceptible towards acidification, with ongoing leakage larger individuals follow. This monitoring only works for very high levels of acidification which might occur directly at a leakage site.

Thresholds could be indicated but are not applicable to all species. Effects on the environment in leakage areas will thus be variable depending on species within environment. Results of this study can be related to potential leakages at Rotterdam CCS sites as species studied in this study do potentially occur in that area.

This study tested elevated levels of pCO_2 for 12 weeks. Mortality increased with longer duration of experiment. From this experiment, we cannot predict the ability of *C. edule* to recover or survive at lower pCO_2 levels for a longer duration. At the event of a leak, the short term influence could possibly harm the cockles beyond recovery. It is thus very important to include a recovery period into the experiment in future research. In addition it is also important to test shorter time periods and a recovery. There is no knowledge on the duration of leakage if leakage occurs. It is possible that duration of leakage is only short but without detection of leakage, leakages could last for longer time periods. The effect of elevated levels of pCO_2, up to 6,600, needs to be tested for longer duration than 12 weeks.

Talmage et al. (2009, 2011) suggest a higher sensitivity of larvae towards acidification. Even though larger individuals can survive stressful conditions, this cannot be transferred as a prediction towards larvae. Reduction in growth rates would be expected, as well as significant increase in mortality. Even if there is no or low influence on adult population, increased mortality in larvae at low levels or already after short term exposure to elevated CO_2 would have an influence on the population in that area. Thus further studies are needed including larvae of species occurring at possible leakage sites.

Clams are important ecosystem engineers and an important food source for other organisms (Taylor et al., 1973; Beukema, 2010). The three species studied make up great amount of the biomass in the Wadden Sea (Beukema, 1976). As suspension feeding organisms (Riisgård, 2011), bivalves filter tremendous amounts of water during their life span and are thus important for water quality. Bivalves do not only have an influence on the plankton communities but as benthic species they also have an impact on the sediment (Flach, 1996). In addition, bivalves serve as food source for different animals like migrating birds (Bibby et al., 2008). It is thus important to know the effects on these organisms as any influence on bivalve communities would have effects on the entire ecosystem. At potential leakage sites, all organisms within the range of influence would be under stressful conditions. It is thus important to not only test bivalve communities but in addition test other communities occurring in that area. Samples were taken in the framework of this thesis for the analysis of meiofaunal and bacterial communities. Those samples were not analyzed in the

framework of this project but will be analyzed in the following months to complement the present study.

From this experiment we know that shells of *C. edule* start to dissolve already at the lowest level tested. In order to titrate the time periods after which dissolution at different levels sets in, shells taken out after 6 weeks need to be analyzed using SEM. If the same dissolution signs are found, ongoing research with new experiments needs to sample individuals in shorter time periods to see when effects set in.

Further measurements of oxidative stress related parameters would in addition be valuable and samples were taken for possible additional analysis. Antioxidant defenses control the influence of oxyradical generation on biological damage. MDA production, which was measured in this study, is thus dependent on the effectiveness and production of antioxidant defenses. In addition to MDA content, further studies would need to include the analysis of antioxidant enzymes such as super oxide dismutase (SOD) or Glutathione peroxidase (GPx). It would also be interesting to include differential transcriptome analysis into further research to identify CO_2 responsive cellular processes.

This experiment investigated feeding rate of clams within the different treatments. From behavioral observations, filtration rate was suggested to be reduced in the two highest treatments. However, no significant results could be achieved. It would be very important to include studies of filtration rates in further research. It is important to include more individuals in filtration rate experiments and to perform filtration rate experiments with sand for *C. edule* to burrow in order to reduce stress.

Even though results of this study lead to new suggestions on further studies, results found are important and give important insights about the impacts of high pCO_2 on the bivalve species tested. *Mya arenaria* and *Macoma balthica* are highly resistant species and do not suffer from mortality during the 12 week experimental duration in response to any of the chosen treatments. *C. edule* is less resistant and suffers from elevated pCO_2. Ocean acidification as it is predicted for the future (< 3,000 µatm) would not impact mortality of *C. edule* during 12 week exposure, it would however, influence calcification of this species. Leakage of sub seabed storage sites would be lethal for all sizes of *C. edule* studied if levels of > 6,000 µatm persist for several weeks. Owing to the dominance of *C. edule* in shallow sandy coastal areas, high

mortality would have a strong secondary impact on the ecosystem in that area, particularly on bioturbation and energy flux (Flach, 1996; Beukema et al., 2010). Leakage would thus be highly stressful for a cockle dominated ecosystem.

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Erklärung

Hiermit bestätige ich, die vorliegende Masterarbeit mit dem Titel „Influence of sea water acidification on benthic bivalve communities“ selbständig und mit keinen anderen als den angegebenen Quellen und Hilfsmitteln angefertigt zu haben.

Ich versichere, dass diese Arbeit nicht an anderer Stelle zur Erlangung eines akademischen Grades vorgelegt wurde.

Mit der Aufnahme der Arbeit in die Bibliotheken des GEOMAR und der Christian-Albrechts-Universität zu Kiel bin ich einverstanden.

Kiel, den

.....
Hanna Schade