The effect of ocean acidification on pelagic bacterial communities: a mesocosm study

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Abstract
The ocean absorbs about 25-30% of the anthropogenic CO$_2$ emissions which has already lowered the pH in the oceans by 0.1 units compared to preindustrial levels. Given that the CO$_2$ in the atmosphere reaches 1000 ppm in year 2100, the ocean pH is predicted to decrease by 0.4 pH units during the coming 90 years. This ocean acidification (OA) is expected to have impacts on processes such as nitrogen and carbon cycling. Bacteria are of great importance for these processes, and effects on the structure and activity of bacterial communities could potentially alter the availability of nitrogen and carbon for other organisms and affect the entire food web. OA is predicted to make certain metals and antibiotics more bioavailable, which would create a stronger selection pressure for resistance. The aim of the project was to investigate the response of bacterial communities to ocean acidification in a mesocosm study. Ten mesocosms were placed in the Gullmar Fjord; five were treated with 1000 µatm CO$_2$ (high) and five were left untreated (control). Samples were collected once a week during the period 8$^{th}$ of March to 15$^{th}$ of May. The responses investigated were: changes in bacterial community composition by PCR-DGGE, enumeration of total bacterial abundance by epifluorescence microscopy of DAPI-stained bacteria, and the prevalence of copper and antibiotic resistance by spreading water on agar plates containing copper or antibiotics. The results showed a change in community composition at the peak of the first phytoplankton bloom and at the decline of the second phytoplankton bloom, although the species richness remained the same. Effects of OA on the community composition have been found in previous mesocosm and microcosm studies and have been proposed to influence the carbon pump in the ocean. There was a decrease in bacterial abundance in the high CO$_2$ mesocosms at the peak of the second spring bloom. No effect of OA could be seen for the prevalence of antibiotic or copper resistance. Which species or functional groups of bacteria shifted in the community composition were not investigated during this project. In future studies, this would be interesting to investigate in order to assess which effect the change in community composition might have on the pelagic ecosystem.
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Introduction

The uptake of anthropogenic CO$_2$ emissions by the oceans has lowered the pH in the oceans by 0.1 units compared to preindustrial levels (Caldeira and Wickett, 2003). If the CO$_2$ concentration in the atmosphere at year 2100 reaches 1000 ppm, the ocean pH is predicted to drop to pH 7.67; this is a reduction in pH of approximately 0.4 units compared to present day values (Caldeira and Wickett, 2003). The most well studied effect of ocean acidification is the detrimental effect on calcification in organisms with a calcium exoskeleton (Doney et al., 2009). Other processes that have been found to be affected by ocean acidification are processes such as nitrogen fixation, denitrification and regeneration of organic carbon (Hutchins et al., 2009). Ocean acidification could lead to less nitrate and nitrite in the ocean and more ammonium due to a reduction in nitrification (Beman et al., 2010). Bacteria are of great importance for the cycling of a variety of nutrients; such as carbon, nitrogen, phosphorous and silica (Hutchins et al., 2009). The marine heterotrophic bacteria utilize dissolved organic matter (DOM) released from phytoplankton and zooplankton. The DOM is recycled into the food chain in the microbial loop by flagellate and ciliate grazing of bacteria (Azam et al., 1983). The flagellates and ciliates are grazed by microzooplankton and thus the DOM enters the higher levels of the food chain (Azam et al., 1983). Effects on the structure and activity of bacterial communities could potentially alter the availability of nutrients for other organisms and affect the entire food web (Hutchins et al., 2009; Yamada et al., 2010).

Ocean acidification could act as a stressor on marine bacteria by changing the levels of H$^+$ in the sea. The reduction in pH due to ocean acidification has been shown to affect the activity of bacterial extracellular enzymes (Arnosti et al. 2011, Maas et al., 2013) and bacterial protein production (Grossart et al., 2006). In every bacterial community, individual bacterial species (or strains) will differ in their sensitivity to a certain stressor. A reduction in pH could thus lead to a shift in the bacterial community composition towards a domination of species most tolerant to pH stress. However, the marine bacteria are already exposed to pH changes in their environment; seasonal and diurnal pH variations are common and during phytoplankton blooms there is normally a temporary increase in pH due to the consumption of CO$_2$ (Joint et al., 2011). The greatest effect of ocean acidification on marine bacteria might not be caused by the reduction of pH but rather by the elevated levels of CO$_2$.

The uptake of CO$_2$ emissions could be viewed as a CO$_2$ fertilization effect that might favour phytoplankton (Hutchins et al., 2009). The phytoplankton utilizes CO$_2$ in their photosynthesis and an increase in the pCO$_2$ of the ocean could therefore be beneficial for phytoplankton by increasing photosynthesis efficiency. This would in theory stimulate growth and increase numbers of marine algae, especially cyanobacteria, which are able to fixe N$_2$ directly from the air if nitrogen is the limiting factor (Hutchins et al., 2009). All phytoplankton will not react to increased CO$_2$ in the same way. A recent study on diatom communities showed that some diatoms were favoured and other disfavoured by high pCO$_2$ treatment (Tatters et al., 2013). Even within the group of cyanobacteria there seem to be divergent responses the higher levels of CO$_2$; the cyanobacterium Trichodesium was favoured by increased pCO$_2$ in the ocean but another cyanobacterium, Nodularia spumigena, decreased its division rate and nitrogen fixation rate under high pCO$_2$ treatment (Czerny et al., 2009 and Levitan et al., 2007).
Phytoplankton produce transparent exopolymer particles (TEP) that heterotrophic bacteria can use as attachment site and nutrient source (Engel, 2002; Passow and Alldredge, 1994). Increased pCO$_2$ has been shown to stimulate the production of TEP by phytoplankton (Engel, 2002), which in turn could increase the number of heterotrophic bacteria in the upper pelagic zone by providing more habitats and available nutrients for the bacteria. Changes in phytoplankton dynamics could affect the bacterial community in terms of abundance, activity and species composition. There has been made several previous studies investigating the effect of ocean acidification on bacterial communities. The results from these studies have shown variable responses to ocean acidification (Liu et al., 2010, Allgaier et al., 2008; Maas et al., 2013; Grossart et al., 2006; Krause et al., 2012; Zhang et al, 2013). In terms of bacterial abundance, the results have shown either no effect or a slight increase with reduced pH (Liu et al., 2010, Allgaier et al., 2008; Maas et al., 2013). Similar results have been found regarding bacterial protein production; one study could see an increase in bacterial protein production and another could see no effect (Grossart et al., 2006; Allgaier et al., 2008). According to some previous findings the community composition will change with increasing ocean acidification (Allgaier et al., 2008; Krause et al., 2012; Zhang et al, 2013).

Another effect of ocean acidification that may influence the bacterial community is the predicted change in speciation of certain metals. Ocean acidification could alter the speciation of copper, from being bound to ions, particles and dissolved organic carbon (DOC) to existing as free Cu$^{2+}$ ions, making the copper more bioavailable and creating a stronger selection pressure for copper resistance (Richards et al., 2011). Copper forms strong complexes with $\text{CO}_3^{2-}$ and $\text{OH}^-$ and both $\text{CO}_3^{2-}$ and $\text{OH}^-$ concentrations are predicted to decrease with increasing pCO$_2$ in the surface waters of the ocean and in estuaries (Millero et al., 2009). An estimation of the change in the Cu$^{2+}$ fraction between the years 2000 and 2100 is an increase by 10.65 percentage units, from 7.67% to 18.32% of the total copper in sea water, assuming a temperature of 25 °C and a salinity of 35 (Millero et al., 2009). The mechanisms for metal resistance can sometimes be effective against antibiotics, examples of such mechanisms are: increased amount of multidrug efflux pumps that transport unwanted compounds out of the organism and reduction of membrane permeability (Sarma et al., 2010).

Some antibiotics will also become more bioavailable with lowered pH (Rendal et al., 2011). These antibiotics may change forms from being charged to having no net charge; the form in which they have no net charge is called a zwitterion (Rendal et al., 2011). The zwitterion is considered to be the most bioavailable form since it passes more easily through cell membranes compared to the charged form (Rendal et al., 2011). One of the antibiotics that are predicted to become more bioavailable is amoxicillin (Alekseev, 2011). Amoxicillin is most bioavailable between pH 7.7 and 3.0 which is the span in which it will be predominantly in its zwitterionic form (Alekseev, 2011). In the future scenario, the pH of the oceans is predicted to be approximately 7.7 which is the pH where amoxicillin starts to become uncharged. This leads to the hypothesis that amoxicillin resistance will increase with ocean acidification. Amoxicillin belongs to the beta-lactam antibiotics that are designed to inhibit cell wall synthesis (Jjemba, 2008). The resistance mechanism to beta-lactams usually involves the ability to produce beta-lactamases, enzymes cleaving the beta-lactam ring, a common
chemical structure for beta-lactam antibiotics (Ijemba, 2008; Rang and Dale, 1991). Among the antibiotics that will not become more bioavailable with increasing ocean acidification we find the fluoroquinolone ciprofloxacin. The reason it will not become more bioavailable with reduced pH is because it is most bioavailable at a pH > 8 (Qiang and Adams, 2004). Ciprofloxacin inhibits the replication of bacteria and is effective against both gram positive and gram negative bacteria (Ijemba, 2008; Rang and Dale, 1991).

To understand the effect of ocean acidification on communities there is a great need to investigate the responses under complex conditions as in e.g. mesocosm studies involving communities of phytoplankton, zooplankton and bacteria in order to get ecologically relevant data. In this study the effect on pelagic bacterial communities was investigated in a mesocosm experiment. The parameters investigated were; total bacterial abundance, community composition and the prevalence of copper, ciprofloxacin and amoxicillin resistance.

This study was conducted as a part of a large international mesocosm project called KOSMOS 2013, Gullmar Fjord. The KOSMOS project has been a part of the BIOACID project (www.bioacid.de) which is coordinated by GEOMAR Helmholtz Centre for Ocean Research Kiel. The abbreviation KOSMOS stands for Kiel Off-Shore Mesocosms for future Ocean Simulations and these mesocosms are designed to allow experimentation in open water environments (Riebesell et al., 2013). The mesocosms are 17 meters tall and can contain about 55,000 litre sea water. Similar KOSMOS-studies have previously been performed in Svalbard (Norway), Bergen (Norway), Hawaii (US) and Tvärminne (Finland) (Riebesell et al., 2013). This year 10 mesocosms were deployed in the Gullmar Fjord on the Swedish west coast. The experiment was started in March and terminated in June, 2013. The duration of the experiment is unique; the previous studies have lasted for approximately 2 months. The main purpose of this year’s KOSMOS project was to get data on how ocean acidification affects the development of a spring bloom, investigating the effects on phytoplankton, zooplankton, bacteria, nutrients et cetera.

Aim
The aim of the project was to investigate the response of bacterial communities to ocean acidification in a mesocosm study.

The null and alternative hypotheses are:

$H_0$ 1: There is no difference in community composition between high pCO$_2$ and control mesocosms

$H_1$ 1: CO$_2$ amendment changes the bacterial community composition

$H_0$ 2: There is no difference in bacterial numbers between high pCO$_2$ and control mesocosms

$H_1$ 2: CO$_2$ amendment affects (leads to an increase in) bacterial numbers

$H_0$ 3: There is no difference in the prevalence of antibiotic/copper resistance between high pCO$_2$ and control mesocosms

$H_1$ 3: CO$_2$ amendment leads to an increase in the prevalence of amoxicillin/copper resistance and a decrease in ciprofloxacin resistance.
Methods

Experimental design and sampling
Ten mesocosms were deployed in the Gullmar Fjord on the Swedish west coast. Five of the mesocosms were treated with 1000 µatm CO$_2$, which is the highest predicted pCO$_2$ at year 2100 and will yield a pH of approximately 7.7. The five other mesocosms were kept at present day pCO$_2$ levels to serve as controls. The experiment was started on 8th of March. This time point served as a t-1 sampling day to obtain data before any pCO$_2$ adjustments had been made. The CO$_2$ addition was performed in 3 steps over 5 days. In connection with the CO$_2$ addition N$_2$O was added once to each mesocosm. Every fourth day each mesocosm was seeded with 20 L of water from the Gullmar Fjord. A water sample was collected every week from the 10 mesocosms and the Fjord. A sample of approximately 5 Litres was obtained with an integrated water sampler that was lowered to 15 meters depth and pulled up to the surface. The last sampling day for my study within the KOSMOS 2013 project was the 15th of May.

Community Composition
Water samples (300 mL) were filtered onto 0.2 µm Cellulose Nitrate membrane filters (Whatman) at -20 kPa. The filters were preserved in 750 µL RNA later (Ambion Inc.) and stored in – 20 °C until the DNA was extracted.

The DNA extraction buffer contained: 20 mL 6.25 M ammonium acetate, 5 mL 1M TRIS pH 8, 2 mL 0.5 M EDTA pH 8, 23 mL H$_2$O and 50 µL CTAB/NaCl per ml buffer. Chloroform-isoamyl alcohol (24:1) was used to achieve a phase separation and the cells were lysed using bead beating with Zirconia/Silica beads of 0.1 and 1.0 mm size. PVPP was added to remove humic substances. The DNA was precipitated overnight in isopropanol and sodium acetate (3 M). The DNA pellet was washed with ice cold ethanol (70 %) which was evaporated on a heating block set to 45 °C and the pellet was dissolved in 25 µL autoclaved MilliQ water. The concentration and purity of the DNA was determined using a NanoDrop 1000 spectrophotometer (Saveen Werner AB).
Genes coding for the β subunit of bacterial RNA polymerase (rpoβ) was amplified for analysis of bacterial community structure according to Dahllöf et al., 2000. Master mix for one sample contained: 2 µL 10x Red Taq PCR Reaction Buffer (Sigma life science), 0.5 µL dNTP (10mM), 0.5 µL Forward primer with GC-clamp (25 µM), 0.5 µL Reverse primer (25 µM), 0.5 µL BSA (Bovine serum albumin, 10 mg/mL), 1 µL RED Taq polymerase (Sigma life science), 13 µL autoclaved MilliQ. Two µL DNA sample (10 ng/µL) was added to 18 µL master mix and then the PCR reaction was started, using the cycle program: 94 °C 5 min, (94 °C 30 sec, 50 °C 1 min, 72 °C 1 min 30 sec)x 30, 72 °C 10 min, 4 °C hold. The forward primer rpoB1698f with GC-clamp had the following sequence: 5´- GCCCGCCGCCCCCGCCCCAACATCGGTTTGATCAAC-3´ and the reverse primer rpoB2041r had the sequence: 5´- CGTTGCATGTTGGTACCCAT-3´ (Dahllöf et al., 2000). PCR products were verified on a 1.2 % agarose gel before further analysis.

To observe possible differences in bacterial community between treatments, the PCR products were run on a denaturing gradient gel electrophoresis (DGGE). The DGGE was run for 15 ½ hours at 60 °C and 75 Volt in 1.25xTAE buffer. The gel was stained with a solution of 1.5 µL SYBRgold nucleic acid gel stain (Invitrogen, 10,000x concentration in DMSO) and 15 mL 1.25x TAE for 20 minutes in darkness. Bands were detected and a number was assigned to each band using the software Quantity One 4.5.0 (BIO-RAD). Each different band was regarded as an operational taxonomic unit (OTU). The different bands were matched across samples with the fjord samples as a reference. The same fjord samples were run on every gel (in total five gels were run). Similarities in the banding patterns were visualized by MDS-plots and analysed by ANOSIM (PRIMER, version 6.0) and the number of OTUs were analysed with one-way ANOVA (IBM SPSS Statistics 21).

**Bacterial Abundance**
The total abundance of pelagic bacteria was determined by staining the bacteria with DAPI (4’, 6- diamidino-2-phenylindole) and counting the numbers of bacteria using epifluorescence microscopy. The DAPI stain emits blue fluorescence when it is bound to DNA and excited with ultra violet light (390 nm). Samples from the mesocosms were fixed with approximately 3.7 % formaldehyde and stored at 8 degrees. To stain the bacteria, 2.5 mL of fixed water sample was mixed with 100 µL of DAPI solution (0.1 µg/mL) and kept dark and cold for at least 15 minutes prior to filtration. The sample was filtered on black polycarbonate filters (Frisenette ApS, pore size 0.2 µm, diameter 25 mm) at a pressure lower than -10 kPa. The filter was placed on a microscope slide on top of a drop of glycerol-Phosphate Buffer Saline (PBS) (7:3). Another drop of glycerol-PBS (7:3) was added above the filter and a cover slip was applied. The bacteria were counted using a Leica Leitz DMRB (Wetzlar, Germany) epifluorescence microscope with a grid ocular (L Plan10x i.e. 10mmx10mm). The grid had an area of 0.01 mm² at 1000x magnification. Approximately 300 bacteria were counted on each filter. The number of bacteria per mL was calculated using the formula: \( N = (n*A*V)/(v*a) \)
N: Bacteria per mL
n: Average number of bacteria per grid
A: Filter area
V: Water volume with dye
a: Grid area
v: Water volume without dye

The bacterial abundance data was analysed using paired t-test (IBM SPSS Statistics 21) with alpha set to 0.05.

**Antibiotic and Copper Resistance**

Water samples of 100 µL from the mesocosms were spread on agar plates containing amoxicillin, ciprofloxacin or copper. The agar medium contained 5 g peptone, 1 g yeast extract and 15 g agar per litre filtered sea water. The medium was autoclaved before the antibiotics and copper solutions were added. The concentrations of the antibiotics in the agar medium were: amoxicillin at 10 µg/mL and 20 µg/mL, and ciprofloxacin at 10 µg/mL and 20 µg/mL. The concentrations of copper in the agar plates were 0.5 mM, 1 mM and 2 mM CuSO₄·5H₂O. The antibiotics and copper salts were dissolved in sterile filtered MilliQ. Plates without metal or antibiotics were used as controls. All plates were incubated at 8 °C in darkness. The number of colonies on each plate was counted every day until ten days had passed since the spreading of the water sample. The data from the plates containing amoxicillin, ciprofloxacin or copper was normalized by dividing the number of colonies on the plates with copper or antibiotics with the number of colonies on the control plate. Analysis of the data was performed using repeated measures ANOVA (IBM SPSS Statistics 21) and alpha was set to 0.05. Co-resistance was examined by isolating colonies from copper plates and spreading them on plates containing either amoxicillin or ciprofloxacin and monitoring the bacterial growth. The colonies were taken from Cu1 mM plates and Cu2 mM plates.
Results

Community composition
The MDS-plot from the day before any CO$_2$ additions had been made (t-1) reveal a random pattern for the mesocosms and there was no significant difference between treatments (Figure 1; ANOSIM: Global R=0.016, p=0.45). The DGGE data revealed a significant difference in community composition between the different treatments at the 11th of April (ANOSIM: Global R=0.615, P=0.001, Figure 2) and 15$^{th}$ of May (ANOSIM: Global R=0.28, p=0.033, Figure 3). There are however two mesocosms from different treatments that overlap in the MDS-plots: mesocosm 2 (high pCO$_2$) and mesocosm 5 (ambient pCO$_2$) at the 15$^{th}$ of May and they are also more similar to each other than the other mesocosms at the 11$^{th}$ of April (Figure 3 and 2). The species richness based on operational taxonomic units (OTUs) in the different mesocosms was the same for all time points (Figure 4).

Figure 1. MDS-plot of the DGGE data from 8$^{th}$ of March before any CO$_2$ additions had been made. The turquoise squares are from the Fjord. The blue triangles are mesocosms that were treated with high pCO$_2$ after this date. The green triangles are mesocosms that were treated with ambient pCO$_2$ after this date. Mesocosm 3, 7 and 9 are missing due to PCR complications.
Figure 2. MDS-plot of the DGGE data from 11th of April at the peak of the first phytoplankton bloom. The turquoise squares are from the Fjord. The blue triangles represent mesocosms that were treated with high pCO$_2$. The green triangles represent mesocosms that were treated with ambient pCO$_2$.

Figure 3. MDS-plot of the DGGE data from 15th of May at the decline of the phytoplankton bloom. The turquoise squares are from the Fjord. The blue triangles are mesocosms that were treated with high pCO$_2$. The green triangles are mesocosms that were treated with ambient pCO$_2$. Mesocosms 6 and 7 are missing due to PCR complications. Mesocosms 2 and 5 overlap and are encircled.
Figure 4. Species richness based on the mean of OTUs for each treatment (high and ambient CO$_2$) and the fjord. Error bars = ±SD, n=5 except for the fjord which had only one replicate.

Bacterial Abundance
There was no general difference in bacterial abundance between high and ambient CO$_2$ mesocosms. One time point, 1$^{st}$ of May, stood out from the general trend and was analysed with an independent samples t-test which revealed that the treatment had a significant effect at this particular time point (p<0.05, independent samples t-test, SPSS). There were fewer bacteria in the high CO$_2$ mesocosms at this time point, which was during the peak of the second phytoplankton bloom (Table 1).

One sampling day (15$^{th}$ of May) was counted two times to check if the numbers declined over time after formaldehyde fixation. These samples were counted after 6 and 19 days after fixation with 3.7 % formaldehyde. The results show that there was a significant decrease in total bacterial abundance between the two measurements (p > 0.05, Paired T-test, IBM SPSS Statistics 21; Figure 6).
Table 1. Bacterial abundance data expressed as mean ± standard deviation. The samples were stored for a varying number of days before counted. The data was analysed with an independent samples t-test. * indicates a significant difference.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Bacterial abundance</th>
<th>SD</th>
<th>Nr of storage days</th>
<th>p-value (t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3/4 high CO₂</td>
<td>740402.9 ± 61193.56</td>
<td>26</td>
<td>0.442</td>
<td></td>
</tr>
<tr>
<td>3/4 ambient CO₂</td>
<td>695229.1 ± 93308.76</td>
<td>26</td>
<td>0.442</td>
<td></td>
</tr>
<tr>
<td>11/4 high CO₂</td>
<td>830800.1 ± 33271.94</td>
<td>12</td>
<td>0.181</td>
<td></td>
</tr>
<tr>
<td>11/4 ambient CO₂</td>
<td>667110.1 ± 220687.6</td>
<td>12</td>
<td>0.181</td>
<td></td>
</tr>
<tr>
<td>17/4 high CO₂</td>
<td>1083031 ± 65117.86</td>
<td>6</td>
<td>0.500</td>
<td></td>
</tr>
<tr>
<td>17/4 ambient CO₂</td>
<td>1217508 ± 375280.7</td>
<td>6</td>
<td>0.500</td>
<td></td>
</tr>
<tr>
<td>25/4 high CO₂</td>
<td>1200526 ± 152250.6</td>
<td>7</td>
<td>0.178</td>
<td></td>
</tr>
<tr>
<td>25/4 ambient CO₂</td>
<td>1037510 ± 159503</td>
<td>7</td>
<td>0.178</td>
<td></td>
</tr>
<tr>
<td>1/5 high CO₂</td>
<td>590581.6 * ± 20646.84</td>
<td>5</td>
<td>0.013</td>
<td></td>
</tr>
<tr>
<td>1/5 ambient CO₂</td>
<td>984974.5 * ± 90781.57</td>
<td>5</td>
<td>0.013</td>
<td></td>
</tr>
<tr>
<td>7/5 high CO₂</td>
<td>652568.5 ± 139389.3</td>
<td>6</td>
<td>0.635</td>
<td></td>
</tr>
<tr>
<td>7/5 ambient CO₂</td>
<td>692820.2 ± 212880.8</td>
<td>6</td>
<td>0.635</td>
<td></td>
</tr>
<tr>
<td>15/5 high CO₂</td>
<td>789764.4 ± 103047.2</td>
<td>8</td>
<td>0.953</td>
<td></td>
</tr>
<tr>
<td>15/5 ambient CO₂</td>
<td>796507.7 ± 195563.1</td>
<td>8</td>
<td>0.953</td>
<td></td>
</tr>
</tbody>
</table>

Figure 6. Bacterial abundance (mean ± SD mL\(^{-1}\), n=5) at the 15\(^{th}\) of May, counted after 6 and 19 days of storage in a refrigerator, respectively.
Antibiotic and copper resistance

The results from the antibiotic and copper resistance experiments showed no difference between the two treatments (high CO$_2$ and ambient CO$_2$) and the variance within treatments were high (Figures 7-10). The pCO$_2$ did not have a significant effect on the number of colonies in any of the treatments: Cu 0.5, Cu 1, Cu 2, Am 10, Am 20 (p> 0.05, Repeated Measures ANOVA, IBM SPSS Statistics 21). There was no growth on the ciprofloxacin plates.

The co-resistance experiments showed that all copper resistant isolates were able to grow on plates containing amoxicillin at 10 µg/mL and 20 µg/mL, and that none of the copper resistant isolates were resistant to ciprofloxacin (Table 2). Due to massive growth covering the plates, the number of colonies could not be counted and the data is presented as presence/absence data (Table 2).

Figure 7. Normalized mean number of colonies from the Cu 1 mM plates of the five replicates from each treatment; high CO$_2$ and ambient CO$_2$. Error bars = ±SD.
Figure 8. Normalized mean number of colonies from the Cu 2 mM plates of the five replicates from each treatment; high CO₂ and ambient CO₂. Until April 11th there was no growth at all on Cu 2 mM plates. Error bars = ±SD.

Figure 9. Normalized mean number of colonies from the amoxicillin 10µg/mL plates of the five replicates from each treatment; high CO₂ and ambient CO₂. Error bars = ±SD.
Isolate type | Amoxicillin 10 | Amoxicillin 20 | Ciprofloxacin 10 | Ciprofloxacin 20
---|---|---|---|---
M3 17/4 Cu1 mM | Yes | Yes | No | No
M4 17/4 Cu1 mM | Yes | Yes | No | No
M6 17/4 Cu2 mM | Yes | Yes | No | No
M9 17/4 Cu1 mM | Yes | Yes | No | No
M10 17/4 Cu1 mM | Yes | Yes | No | No

Table 2. Results from the co-resistance experiment. Isolates from different copper plates were spread on plates containing antibiotics, either 10 µg/mL or 20 µg/mL. The number of colonies could not be counted. Because of this, the data is in present/absent form. Where there was bacterial growth present, indicated with “yes”, it was always abundant over the entire plate.

Figure 10. Normalized mean number of colonies from the amoxicillin 20 µg/mL plates of the five replicates from each treatment; high CO₂ and ambient CO₂. Error bars = ±SD.
The results from the bacterial community composition data showed that there was a significant difference between treatments in the community at the peak of the first bloom (11th of April) and at the last sampling date, (15th of May). The 15th of May coincided with the decline of the second spring bloom. In two other mesocosm studies similar results have been found, both Sperling et al., 2013 and Allgaier et al., 2008 could see a change in the community composition due to high pCO2 treatment. These two studies separated the bacterial community in two size fractions: particle associated and free living (Sperling et al., 2013; Allgaier et al., 2008). Sperling et al. 2013 found a change in the particle associated community at the decline of the picophytopankton bloom and Allgaier et al., 2008 noticed a change in the free living bacterial community. It is difficult to assess the implications of such findings without information about which species are changing. A microcosm study found that γ-proteobacteria, a group containing many pathogens, were dominant in the high pCO2 communities (Krause et al., 2012). On the other hand, an OA study conducted in mesocosms did not detect a change in γ-proteobacteria; instead they saw a decrease in Bacteroidetes (Zhang et al., 2013). Zhang et al., 2013 speculate that the decline in Bacteroidetes could affect the carbon pump of the ocean due to less consumption of DOM. It would have been interesting to sequence the bands from the 11th of April and 15th of May in order to get a picture of which species are shifting and see if any conclusions about the impacts on the ecosystem function could be made from these data and to be able to compare my results to the other studies. However, there seem to be a lack of consensus between the different previous studies regarding which species are the most dominant during high CO2 conditions. This is not altogether surprising considering the complex systems that are being evaluated. The initial community composition will surely affect the outcome of the experiment and the initial community in any experiment is dependent on the location and season. The method used to analyse the community composition will also determine to some degree what differences can be deduced.

In this study there was no difference in bacterial species richness between treatments at any time point. Zhang et al., 2013 could see a reduction in species richness due to OA in a mesocosm study conducted in Svalbard, while both Sperling et al., 2013 and Krause et al., 2012 found higher species richness under high pCO2 treatment compared to controls. In this study the OTUs were based on the number of bands on the DGGE gel. The number of bands in each sample was rather low; the maximum was 11 bands in one sample. In previous studies using the DGGE method, the number of OTUs found in water samples is in the range of 11-40 bands (Murray et al., 1996; Kan et al., 2006; Casamayor et al., 2000; Allgaier et al., 2008). The low numbers found in this study could be due to difficulties in detecting the bands or alternatively due to an actual low bacterial diversity. The other studies have used the 16S rRNA gene in their analyses which is a multiple copy gene in contrast to the gene used in this study, rpoB, which is a single copy gene (Murray et al., 1996; Kan et al., 2006; Casamayor et al., 2000; Allgaier et al., 2008; Dahllöf, et al., 2000). This could also be an explanation for the low number of bands in this study in comparison to the previous ones. The relative abundance of bacteria influences which species can be detected by DGGE. Higher concentrations of DNA will give strong bands and less DNA will give weaker bands; at some point the DNA
will not be detected due to scarcity of DNA (Murray et al., 1996). Previous studies have found that the DGGE method will not detect DNA from bacteria that make up less than 0.3 – 1.6% of the entire bacterial community under optimized conditions, and due to this fact, the diversity is probably underestimated when using DGGE to assess biodiversity in bacterial communities (Murray et al., 1996; Casamayor et al., 2000; Kan, 2006). The biodiversity might also be underestimated due to primer bias during the PCR and incomplete separation of the DNA products on the gel (Casamayor et al., 2000; de Araujo and Schneider, 2008). Because of these shortcomings that all lead to an underestimation of the diversity I propose that the community composition and species richness results should be regarded as “the most abundant species” composition and richness. The complete picture will most likely involve more species and the patterns that could be seen in this study might not reflect what is happening to the entire community. Despite these shortcomings, the changes in “the most abundant species” composition could still have an effect on the ecosystem. The most abundant species are probably the most important ones for the ecosystem and a change in the composition of the minor species is probably less detrimental than changes in the dominant bacterial species. However, the functional effect on the ecosystem can only be evaluated if we know which of the most abundant species are shifting and this was not resolved in the present study. An unknown part of the total DNA extracted from the filtered water samples originates from inactive or dead bacteria; this will not be discriminated in the DGGE method. There are methods to screen for the metabolically active bacteria in a sample, such as labelling active cells and counting by flow cytometry (Bernard et al., 2000) or community analysis based on RNA extract instead of DNA (Maas et al., 2013). This parameter would be interesting to measure in future mesocosm experiments under different pCO\textsubscript{2} conditions in order to assess which type of bacteria would be most active in an ocean of pH 7.67.

The method used to assess the total bacterial abundance involved fixing water samples with 4% formaldehyde and storing the fixed samples in the fridge for sometimes up to several weeks. This proved to be a mistake because the number of bacteria in the fixed water sample declines over time (Figure 6). According to a previous study, the number of bacteria will decrease by 55% after only 7 days in 4% formaldehyde and by 65.4% after 25 days in 4% formaldehyde (Pravathi et al., 2011). My data cannot be compared over time because the samples were analysed after a varying amount of days after fixation. Some samples were counted within a few days after fixation and some after several weeks. It is possible, however, to compare between the different treatments at the same time point. There was an effect of high pCO\textsubscript{2} on total bacterial abundance at one time point; first of May (Table 1). The first of May was during the second phytoplankton bloom and the Chlorophyll a data (not shown) from the second bloom indicate that there was a stronger bloom in the mesocosms treated with high pCO\textsubscript{2} compared to the mesocosms treated with ambient pCO\textsubscript{2}. The hypothesis was that the bacterial abundance would increase with higher pCO\textsubscript{2} due to an increase in phytoplankton, which would lead to an increase in TEP that bacteria would attach to and use as a nutrient source. Contrary to the hypothesis, the bacterial abundance data at this time point shows that there are fewer bacteria in the high pCO\textsubscript{2} mesocosms. Allgaier et al., 2008 could not find an effect of ocean acidification on bacterial abundance but found a positive correlation between TEP and bacterial abundance. Another study did see an effect on the
abundance of attached bacteria but not on the free-living bacteria (Grossart et al., 2006). The abundance of attached bacteria was significantly higher in their study (Grossart et al., 2006). The phytoplankton blooms in the mesocosms were highly dominated by diatoms, mainly *Coscinodicus* species. According to Wolter, 1982 bacteria assimilate only 10% of the exudates released by diatoms. When the phytoplankton community was dominated by other phytoplankton, the bacteria assimilated up to 90% of the phytoplankton exudates (Wolter, 1982). This might be an explanation why the bacterial abundance did not increase with increasing pCO$_2$ in the mesocosms, although the exact numbers and composition of phytoplankton is not resolved. More diatoms in the high pCO$_2$ would not necessarily stimulate bacterial growth and abundance. Still, this does not explain why there is a decrease in bacterial abundance at this time point. Usually when there is a sudden decline in bacterial numbers, it is associated with a peak in viral abundance (Grossart et al, 2006), but in this study the viral abundance is not known.

The resistance to amoxicillin increased in at least some of the mesocosms during the decline of the first algal bloom. This is probably due to an increase in bacterial numbers, which could lead to an increase in resistance. Growth on the 2 mM copper plates was seen only after the first bloom and is most likely also a result of higher bacterial numbers. The results from the antibiotic and copper resistance experiments indicated that there was no difference in resistance between ambient and high pCO$_2$ treated mesocosms. This could be due to low concentrations of copper and antibiotics in the mesocosms. In order to get a selection pressure for copper and antibiotic resistance there has to be a sufficiently high concentration. A low concentration of copper is not toxic but rather a necessity to life because copper is an essential metal. The results could also indicate that the bioavailability of the selected compounds did not change with higher pCO$_2$ if the concentrations of copper, ciprofloxacin and amoxicillin indeed were high in the mesocosms. In this experiment the initial concentrations was unknown. The concentration of copper could not be measured due to a malfunctioning graphite furnace of the atomic absorbance spectrometer. No data for the concentration of copper in the Gullmar Fjord could be found for earlier years. The concentration of copper in the marine environment is dependent on factors such as distance to sewage treatment plants (STP), industrial areas and harbours. The mesocosms were placed in the Gullmar Fjord in the vicinity to a small industrial harbour and a small marina. The time when the mesocosms are closed will also affect the amount of copper that is trapped inside them. In this experiment the mesocosms were closed 8$^{th}$ of March which was also the first sampling day. The most likely source of copper pollution in the Gullmar Fjord is antifouling paint and at the 8$^{th}$ of March most people still keep their boats on land. The Swedish Chemicals Agency has found copper concentrations in the sea around Bullandö Marina outside Stockholm, Sweden to be in the range of 10.2 nM – 104 nM (Swedish Chemicals Agency). Copper concentrations between 44.5 µM – 0.41 mM were found in Swedish STP effluents (Lilja et al., 2010). In highly polluted industrial wastewater the concentration of copper in the effluents can reach as high concentrations as 8 mg/L (0.56 M) (Kamika and Momba, 2013). The concentration of copper in the mesocosms that were placed in the Gullmar Fjord is probably in the range found in the sea around the marina or slightly higher because there was a marina in the area but also a
small industrial harbour, but there are no STP effluents or highly polluted wastewater entering the fjord.

The results from the co-resistance experiment revealed that all copper resistant strains that were tested also exhibited resistance against amoxicillin and none of them exhibited resistance against ciprofloxacin (Table 2). This was quite expected considering the results from the other resistance experiment where amoxicillin resistance was fairly common and ciprofloxacin resistance was extremely rare. Because the resistance towards copper was not linked to resistance against both antibiotics it is unlikely that the mechanism is a general one like a broad spectrum multi-drug efflux pump. The isolates that were chosen for the co-resistance experiment probably have two different mechanisms of resistance, one for copper and one for amoxicillin. The most common mechanism for amoxicillin resistance is the production of beta-lactamases (Rang and Dale, 1991) and resistance towards copper can be provided by proteins for copper sequestration and transport (Kamika and Momba, 2013).

The potentially detrimental effects of ocean acidification on the bacterial community, in terms of changes in community composition, abundance and prevalence of resistance to copper and antibiotics, would cause damage to the coastal marine ecosystems. These ecosystems are highly valuable for the human society as a resource for food, raw material and ecosystem services; for example nutrient cycling, providing areas for reproduction and nursery, photosynthesis and opportunities for recreation and tourism (Salomidi et al, 2012). Costanza et al, 1997 estimated the value of ecosystem services of different ecosystems around the globe and they claim that coastal ecosystem services contribute with services worth about 10.6 trillion US$ per year, which is 32% of the value of all ecosystems on earth. The threat to marine coastal ecosystems is not limited to ocean acidification, other aspects of climate change; such as the rise in sea water temperature and increase in UV radiation, combined with over-fishing and pollution all contribute to the detrimental effects that can be seen in many coastal ecosystems (Harley et al., 2006). The effect of the rising average temperature might even be the main factor for changes in community composition (Tatters et al., 2013). More studies combining the multiple factors that pose a threat to marine ecosystems are needed to get better predictions and to assess which species, communities and ecosystems are the most vulnerable. This could help mitigating the adverse effects and direct the prioritization of conservation measures.

Conclusions
Ocean acidification in the mesocosms led to a shift in the most dominant species of the bacterial community. What implications this shift will have on the ecosystem function was not determined in this study since the community composition on the DGGE gels does not reveal which species are changing. The bacterial abundance decreased at one time point in the high pCO₂ mesocosms during the second phytoplankton bloom. The prevalence of amoxicillin, ciprofloxacin and copper resistance was not affected by pCO₂ but the results are difficult to interpret due to the lack of data on the concentration of copper and antibiotics in the mesocosms. In future mesocosm studies investigating the effect on ocean acidification on the bacterial community, it would be interesting to screen for active bacteria and if possible combine effects like temperature and acidification.
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