Spring bloom development of *Vibrio spp.* abundance and activity influenced by ocean acidification; a mesocosm study

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Abstract
Oceanic surface pH is expected to drop by approximately 0.4 units in the coming 100 years. This anthropogenically induced phenomenon, known as ocean acidification, is thought to have a large impact on our oceans. So far, most studies on this subject have concentrated on microcosms or single species, which have given rise to results not necessarily representative for whole ecosystem responses to this stressor. Therefore it is important to further study microbes on an ecosystem level. This study particularly focuses on *Vibrio* bacteria in an integrated ecosystem. *Vibrio* spp. is considered a group of bacteria potentially benefitting from ocean acidification. They are opportunistic and highly resilient to environmental stressors. Some *Vibrio* species are potent human, as well as fish, bivalve and coral pathogens, which could have severe impact on societal economics and health in case large blooms regularly emerge in temperate waters. The aim of this study was to investigate the abundance and culturability of *Vibrios* when exposed to pH levels expected for the 22nd century, with the hypothesis that these bacteria will benefit from decreasing pH. The *Vibrio* were investigated, as a small part, in a large scale mesocosm project carried out on the west coast of Sweden. During the whole spring of 2013 the pelagic ecosystem within each mesocosm was monitored along with adjacent monitoring of the surrounding fjord water. Half of the mesocosms were treated with carbon dioxide to reach pH levels expected for the 22nd century while the other half were left untreated serving as controls. Samples taken from the mesocosms were filtered, culturability on *Vibrio* selective agar-plates were performed and after DNA extraction *Vibrio* abundance were measured using a qPCR assay. Results indicate that the *Vibrio* bacteria are directly unaffected by increased CO$_2$, however, indirectly, e.g. coupled to phytoplankton blooms, they show a strong positive response. Peaks in the phytoplankton spring bloom were quickly followed by a peak in *Vibrio* abundance and culturability. This trend was also observed in the fjord although earlier than in the mesocosms. There is further need to pursue these findings during both cold and warm seasons since increased temperature is, for most species, more favorable to *Vibrio* growth and spring in Sweden naturally is too cold for most *Vibrio* to potentially fully exploit the impact of ocean acidification.

Keywords: Ocean acidification, *Vibrio*, Phytoplankton coupling, Spring bloom, Mesocosm, Cultures, Quantitative PCR, Sweden, BIOACID, KOSMOS 2013.
Table of contents

Abstract ................................................................................................................................................... 1
Table of contents ..................................................................................................................................... 2
Introduction ............................................................................................................................................. 3
Material and Methods ............................................................................................................................. 4
  Experimental system and sampling ....................................................................................................... 4
  Vibrio Culturing ..................................................................................................................................... 6
  Bacterial smears .................................................................................................................................... 7
  DNA extraction and qPCR ....................................................................................................................... 7
  Statistics .............................................................................................................................................. 8
Results ..................................................................................................................................................... 8
  Bacterial identification ............................................................................................................................ 8
  Vibrio culturability ................................................................................................................................. 9
  Vibrio abundance ................................................................................................................................. 11
Discussion .............................................................................................................................................. 13
  Abundance variability ............................................................................................................................ 14
  Phytoplankton coupling ......................................................................................................................... 14
  Temperature .......................................................................................................................................... 15
Conclusion ............................................................................................................................................. 15
Acknowledgments ..................................................................................................................................... 15
References ............................................................................................................................................. 16
Introduction

Today, ocean acidification (OA) is beside global warming one of the major climate issues for modern society. Approximately one third of all anthropogenic CO$_2$ has been absorbed by the oceans during the last 250 years (Sabine et al. 2004), which have led to a global decline in ocean surface pH by 0.1 units (Caldeira & Wicket, 2003). Even though much research has been carried out, and is currently being performed on the subject, there is little consensus on how our oceans will respond to the increasing levels of CO$_2$ (Riebesell et al. 2004, Riebesell et al. 2010, Liu et al. 2010, Joint et al. 2011, Kraus et al. 2012, Sperling et al. 2013, Asplund. 2013). Recent reports suggest that the global ocean pH will drop by approximately 0.4 units in the coming 100 years (Wolf-Gladrow et al. 1999, IPCC. 2007). Considering the logarithmic pH scale this means a four-fold more acidic environment than today for all marine organisms. This decline in pH could lead to a significant decline of biogenetic calcification (Riebesell et al. 2000) and a higher sedimentation of particulate organic carbon (POC), which is then lost from the pelagic environment (Engel et al. 2005). An increasing number of peer-reviews on e.g. Vibrio and marine environments have been conducted since the 1980’s (Asplund 2013). Although, as our oceans change there is further need for pursuing microbes which could pose an increased threat to societal economics and health in temperate waters. In case lowered pH will have a beneficial effect on Vibrio, it will not be the only genus competing for e.g. more available space by TEP (transparent exopolymer particles) formation as a result of lowered pH (Mari. 2008) or nutrients. Therefore, what is important to study is the plankton community response when low pH environments become a common condition for all of the oceans species. This means that previous studies like Labare et al. (2010), Yamada et al. (2010) and Krause et al. (2012) microcosm experiment on isolated bacteria might not be representative for what will actually happen in the mesocosms or entire ecosystems in the ocean.

Vibrio is a large group of bacteria, which naturally occurs worldwide in estuarine and marine environments, where they are either free-living or substrate attached (Thompson et al. 2004, Farmer et al. 2005). Most of them are important decomposers and thus crucial for nutrient recycling in the ocean (Fukami et al. 1985). Some Vibrio species are potent human pathogens, the most notorious being the Vibrio cholera, which causes severe diarrhea (Kaper et al. 1995). Vibrio cholera constitute together with Vibrio parahemolyticus and Vibrio vulnificus, one of the main bacterial groups causing foodborne disease globally (Daniels & Shafaie. 2000, Su & Liu. 2007, Jones & Oliver. 2009). There are also known fish, bivalve and coral pathogens among the Vibrio bacteria, making them a group having an impact on a wide range of marine organisms (Pruzzo et al. 2005b). Many Vibrios are known to readily enter a viable-but-not-cultureable state (VBNC) when environmental conditions become poor e.g. due to nutrient depletion. In this way they can survive in hostile environments and emerge when conditions become more favorable (Colwell et al. 1985, Roszak & Colwell. 1987, McDougald & Kjelleberg. 2006). On the Swedish west coast, low temperature could be one of the stressors triggering the VBNC state. The optimal temperature for most, but far from all, Vibrio is above 17 °C, occurring only during the summer months (Kaneko & Colwell. 1978, Kaspar & Tramplin. 1993, Motes et al. 1998, Jiang & Fu. 2001, Thompson et al. 2004). Coastal sediments are a possible site where dormant Vibrio may reside and again be reintroduced to the water column during storm events giving rise to blooms in favorable seasons (Vanoy et al. 1992, Randa et al. 2004, Oberbeckman et al.
Vibrios are generally extremely adaptable to environmental change (Oberbeckman et al. 2011) and there are some indications that Vibrio can effectively recover from lowered pH exposure or adapt altogether (Labare et al. 2010). They are also extremely opportunistic and many strains have the ability to produce enzymes for using both chitin and agar as carbon sources (Sugano et al. 1994, Riemann & Azam. 2002, Pruzzo et al. 2008). This ability provides Vibrio with the means to readily utilize phytoplankton blooms as well as their grazers as carbon sources.

The aim of this study is to investigate how Vibrios are affected by ocean acidification as a part of the natural plankton community, by measuring culturability and abundance while comparing with biotic and abiotic parameters in a mesocosm environment. This way the whole system will be taken into account.

This study has been part of a large mesocosm study, KOSMOS 2013 (BIOACID) in the Gullmar fjord, Sweden, where numerous parameters (e.g. chlorophyll a, salinity, temperature and bacterial protein production) were measured simultaneously. These parameters were shared internally to give a more complete picture of what actually happened in the community inside the mesocosms.

The null-hypothesis of this study was that there will be no change in the abundance and culturability of Vibrio bacteria as a result of ocean acidification. The hypothesis tested is thus that Vibrio abundance and culturability will benefit from rising CO₂ levels in the ocean.

**Material and Methods**

*Experimental system and sampling*

All exposures and samplings took place in mesocosms in the Gullmar fjord (N 58° 15.901', E 11° 28.567') (Fig. 1), which were deployed on the 21/01/13 and taken up again on the 28/6/13. The mesocosms consisted of 17 m long plastic bags (4 m diameter), sealed with a sedimentation trap in the bottom, each held stable with sturdy plastic constructions, which in turn connected all the mesocosms together as well as anchored them to the bottom with ropes and weights. In this way the mesocosms stayed in place in the fjord. The mesocosms were sealed on the 12/02/13 enclosing 55 m³ of seawater. The openings of the mesocosms were held above the water surface allowing easy sampling. Further, each mesocosm was equipped with a small roof to prevent possible dilution or pollution. After sealing the mesocosms CO₂ was added in 3 steps, over 3 days, as to reduce the shock on the plankton community. This was done by adding acidified seawater to each designated mesocosm to reach the intended pCO₂ level of approximately 1000 ppm. Mesocosms 2, 4, 6, 7, and 8, were OA treated while mesocosms 1, 3, 5, 9 and 10 were controls with an ambient pCO₂ level. After a hard-reset of the mesocosm systems, because of persistent unfavorable fjord conditions, CO₂ was re-added on the 7/3/13.
Figure 1. The red cross marks the position of the mesocosms in the Gullmar fjord, Sweden (N 58° 15.901', E 11° 28.567'). A 5 min boat ride from the marine research station SLC-Kristineberg (Map from https://maps.google.se).

Sampling of mesocosms (Fig. 2) was conducted using an IWS integrating water sampler (Hydro-Bios, DE). The sampler recorded the depth as it was slowly lowered down to 17 m in each mesocosm using an attached wire. When registering a certain pre-programed depth the sampler retracted the bottom plastic disc connected to a piston, letting water pour in and rendering the piston fully retracted and the sampler full at 17 m of depth. This gave an integrated water sample from the whole water column down to the predetermined depth. This sampling was performed twice a week from the 8/3/13 to the 15/5/13, acquiring approximately 1 liter of sample water from each mesocosm during each sampling. The reason for this sampling interval was to avoid overlapping of *Vibrio* culture incubation for the sampling days.
Sample water from each mesocosm was brought into the lab as soon as possible, and stored cold and dark in designated plastic bottles, one for each mesocosm as well as a fjord reference sample, awaiting filtration. Three hundred ml of sample water was filtered through 0.2 µm membrane filters (Whatman Cyclopore, UK) to catch everything down to free-living bacteria. Larger phytoplankton and zooplankton would also be trapped on the filter which is essential since they are possible attachment sites for bacteria. Thus, the whole *Vibrio* bacteria community was studied. The filtering volume was decided after testing bacterial growth on TCBS-agar (HiMedia Laboratories, India) plates with a series of fjord water volumes (50, 100 and 300 ml). After filtration, filters were placed in 2 ml tubes. The one filter used for bacterial culturing on TCBS-agar plates was immersed in 1 ml ultra filtered seawater buffered to the respective mesocosm pH. The two remaining filters were covered with RNA later (Ambion, UK) and stored frozen (-18 °C) until DNA extraction.

**Vibrio Culturing**

Bacteria and plankton were initially detached from the filters by vortexing the sample tubes for approximately 60 seconds. From the solution 100 µl was taken and spread on TCBS (Thiosulfate citrate bile salts sucrose)-agar plates, in duplicates (A and B plates). The finished plates were incubated in darkness at the temperature best mimicking the temperature in the fjord, i.e. in the beginning at 6 °C and then 10 °C and 15 °C respectively. In the beginning of the project the plates were ready for analysis after approximately a week. At the end of the project, however, when the temperature in the fjord had more than doubled, this time was reduced to approximately 3 days.

After incubation, colonies appearing on both agar plates (A and B) from each mesocosm as well as from the fjord were counted and colonies were characterized as either small or large. All colonies which had an identifiable color were recorded as large and the rest small. Additionally, the color for each large colony was noted as well as the total incubation time for the plates. From the A plates a maximum of six colonies were isolated in cryotubes filled with one ml of growth medium containing meat peptone (Sigma-Aldrich, USA), yeast extract (Sigma-Aldrich, USA) and seawater with the
appropriate pH (8.1 for untreated and 7.7 for OA treated mesocosm samples). Prior to isolation, the colony was identified using an oxidase reagent indicative of *Vibrio* bacteria. However, API-tests (as described further down) suggested that *Vibrio fluvialis*, a common *Vibrio* bacterium in our tests, was in fact oxidase negative. Additionally, API-tests (bioMérieux, SA) were conducted on 3 to 4 remaining colonies on the A plates. Since other bacteria than *Vibrio* (e.g. *Aeromonas* and *Pseudomonas*) also may grow on TCBS-agar (although not as efficient as the *Vibrio* which usually grow larger colonies), the API test was useful to get an indication of the growing bacteria identity. Results of the API-tests give number codes which are compared in a database (APIweb 1.2.1) online and presented a probability of bacterial species.

After incubating the isolates from the A-plates, glycerol was added to preserve them stored in a -80 °C freezer for later MALDI-TOF (Matrix Assisted Laser Desorption/Ionization-Time of Flight) detection of peptides produced by the isolated bacteria using a protocol and database distinguishing different *Vibrio* bacteria and potential pathogens (Oberbeckmann et al 2011). This last incubation varied a lot in time between every individual tube and some never grew at all.

**Bacterial smears**

The B plates were used exclusively for obtaining bacterial smears for DNA analysis. All colonies on each plate were swiped up using a sterile inoculation loop, put into a tube with sterile filtered milliQ water, for (10 min), centrifuged (5 min, 22 °C, 16.0 RCF) and the supernatant transferred to another set of tubes for storage in a freezer (-18 °C). These samples were backups with bacterial DNA in case the isolates or DNA filters failed to give any results.

**DNA extraction and qPCR**

DNA was extracted from filtered samples in order to perform qPCR to determine the amount of *Vibrio* bacteria present. After thawing the filters, RNA later (Ambion) was transferred to new 2 ml tubes (Eppendorf) and the filters cut in tiny pieces. The RNA later was centrifuged (16.1 RCF, 21 °C, 3 min) and the supernatant removed. Next, 0.9 ml of extraction buffer and 20 µl proteinas-K was added to the tubes with the pellet and the whole content was, after vortexing (3x15 sec), transferred to the tubes with the cut filters. The filled tubes were vortexed (3x15 sec) before being placed in a heating block (56 °C) for 60 min. After heating, big and small silica beads and PVPP was added to each tube together with 300 µl of chloroform:isoamylalcohol (24:1) and then “bead-beaten” in a Tissue Lyser II (Qiagen) (frequency 30 for 30 sec). The tubes were then centrifuged again (15.0 RCF, 21 °C, 3 min) and the supernatant transferred to new tubes. 10 % of the volume, with NaAC (3M) and at least 60 % of the volume with isopropanol was added and the samples were gently mixed. The samples were then put away to settle in darkness in the refrigerator overnight. After settling the tubes were centrifuged (15.0 RCF, 15 °C, 30 min) and the supernatant discarded. Next, 1 ml of ice cold ethanol (70-96 %) was added to the pellet and vortexed briefly. Once again the tubes were centrifuged (14.0 RCF, 4 °C, 10 min) and the supernatant discarded. The samples were then put on a heating block (45 °C) until dry. Lastly, 25 µl of sterile filtered water was added, the tubes vortexed briefly and centrifuged one last time (14 RCF, 4 °C, 1 min). DNA was quantified and quality controlled using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA), thus giving a value of DNA concentration (ng/µl) and purity, as well as by the use of a 1.2 % agarose gel electrophoresis. The purity value from the spectrophotometer was used for determining how successful the DNA extraction was and if the sample was viable for further processing. The agarose-gels visualized the approximate length of DNA and whether we had one clear band or if the DNA was fragmented,
producing a smear on the gel. Total abundance of *Vibrio* spp. was measured using a SYBRgreen quantitative PCR (Polymerase Chain Reaction) assay targeting the 16S rDNA gene (Thompson et al. 2004) modified by Asplund et al (2011). Samples were run in triplicates together with a standard dilution series of DNA extract from a *V. parahaemolyticus* (CCUG 43364) culture and non-template controls. *Vibrio* cells per liter of seawater were estimated from the standard curve as *V. parahaemolyticus* cell equivalents, based on the fact that they contain 11 16S rDNA copies, using the formula provided by the real-Time PCR supplier (Applied Biosystems, USA). Outliers among the triplicates were discarded.

**Statistics**

All bacterial data were log10 transformed prior to statistical analysis. The data were tested using one-way ANOVAs to discern effects of treatment at each sampling date. All statistical analyses were made using the software IBM SPSS 20.

**Results**

The *Vibrio* abundance and culturability measurements gave the same general trends for both OA treated mesocosms and controls. Fjord samples also displayed the same patterns in *Vibrio* abundance and culturability, although the mesocosms were delayed in time. Generally, there was a high variation in total number of TCBS-cultured bacteria (Fig. 3-5) and in total abundance of *Vibrio* (generated from the real-Time PCR assay, Fig. 6-8) among the mesocosms independent of treatment, and sometimes even among replicates. This is easily discernable by the large error bars (Fig. 2 and 5). However, variation was greater among the control samples of the experiment than of the OA treated ones (Fig. 4 and 7). Fjord samples of cultures and qPCR (Fig. 5 and 8) are presented mainly for comparison of community response between the artificial mesocosm system and the natural fjord.

By use of external data, shared within the KOSMOS 2013 team, approximate dates of the spring bloom could be determined and used for identifying possible dynamics between phytoplankton blooms and the abundance and culturability of *Vibrio*.

**Bacterial identification**

A number of API-tests were conducted but only 21 tests were comparable since the remaining 14 tests were conducted on plates with different incubation temperatures. The results of these tests are presented (Table 1) with the most common bacteria and the highest probability for each of them and in what samples they occurred. *Vibrio fluvialis* was the most common bacterium on the TCBS-agar plates followed by *Aeromonas* and *Pseudomonas* species as well as *Chromobacterium violaceum*. 
Table 1. The most commonly identified species or genesis found in the samples using an API-test (n=21). Samples were distributed among OA treated samples (10), control samples (9) and fjord samples (2).

<table>
<thead>
<tr>
<th>Species</th>
<th>Highest Probability (%)</th>
<th>No. In OA samples</th>
<th>No. In Control samples</th>
<th>No. In Fjord samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vibrio fluvialis</td>
<td>98.3</td>
<td>5</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Aeromonas salmonicida ssp. salmonicida</td>
<td>95.5</td>
<td>1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Chromobacterium violaceum</td>
<td>81.3</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas oryzihabitans</td>
<td>81.1</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Aeromonas hydrophila/caviae/sobria 2</td>
<td>81.0</td>
<td>4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Stenotrophomonas maltophilia</td>
<td>80.4</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>77.5</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Non-fermenter spp</td>
<td>62.4</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Myroides spp/Chryseobacterium indolgenes</td>
<td>42</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Ochrobactrum anthropl</td>
<td>25.6</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Bordetella/Alcaligenes/Moraxella spp</td>
<td>24.4</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Aeromonas hydrophila/caviae/sobria 1</td>
<td>16.6</td>
<td>5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas fluorescens/putida</td>
<td>15.6</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Burkholderia cepacia</td>
<td>11.1</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

**Vibrio culturability**

The amount of colonies and the variation both among treatments and sampling dates was initially comparably small (Fig. 3). After the spring bloom peaked, the bacterial culturability increased in most mesocosms in particular mesocosms 1, 2, 4, 6, 7, and 10. Most of these mesocosms (i.e. 2, 4, 6 and 7) are OA treated. However, mesocosm 10 obscures the picture by consistently containing higher bacterial numbers than the other mesocosms. This makes the picture even more complicated when comparing averages between OA treatment and control. This is most notable during the last sampling date (May 15th) when comparing averages of the OA treatment and control (Fig. 4). The same is true for sampling dates April 25th and May 7th for mesocosms 1 and 7 respectively.

In spite of these single mesocosm deviations both OA treated and control mesocosm bacteria follow the same pattern. However, the variation of control mesocosms is consistently larger than that of OA treated mesocosms. There was a significant difference among treatments for *Vibrio*-like bacteria culturability on April 11th, however, treatments did not differ from each other on the other dates (Table 2).
Figure 4. Average number of colonies, small and large (grown on TCBS-agar), of OA treated and control mesocosms (n=5) at every sampling day of the experiment. The error bars are standard deviations. Number of colonies is equivalent to number of culturable *Vibrio*-like bacteria.

The fjord samples which were acquired during mesocosm sampling showed the same general trends as the mesocosms in terms of spring bloom response (Fig. 5) but varied more among sampling days.

Figure 5. Total number of colonies, small and large (grown on TCBS-agar), of both replicates from the Gullmar fjord at every sampling day during the experiment. Number of colonies is equivalent to number of active *Vibrio*-like bacteria.
Table 2. One-way ANOVA for Vibrio-like bacteria culturability between OA treated mesocosms and controls (n=5).

<table>
<thead>
<tr>
<th>Date</th>
<th>One-Way ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>March 14th</td>
<td>$F_{1,8} = 0.483$, $p = 0.507$</td>
</tr>
<tr>
<td>March 28th</td>
<td>$F_{1,8} = 2.282$, $p = 0.169$</td>
</tr>
<tr>
<td>April 11th</td>
<td>$F_{1,8} = 7.331$, $p = 0.027$</td>
</tr>
<tr>
<td>April 17th</td>
<td>$F_{1,8} = 1.374$, $p = 0.275$</td>
</tr>
<tr>
<td>April 25th</td>
<td>$F_{1,8} = 2.868$, $p = 0.129$</td>
</tr>
<tr>
<td>May 1st</td>
<td>$F_{1,8} = 0.756$, $p = 0.410$</td>
</tr>
<tr>
<td>May 15th</td>
<td>$F_{1,8} = 0.160$, $p = 0.699$</td>
</tr>
</tbody>
</table>

**Vibrio abundance**

The total number of Vibrio cells per liter of sea water was very low in all mesocosms throughout the whole experiment, but there are two easily discernable peaks (Fig. 6). During most of the sampling dates Vibrio cells per liter of sea water did not exceed 2000 and often ended up below 1000. The first peak right before the spring bloom starts is not uniform for all mesocosms and not limited to either OA treated or control. The second peak, right after the spring bloom, however, affects all mesocosms approximately equal. The one exception is mesocosm 10 which had Vibrio abundance more than double the second most abundant, ending up on a still low value of 21000 Vibrio cells per liter of sea water.

![Figure 6. Number of Vibrio cells per liter of sea water for each mesocosm OA treated and control. Each data point is an average of the real-Time PCR triplicates.](image)

When presenting the data grouped into treated and untreated mesocosms (Fig. 7), the abundance is almost identical on most sampling days. However, the variation is larger in the control group. During the peak right after the spring bloom, data suggest that control mesocosms have a higher abundance...
on average, but most of this is obviously due to mesocosm 10. When the temperature rises during
the last sampling day (15\textsuperscript{th} of May) the trend is reversed and OA treated mesocosms have a higher
abundance of \textit{Vibrio} without a possible outlier. There were no significant differences in \textit{Vibrio}
abundance between OA treated and control mesocosms for all sampling dates analyzed (Table 3).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure7.png}
\caption{Average values for \textit{Vibrio} cells per liter of sea water divided into OA treated and control mesocosms (n=5). The error bars are standard deviations.}
\end{figure}

The fjord samples had a very low amount of \textit{Vibrio} cells per liter of sea water even though the spring
bloom response, calculated as a percentage, was high and easily detectable (Fig. 8). The abundance
was mostly below 1500 \textit{Vibrio} cells per liter of sea water. A fairly large increase was noted at the end
of the experiment but still did not reach above 5000 \textit{Vibrio} cells per liter of sea water.
Figure 8. The fjord samples, which were taken parallel to the mesocosm samples, where each data point is an average of the qPCR triplicates.

Table 3. One-way ANOVA for *Vibrio* abundance between OA treated and control mesocosms (n=5).

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**Discussion**

Mesocosms, despite all their technical advantages and well monitored biotic and abiotic factors, are still closed artificial systems that over time differs from the actual environment that they are sought to mimic. However, this is still the best indication of natural ecosystem response to OA which is achievable with modern day technology and knowledge, and the general trend of interaction was observed in both the mesocosms and the fjord. Further, other mesocosm experiments recently conducted like Allgaier et al. (2008) and Newbold et al. (2012) did observe similar plankton communities within the mesocosms compared to natural local and global ecosystems. When it comes to OA we already know that many coastal and ocean habitats naturally experience large
fluctuations and seasonal variations in pH. Thus, all species living in these environments have already adapted to these changes and in some cases they are more extreme than what is estimated for the 22nd century (Joint et al. 2011).

In total this mesocosm project indicates that Vibrio bacteria will not be adversely affected by ocean acidification, either positively or negatively, on the west coast of Sweden. The hypothesis that Vibrio bacteria, with their naturally strong resistance to environmental changes as shown in microcosm experiments in lab environments (Labare et al. 2010), will benefit from an increased CO$_2$ concentration is rejected. However, this is only true for the time of year that this study was conducted and at the very end of the project there was an indication of an interaction between temperature and increased pCO$_2$ leading to higher average numbers of Vibrio in the OA treated mesocosms (Fig. 7). Further, the result covers the total amount of Vibrio bacteria regardless of particle attachment or free-living strategies. A study conducted by Eiler & Bertilsson (2006), geographically close to where this study was made, suggests that the particle attached Vibrio bacteria only constitute 10% of the total abundance. This might mean that the results can be applied on free-living bacteria in addition to the total picture. Further, Sperling et al. (2012) showed that OA influenced particle attached marine bacteria to a larger extent than free-living.

**Abundance variability**

There was a smaller difference in variability within OA treated samples compared to control samples concerning both the Vibrio abundance and their culturability. This is an indication that even though the total Vibrio abundance and culturability were largely unaffected by increased CO$_2$ concentration, OA is still a stressor that may lower the diversity, leaving the community open for the domination of a few exceptionally durable and perhaps opportunistic Vibrio species or strains. This is true for e.g. temperature as shown by Eiler et al. (2006) and for OA when looking at total bacterial community as shown by Sperling et al. (2012) and Allgaier et al. (2008). Furthermore, there is a chance that we get increased domination of species or strains that are potentially dangerous to human, fish, crustaceans and bivalves alike, since they are the most durable and could reach a higher abundance when the community structure shifts. This could explain why some isolated colonies had grown enough after one day while others needed a week, and why some isolates never grew, which in the end meant they had to be discarded. To confirm this, a community structure analysis would have had to be made. Within the mesocosm project such an analysis, in the form of a DGGE and MALDI-TOF, will be performed, but unfortunately the DGGE was initially unsuccessful and the MALDI-TOF had to be postponed.

**Phytoplankton coupling**

Results from this study suggest a strong interaction between the bloom stages of phytoplankton and the abundance of Vibrio. As seen for all mesocosms, both OA treated and controls, as well as for the fjord samples (Fig. 6-8), the Vibrio bacteria drastically increased in abundance and culturability after each peak of the bloom. This is the times of nutrient richness for the bacteria, since dead or dying phytoplankton is an excellent and readily available source of carbon and nitrogen. This finding is in line with previous observations made by Asplund et al. (2011) and Rehnstam-Holm et al. (2010) in tropical regions and also in temperate and boreal regions as shown by Eiler et al. (2006) and Asplund (2013). Since some species of Vibrio possess the ability to produce enzymes for dissolving chitin and agarose alike (Pruzzo et al. 2008, Riemann & Azam. 2002, Sugano et al. 1994), an increase in size and magnitude as well as frequency of phytoplankton blooms due to ocean acidification could be an
indirect positive effect for the Vibrio bacteria, and marine bacteria in general (Allgaier et al. 2008, Newbold et al. 2012), that will cause increasing problems in temperate and boreal regions.

**Temperature**

For all but a few samples there were large increases in Vibrio abundance and culturability during the final two sampling days. However, the increase was more profound in the OA treated mesocosms. These two days saw in total more than a double increase in the fjord surface water temperature and were currently at approximately 14 °C at the time of closure for my project. Since Vibrio bacteria are known to have a temperature optimum above 17 °C (Thompson. 2004), there is a possibility that the suggested benefits from increased CO₂ concentrations will be played out when the bacteria grow at their best. Focusing on the Vibrio abundance, it was on average 10 %, and the sample with highest abundance approximately 66 % of the abundance observed by Eiler et al. (2006) in Skagerrak during the summer months. It has been shown by Burnham et al. (2009) and Nishina et al. (2004) that certain strains of Vibrio grow significantly faster with increasing temperature. This makes tropical and subtropical regions more vulnerable to Vibrio epidemics. Furthermore, this couples ocean acidification effects with global warming since increasing temperatures will more readily, more often and for a prolonged period of time render temperate and boreal regions within the temperature range of optimum Vibrio bacteria growth. This is, however, not true for all Vibrio (Popovic et al. 2007), and this is therefore in great need of further investigation. To be able to confirm this, a project would have to span across the summer months in addition to spring.

**Conclusion**

It is important to keep studying the delicate interactions within mesocosm systems even though they might not develop entirely along the lines of a natural ecosystem. As far as the results of this study are concerned, the same patterns could be identified in the natural environment and the mesocosms for the Vibrio bacteria. This strengthens the credibility of the methods and suggests that Vibrio bacteria abundance, as a whole, will not be directly affected by ocean acidification levels in the upcoming 100 years. Therefore, it is important to keep investigating possible community interactions and indirect effects of OA, preferably in CO₂ gradients since it is a common environment for marine bacteria.

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