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\"Bottom up\" ocean acidification: A study on the effects of CO2 on the bacterial community in sediments

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Environmental Toxicology and Chemistry
Submission date: May 2011
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ACKNOWLEDGEMENTS

This project is collaboration between NTNU, SINTEF and Statoil. Thank you to all of those who have been involved in this project.

A special thanks to my supervisor, Murat Van Ardelan, at department of Chemistry, NTNU, for always being available and helpful. I appreciate all knowledge and encouragement you have given me during this process. Thank you for giving me the chance to participate in such an exciting project. Also I want to thank my supervisor Bjørn Munro Jenssen, NTNU department of Biology, for being very helpful.

A special thanks to Ingrid Bakke, NTNU department of Biotechnology, whose knowledge and help has been invaluable during the analysis of my samples, and for always being available to help and being cheerful in doing so.

Also Lasse Olsen knowledge in statistics and the help I received with this was most appreciated.

Thanks to Trond Nordtug and Anders Johny Olsen for helping with everything practical during the experiments and being alert.

Also I want to give a big thanks to my partners Gøril Aasen Slinde and Kathrine Helen Sundeng. Your company and cooperation has been essential for me. Thank you for shearing and listening to all annoyances and troubles, and also shearing the success and joy these past two years has brought.

Finally a personal thanks to my family. My parents and my sisters has been a massive support during all of my years as a student, thank you for always encouraging me. Also I want to thank my boyfriend for motivating and being a great listener.

SAMMENDRAG

Etter hvert som konsentrasjonen av CO_2 i atmosfæren fortsetter å øke har alternativer i hvordan dempe og redusere hastigheten av denne utviklingen fått mye oppmerksomhet. CCS gjør akkurat dette ved å lagre CO_2 som til vanlig ville blitt avgitt til atmosfæren. Ved å lagre CO_2 i geologiske lagrer, isoleres det for en lengre tidsperiode på tusener av år. Selv om denne type lagring vurderes som trygg og risikoen for lekkasjer er liten, kan en aldri være helt sikker på at det holder. Risikoen for store lekkasjer er svært små, men risikoen for relativt små lekkasjer er noe usikkert. Dersom en slik liten lekkasje skulle forekomme, hva er konsekvensene og hvordan kan den detekteres? Dette er vanskelige spørsmål å svare på, men der er et stort behov for å kunne besvare dem. Internasjonale retningslinjer (London protokollen og OSPAR) er laget nettopp for å besvare denne type spørsmål, og etter hvert må de også kunne følges opp. Det langsiktige målet med dette prosjektet er å utvikle overvåknings- og deteksjonsmetoder for denne type små lekkasjer og vurdere konsekvensene dette har for miljøet.

Det marine økosystemet er ekstremt viktig så alle biologiske konsekvenser av lekkasje er viktig å studere. Bakterier utgjør grunnlaget for et godt fungerende økosystem og med sine mange oppgaver er de en viktig del av dette økosystemet. Dette studiet undersøker hvordan CO_2 lekkasje gjennom sedimentet påvirker det bakterielle samfunnet i sediment.
Ved å studere det bakterielle samfunnet i sediment og om det responderer til en CO_2 lekkasje kan et mer realistisk anslag om hvordan naturlige system reagerer, og indirekte eller direkte konsekvenser av dette på andre aspekter av økosystemet, anslås.

Det eksperimentelle oppsetter er designet for å være så likt sjøbunnen som mulig. Titanium tanken fungerer som et akvatisk mesocosm der temperatur, lys, trykk, naturlige sediment og kontinuerlig tilførsel av sjøvann bidrar til en realistisk imitasjon av det naturlige miljøet til det bakterielle samfunnet. To eksperiment ble utført ved å injisere CO_2 inn til systemet via sedimentene. Det første eksperimentet varte to uker, det andre i en måned. Effektene av CO_2 på det bakterielle samfunnet ble testet ved å bruke metoden PCR-DGGE. Metoden gir et overblikk over de mest dominerende bakteriepopulasjonene som samfunnet er bygd opp av. Derfor brukes metoden til å undersøke hvordan bakteriesamfunnet i prøver, i dette tilfellet seidmentprøver, responderer ved å detektere endringer i samfunnsstrukturen. Kun eksperiment 2 var en suksess. Resultatene fra dette eksperimentet viser at det bakterielle samfunnet i det øverste sedimentlaget ikke endres selv etter en måned med CO_2 behandling.

Dette var ikke tilfelle for det bakterielle samfunnet i dypere sedimentlag, dvs. lagene (2-9 cm) under toppsedimentet, som ble signifikant endret som en konsekvens av CO_2 behandlingen.

Om denne endringen i samfunnsstruktur er et resultat av CO_2, pH eller endring i sedimentets kjemi, spesielt med tanke på metall mobilitet og løselighet, er diskutert. Der er likevel ingen måte å separere effektene av disse fra hverandre med denne type eksperimentelt oppsett. De observerte effektene på det bakterielle samfunnet er en konsekvens av forholdene i tanken som helhet og ikke kun CO_2, pH eller økt metallkonsentrasjoner.
Mer forskning trengs før en vet hva effektene av CO_2 lekkasje er, men det er forslått mange forbedringer for hvordan gå frem med dette. Ved å gjøre disse eksperimentene vet en nå mer om responsen til ett naturlig bakteriesamfunn i sediment når det står ovenfor en CO_2 lekkasje.

ABSTRACT

As atmospheric concentration of CO_2 continues to increase, alternatives on how to mitigate and reduce the rate of this development has received much attention. Carbon Capture and Storage (CCS) is doing just this by storing CO_2 that ordinarily would have been emitted into the atmosphere. By storing the CO_2 in geological storages it is isolated for a long period of time, thousands of years. Even though this type of storage is considered safe and the risk of leakage small, one can never be absolutely sure of it holding. The risk of large leakages is considered negligible, but the risk of relative small leakages is uncertain. If such a small leakage were to occur, what are the consequences and how such a leakage could be detected? These are difficult questions to answer, but the need to be able eventually answer them is important, especially considering that international guidelines (London protocol and OSPAR) has been developed so that these questions can be answered, and they eventually need to be followed. The long term aims of this project are to developing monitoring and detection methods for small leakages and assess the environmental impacts of this type of leakage.

Biological impacts are important to study since the marine ecosystem is extremely important. Bacteria are an important part of this ecosystem, and have many important tasks and constitute the foundation of a well functioning ecosystem. This study investigates the influence of increased CO_2 concentration, as a result of CO_2 leakage through sediments, on bacterial community structure in sediments. By studying the bacterial community in sediments and if it responds to a CO_2 leakage a more realistic assumption on how natural system might react is acquired, and if consequences are observed here, then it might indirectly or directly affect other aspects of the ecosystem.

The experimental setup is designed to be as genuinely similar to the seafloor as possible. The titanium tank functions as an aquatic mesocosm where temperature, light, pressure, continuous supply of seawater, natural sediments contributes to a realistic imitation of the natural environment to the bacterial community. Two experiments were performed by injecting CO_2 into the system through the sediment. The first experiment lasted two weeks, the second a month. The effects of CO_2 on the bacterial community were tested by using the method PCR-DGGE. The method gives an overview of the most dominant bacterial populations that the community is constituted of. Therefore it's used to establish how the bacteria community in samples, in this case sediment samples, responds by detecting changes in community structure.

Only experiment 2 was a success. Results from this experiment show that the bacterial community structure in the topmost layer in sediments is resisting changes even after a month with CO_2 treatment. This was not the case for the bacterial communities in deeper sediment layers, meaning layers beneath (2-9 cm) of the top sediment, which was significantly changed due to CO_2 treatment.

Whether this change in community structure is a result of CO_2 itself, pH or a result of CO_2 changing the chemistry in sediments, especially metal mobility and solubility, is discussed. However there is no way to separate these two effects with this type of experimental setup, the observe effects on the bacterial community are a consequence of the conditions in the tank as a whole and not only CO_2 or increased metal concentrations.
More research is needed before one knows what the effects of leakages are, but many improvements on how to proceed with this have been suggested. By doing these experiments some basic knowledge of how a natural bacterial community in sediments might react when faced with a CO_2 leakage is obtained.

ABBREVATIONS

ANOSIM – analysis of similarities
APS – ammonium persulfate
CCS – Carbon Capture and Storage
CO_2 – Carbon Dioxide
DGGE – Denaturing Gradient Gel Electrophoresis
DGT – diffusive gradients in thin-films
DNA – deoxyribonucleic acid
FISH – fluorescent *in situ* hybridization
NMDS – Non-metric Multidimensional Scaling
PCR – polymerase chain reaction
RDP – ribosomal database project
rDNA – ribosomal DNA
rRNA – ribosomal ribonucleic acid
TAE – tris base, acetic acid and EDTA
TEMED – tetramethylethylenediamine

Sediment sample abbreviations

$$X_1\text{-}X_2\text{-}X_3$$

X_1 = Sediment layer
B = bottom layer
SB = second from bottom layer
ST = second from top layer
T = top layer

X_2 = Treatment
B = before CO_2 exposure
A = after CO_2 exposure
C = after "control" samples
N = samples taken from natural environment

X_3 = sediment chamber*
D = direct CO_2chamber
TD = top-down chamber

*X_3 samples are A samples, taken from the two sediment chambers after CO_2 experiment was finished. More details see 2.1.2 and 2.1.3

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1. INTRODUCTION

1.1 Background

1.1.1 Anthropological CO_2

Global warming, ocean acidification, greenhouse gasses and pollution are words that most people have become familiar with. In the centre of it all we find carbon dioxide (CO_2). Concentration of atmospheric CO_2 is increasing and has been for some time. In 1958 Charles David Keeling and his colleagues started continual measurements of atmospheric carbon dioxide concentration in Mauna Loa Observatory in Hawaii [Tans 2010]. The results, known as the Keeling Curve (Figure 1.1), clearly confirms the increasing trend, and has become an important evidence when arguing for that anthropogenic emissions of CO_2 are an important cause for this observed trend. Petit reported in 1999 that the current CO_2 levels was the highest recorded the past 420 000 years (Figure 1.2) [Petit et al. 1999]. In 2005 CO_2 measurements from air extracted from ice core samples at Dom Concordia in Antarctica revealed that the CO_2 concentration the past 600 000 years did not exceed 300 µatm [Siegenthaler et al. 2005]. As of December 2010 the atmospheric concentration of CO_2 measures 389 µatm [Tans 2010]. Whether this trend continues or halts in near or distant future and what the consequences are, is a topic not only confined to the scientific community. It seems like everyone, scientists, politicians and regular commoners, is engaged in this discussion. Though a difficult problem to solve, there

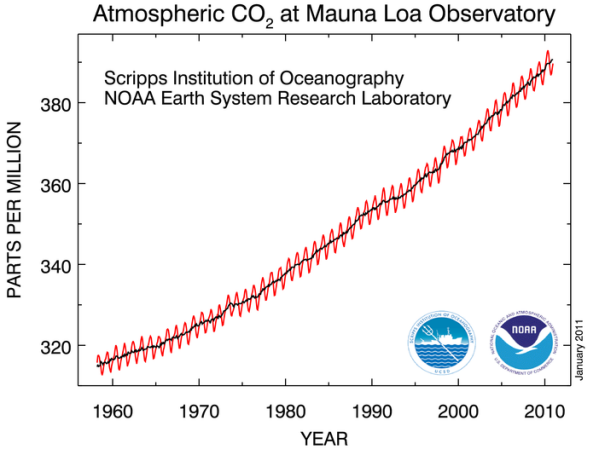

Figure 1.1: **The Keeling Curve** shows how the concentration of atmospheric CO_2 ppm (parts per million) or µatm has increased the past 50 years. CO_2 is measured directly from the atmosphere. [Tans 2010]

Figure 1.2: **Long-term CO_2 concentration** ppm or µatm the past 600 000 years [NASA 2011].

seems to be no lack of suggestions on how to “fix it”. No single action can solve the whole problem, but some measurements have been taken to try to reverse this trend. One of them is known as carbon capture and storage (CCS).

1.1.2 Carbon Capture and Storage (CCS) and leakages

CCS is a process where CO_2 is captured from a CO_2 source, mostly from large point sources, separated from other gasses and stored. When stored, the CO_2 is prevented from reaching the atmosphere. There are different types of storages but the most relevant in Norway is geological storages, as old oil fields etc. CO_2 injection in the Utsira Sand at Sleipner in Norway, by Statoil ant its partners, started in 1996 and it was the first large-scale project of its kind [IPCC 2005]. London Protocol, a global agreement regulating dumping of wastes at sea, and OSPAR, the convention protecting the marine environment in the North East Atlantic, has produced certain guidelines which has implications for CCS. Included among the guidelines is monitoring. This monitoring does not only include leakages, but also monitoring of ecosystem and chemical processes. Developing site-specific monitoring techniques are one of the requirements for CCS activity to continue [Dixon et al. 2009].

The benefits of CCS are many, especially keeping CO_2 from reaching the atmosphere, but one must keep in mind that eventually the CO_2 migrates out of these formations. How long the CO_2 is kept in these storages depends on a number of factors, especially the topography of the top rock is important. Research tells that the storages will hold the CO_2 captured for thousands or even hundreds of thousands years [Lindeberg et al. 2003]. CO_2 will eventually migrate out through molecular diffusion, a slow process, and when reaching the ocean it can take hundreds of years before reaching the atmosphere. All in all these storages are considered safe, but still, these are no guarantees against leakages. Leakages can happen in two ways: abrupt and gradual [IPCC 2005]. Geological storages are continually monitored, so any big abrupt leakages will quickly be discovered and fixed. It is the small gradual leakages that are difficult to discover. If not discovered, these leakages might lead to a chronic release of CO_2. What the consequences of these leakages are is largely unknown. Constant input of CO_2 will first of all lead to a local acidification. How this will affect the living organisms in this environment is uncertain and dependent on the environment around the storage. This master thesis focuses on the bacterial community in sediment and of its composition is changed by CO_2. In the sediments a complex community of bacteria lays the foundation of the benthic ecosystem. When CO_2 is added continually it is reasonable to believe that this could cause a change in the bacterial community.

1.2 Bacteria

1.2.1 Bacteria in marine sediments

In general the sediments are heterotrophic systems in which recycling, decomposition and mineralisation of organic and inorganic matter is important. Products of photosynthesis are in this way reintroduced, completing the cycle. Marine sediments cover a large part of the earth surface, and its composition varies immensely [Fenchel et al. 1998]. This means that the diversity of bacteria in sediments will vary even more. Bacterial actions lay the foundation of the entire ecosystem by performing various metabolic tasks. Their roles in sediments range from being primary producers, decomposers, symbiants, pathogens and modifiers of marine sediments. They are a key organism in biogeochemical cycles, but are also food for other marine organisms. Having this many tasks require enormous diversity, especially in terms of metabolism [Karleskint et al. 2010].

Sediments are continually in contact with seawater, which chemistry can vary to a great extent. The oxygen content of the water diminishes as depth increases. Oxygen will in an aerobe environment function as the most important electron acceptor for heterotrophic organisms, and the reduced availability of molecular oxygen in sediments has forced prokaryotes to evolve into being able to utilize a wide variety of electron acceptors. The most important ones in sediments being O_2, NO_3^-, Mn (IV), Fe(III), SO_4^{2-}, S^0, CO_2 and organic carbon. The energy sources available also vary and prokaryotes can utilize organic as well as inorganic energy sources. Because of this, several metabolic groups of prokaryotes exists based on their energy sources. Heterotroph prokaryotes, including aerobes, denitrifiers, Mn reducers, Fe, reducers, sulphate-reducing bacteria, methanogens, syntrophs, acetogens and fermentors, uses organic carbon as energy source and the different subgroups uses a variety of electron acceptors. Phototrophs uses light as an energy source while lithotrophs uses inorganic compounds as energy sources. The availability of electron acceptors and energy sources varies with depth within the sediments and thus forming horizontal gradients. The types of energy sources and electron acceptors available will be important for the bacteria diversity and which type of bacteria inhabits the sediment [Nealson 1997]. Much is known about the general function of bacteria in sediments and what types exists, but when starting to map the bacterial diversity in a specific location one start from scratch. Several methods can be used to do this. Denaturing gradient gel electrophoresis (DGGE) is a much used method when trying to examine changes in the bacterial community.

1.2.2 Environmental bacterial communities

Bacteria evolved from the first simple cells and has continued to inhabit this earth for more than 3,5 billion years. Even after all this time bacteria is still small and simple, expressing their diversity in terms of differences in physiology and metabolism [Nealson 1997].
It`s general knowledge that most of bacterial species has yet to be identified. One can only try to imagine the vast diversity of bacteria that exists in this world. A few thousand species are identified and classified into approximately 18 major phyla, but one suspect 100 000 – 1 000 000, or even more, species exists in total. The diversity of bacteria is endless and varies tremendously from habitat to habitat. Different populations of bacteria interact to form a community, which in turn interacts with other communities to form an ecosystem [Madigan & Martinko 2006]. Development of new scientific tools and knowledge, especially in molecular biology, has lead to a greater understanding of microbe's interaction with the environment and their diversity. Some of these techniques are based on the isolation and analysis of nucleic acids. Development of the polymerase chain reaction (PCR) forms the foundation for culture independent methods and has made it easy to amplify 16S rDNA as a target gene. Genetic material can in this was be extracted from the environment, or a sample, without culturing and analysed directly [Munn 2004]. Environmental samples are in general more difficult to handle compared to pure culture samples. Among the many types of environmental samples soil and sediment sample are considered to be the most difficult to perform PCR on. The reason for this is that these samples often contain physical and chemical inhibitors, which can inhibit or disrupt PCR. Physical inhibitors, for instance soil colloids, can disrupt primer annealing or increase formation of undesirable primer dimers. Chemical inhibitors, inorganic (iron) or organic (humic acids) is present in various concentrations and if not properly removed during DNA extraction phase, they provide a major obstacle during PCR. Well functioning PCR conditions are very important to amplification, and thus further analyses with DGGE [Hurst et al. 2002]. Noller & Woese (1981) was the first to attempt characterization of microbial diversity in marine samples. This was done by isolation ribosomal RNA (rRNA).
When studying prokaryotic diversity the small ribosomal subunit 16 S soon became a popular choice. This is because 16S rRNA are universally present, evolution occurs slowly and it have highly conserved and variable regions. It is now common to isolate total-DNA and amplify regions of 16S rDNA, the DNA corresponding 16S rRNA, to evaluate microbial diversity [Munn 2004]. Denaturing gradient gel electrophoresis (DGGE) separates PCR products by sequence and is used to examine the bacterial community [Muyzer et al. 1993].

1.2.3 Consequences of increased CO_2 and/or reduced pH on bacteria-(primary effects)

When enriching sediment with CO_2 the conditions, in which the bacteria normally live in, are changed drastically. The CO_2 functions as a disturbance and can probably cause the microbial community to respond in several different ways, or none at all (Figure 1.3). If the microbial community remains unchanged despite the disturbance, it is resistant. In cases where the disturbance leads to a change in the microbial composition, but eventually returns to its original composition, makes it resilient. Resilient communities are considered sensitive but have the capacity to recover. If a microbial community is sensitive and not resilient it will remain altered, but if it despite this can carry out the same processes at the same rate the microbial community is considered functionally redundant and performs the way it used to before the disturbance. Alternatively it cannot carry out the same way as before and it will perform differently [Allison & Martiny 2008].

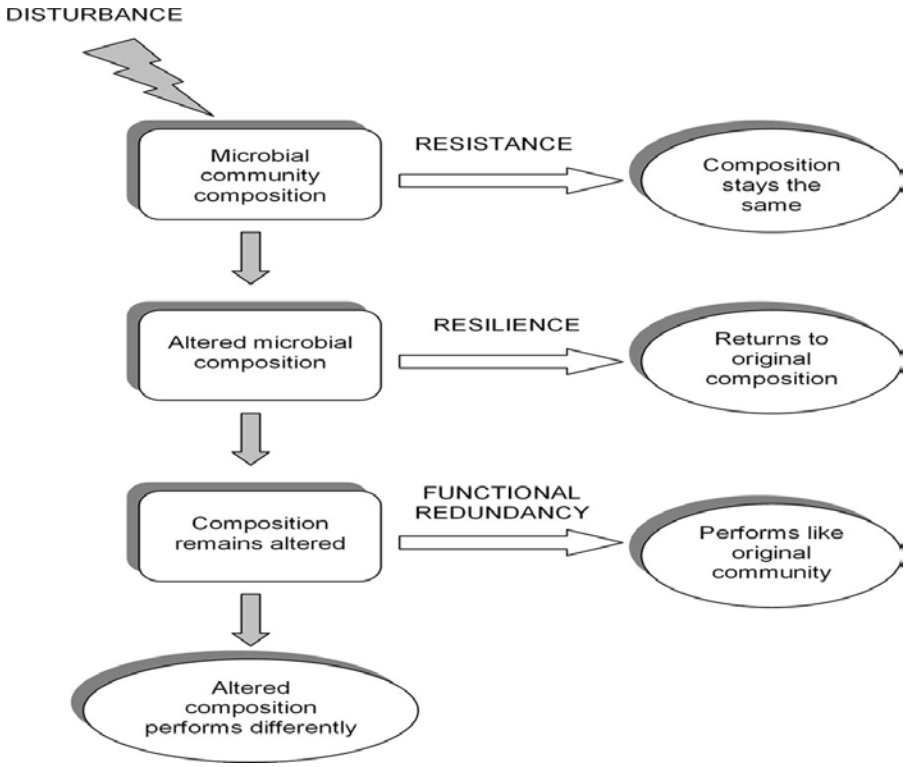

Figure 1.3: **Responses of the microbial community** composition in the face of a disturbance. A resistant microbial community stays the same while sensitive microbial communities can be resilient or functionally redundant. If a sensitive microbial community fails to regain its function and composition, its function will be altered [Allison & Martiny 2008].

Most microbial groups are sensitive but also resistant groups are normal. There are several reasons to why the microbes often recover so easily. Generally the abundance, widespread dispersal and diversity all favours a quick recovery. They have a high growth potential so when the abundance is reduces they can recover quickly. High physiological flexibility makes

acclimation less troublesome. Also their rapid evolutionary adaption and short generation time favours recovery [Allison & Martiny 2008].

Several studies focusing on CO_2 enrichment and microbial response have been done, but most focuses on terrestrial soil and global changes in CO_2 concentrations. Opposed to this thesis, these studies often focus on relatively small change in CO_2 concentration. A study considered by Allison & Martiny (2008) concludes with that in approximately 60 % of the studies, the microbial communities were found to be sensitive to a CO_2 increase. Of course the methodologies and the focus of the studies differ, making comparison with this thesis difficult. Also the strength and how often the disturbance is applied vary. These are all factors important for how the microbial community responds. When investigating further it was soon clear that studies on how high concentrations affect marine organisms has also been looked into by several scientists. Takeuchi et al (1997) investigated the effects of reduction in pH and increased CO_2 concentration on marine organisms, among them bacteria. This study tested 11 species of cultured bacteria, among them one deep sea sediment species, exposing them to various pH and CO_2 concentrations similar to what this master thesis focuses on. Still, the experiment was only a few hours long and in general more acidic conditions were used. Impacts on bacterial growth were observed, but the study concluded with that more research is needed. In the same study the effect under pressure were tested. The results indicated that deep sea species are not necessarily more sensitive to environmental change just because they normally live under extremely stable conditions compared to shallow water species. The study also concluded with that more research on effects on the community structure is necessary [Takeuchi et al. 1997]. Yamada et al. (2008) investigated effects of high CO_2 and low pH on bacterial abundance and production in relation to CO_2 sequestration in deep ocean waters. This study suggested relatively high tolerance of bacteria to increased CO_2. The effect on a natural assemblage of bathypelagic bacteria under pH 6.8-7.4 was found to be relatively insignificant. Only under the highest concentrations of CO_2 was bacterial production rate depressed. Another study [Coffin et al. 2004] tests the impacts of CO_2 on bacterial production and found it to be moderately sensitive to seawater acidification. It seems that a drastic change in pH or CO_2 is necessary to produce a response, while mild injections of CO_2 might cause no inhibition of production or it might even enhance it. Just as with other studies it hints about the fact that more research is needed and especially to investigate the effects on the microbial community.

Studies that investigates alterations in bacterial production or abundance, whether it is positive or negative, in response to CO_2 gives a little information about community responses in face of a disturbance. Studying the microbial community is important because an alteration there can cause consequences to several ecosystem- and biogeochemical processes and so cause troubles in higher organisms.

1.2.4 Consequences of changes in metal concentration (secondary effects)

Changes in pCO_2 and pH are not the only consequences of CO_2 leakages. These are primary consequences. Changing pH and pE will affect the chemistry of the water and sediment thus causing changes, which in turn might affect the organisms living there, resulting in secondary consequences or effects. Bacteria, as opposed to fish, are stationary and therefore have no choice but to face these changes. The changes in pH and pE following release of CO_2 causes trace metals, like Al, Cr, Ni, Pb, Cd, Cu, and Zn to dissolve into the water and sediment. Ardelan et al. (2009) reported that increases in concentration for these metals was highest for most of them during the first phase of an experiment similar to this project, but only Pb concentration continued to increase at a faster rate. Ni and Cu also increased, but at a slower rate. The rest of the trace metals were partially removed. When the concentration of these metals increases, this will probably cause some sort of effect on the biota [Ardelan et al 2009]. Especially Pb and Cd are toxic. Other metals that are mobilized by CO_2 include Fe, Mn and Co. Also here the increase in concentration is highest during the early phase of the experiment but continued to dissolve further out in the experiment [Ardelan & Steinnes 2010]. In sediments, where O_2 is often scarce, Fe and Mn play an important role in anaerobic respiration. Fe (III) and Mn (IV) function as electron acceptors in metabolism and are reduced to Fe (II) and Mn (II) [Lovley 1991]. Both forms for respiration are among the most important contributors to anaerobic respiration in anoxic environments, like sediments, because of their abundance and high reduction potential [Madigan & Martinko 2006].

Whether the metals are essential or non-essential, the chemistry is changing when CO_2 is introduced. The foundation of a bacterial community is dependent on, among many factors, the chemistry of the water and sediment. If this changes it is logical to assume the bacterial community will be affected in some way or another.

AIM OF STUDY

This thesis is a part of a larger project where the main goal is to investigate what happens chemically and biologically when CO_2 is introduced to an environment similar to the seafloor, by leaking through sediments. The biological part is covered in this thesis by studying the bacterial communities in sediments and if it changes as a result of a CO_2 seepage into the system. This is accomplished by using the method PCR-DGGE to get an overview of changes at community level. A simply hypothesis is formed:
CO_2 leakage causes the bacterial community structure in sediments to either change, indicating sensitivity, or not change, indicating resistance.

The hypothesis is tested by setting up two experiments, imitating seepage at the seafloor, and taking before and after sediment core samples. There are several objectives in this thesis:

- Compare the bacterial community in sediment samples from before and after CO_2 exposure experiments to confirm if the community structure undergoes significant changes in response to CO_2 seepage.
  - Differences between the sediment layers are taken into account during evaluating effects of CO_2 treatment.
  - Differences between the two sediment chambers (Direct/CO_2- and Top-Down chamber), to check if being near or at the seepage source is of importance, are investigated.
  - Discuss whether changes of the bacterial community could be caused directly by CO_2 or indirectly by change in metal concentrations, or both.

- Sequencing bands from the DGGE analysis to hopefully reveal what population of bacteria the DGGE gel bands represent.

- Tips and recommendations for further work in this project.

If a change in the bacterial community occurs, further studies to identify what is changing can be made. But since this is a pilot experiment, most analysis will be superficial, since the knowledge that is required to go into depth is lacking at the moment. Hopefully, this project will contribute to reduce this lack of knowledge.

2. MATHERIALS AND METHODS

2.1 Experiment and setup

2.1.1 Gathering of sediments

The sediments used in this project were gathered at the exact same location (at N°63.28026, E°10.30977) in the Trondheimsfjord at approximately 250 m depth, using a box corer on the research vessel Gunnerus. Two trips were made: 19.02.2010 and 14.09.2010. Sediments were collected using a box corer as shown in figure 2.1. This box corer is made from metal, and since the sediments needed to be clean from any metal contamination, the outer layer was removed by placing a clean plastic box (27x50x27 cm) in the middle of the sediment. This is shown in figure 2.2. Two sediment samples were gathered. The leftover sediments were disposed of, and the plastic boxes containing the remaining sediments were wrapped in plastic to prevent contamination from air and water until the experiments began.

Figure 2.1: **Gathering of sediments.** Sediments from 250 m depth were collected using a box corer [Photo: Kathrine Helen Sundeng].

Figure 2.2: **Removing contamination.** The contaminated outer sediment layer was removed by using a plastic box [Photo: Kathrine Helen Sundeng].

2.1.2 Treatment of sediments

Sediment taken 19.02.2010 were immediately upon arrival placed in the deep freezer (-22°C) until they were needed, and 16th June they were taken out of the freezer and thawed to be used in the first experiment. During thawing the structure of the sediments appeared to have changed as a result of freezing. Instead of a being smooth and even, like when the sediments were gathered, it was now cracked and the water was unevenly distributed. It was assumed that the chemistry and the bacteria in the sediments might not have been affected much by freezing, but to be sure the next experiment was going to have fresh sediments. The next day both thawed sediments were placed in another and larger plastic box containing two chambers (each chamber 27.2x55x27.2 cm) specially designed for this project (Figure 2.3). The two chambers containing the sediments are separated by a plastic wall. The direct chamber (denoted D) has CO_2 pumped in through small tubes connected to the bottom of the box, thereby exposing the sediment CO_2 all the way through. In this way a leakage from a sub-seabed storage site through the sediments is imitated. Top-down chamber (denoted T-D) is not connected to these tubes, but it`s function is to investigate the top-down effects, meaning how elevated CO_2 in the surrounding seawater affects the sediment located near the CO_2 source. This setup was chosen hopefully to establish if the area around the seepage is equally affected. As shown in figure 2.3, there are placed sediment DGT (diffusive gradients in thin-films) to measure concentration of metals in the sediment and water. The sediment box was placed inside the titanium pressure tank (Figure 2.4) and the door shut close before pumping seawater from outside Sealab into the tank.

Figure 2.3: **Sediment box** used in both experiments. This is from the second experiment.
The box consists of two separate chambers:
Direct chamber: at the top in the picture, and has CO_2 running through the sediment.
Top-down chamber: bottom part of the picture, has no CO_2 running through it.
DGTs are placed in or nearby the sediment to measure metal concentrations [Photo: Kathrine Helen Sundeng].

The second experiment started 14.09.2010 by collecting sediments that were to be used. Collected sediments were put in the sediment box, and placed in the titanium tank. Until the experiments started, a week later, the sediment was stored inside the tank. To keep the bacterial community from suffering effects of this, the sediment was kept cold and dark inside the tank, and seawater was continually supplied (1 L/min) to keep the sediments partially immersed in water.

2.1.3 Titanium tank experiment

SHORT INTRODUCTION TO THE TANK

Titanium is known to be an extremely corrosion resistant metal [Van Noort 1987], also against seawater. Because of its corrosion resistance and chemical stability it makes the perfect metal to use in this kind of experiment. CO_2 is added to the seawater, therefore enhancing the corrosion. Scientific 5.2 pure CO2 (HiQ, AGA) was pumped in using 16 % of pump capacity. Because of this iron was avoided when building this tank (Figure 2.4) and so only titanium was used. Pressure was added to imitate the conditions on the seafloor. A pressure of 10 atm was used. This is the pressure at the seafloor around 100 m depth. 30 atm was the desired pressure, since this is similar to the pressure where the sediment samples were collected. The tank was designed to be able to do experiments under this pressure but this was not yet tested on the tank, so 10 was the maximum pressure allowed to use at the moment. The tank is located at SINTEF SeaLab in Trondheim in a room where the temperature was kept around 7°C. The only exception was when taking samples. Then the air-conditioning was turned off for a short period of time. Seawater from the harbour area (approximately 90 m depth) was supplied to the tank continually, with a flow rate of 1L/min.

Figure 2.4: **The titanium tank** with the sediment box inside. This picture was taken on the last day of the second experiment, after the water was emptied. During the experiments the tank contains 1500 L, or 1.5 m^3 seawater, [Photo: Kathrine Helen Sundeng].

EXPERIMENT SETUP

Two experiments were performed (Figure 2.5) with two different setups.

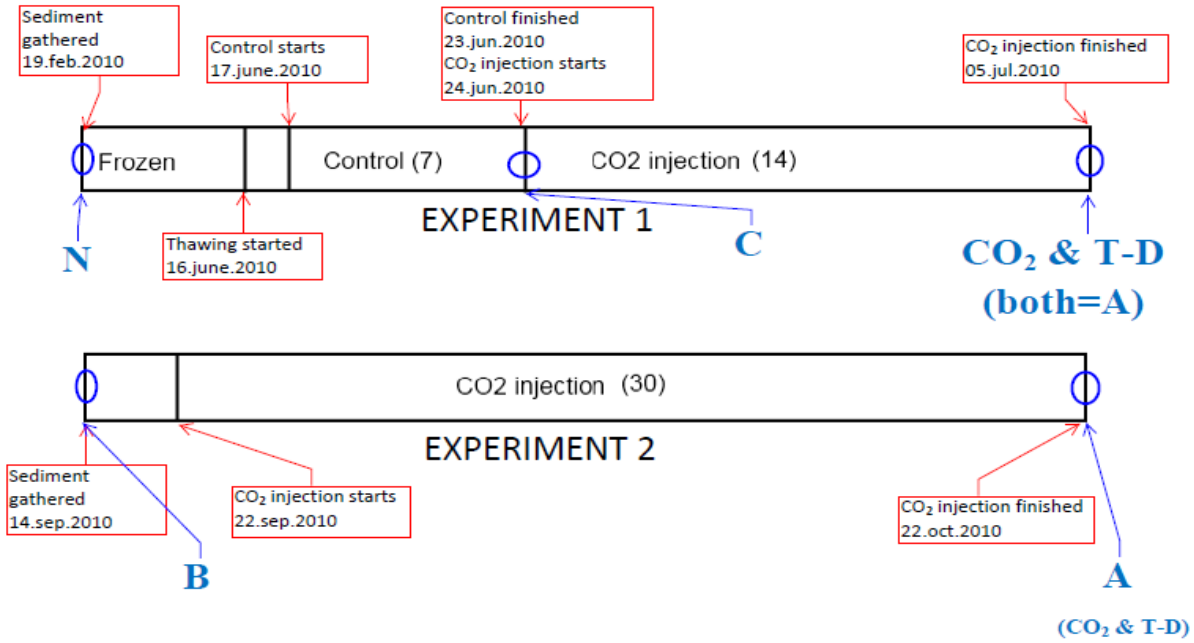

Figure 2.5: **Experimental setup of experiment 1 and 2**. The blue rings indicate at what date the sediment samples were taken and the blue letters represents the abbreviations given for these samples. The numbers in brackets represent number of days.

Experiment 1: This was the first experiment performed, and included a control experiment in addition to a two week CO_2 leakage experiment. Sediment samples taken included:

- N = natural samples, meaning samples collected immediately after gathering of sediments, before freezing them.
- C = control samples, is samples taken after the control experiment lasting a week. The control experiment has all the experimental conditions of a CO_2 experiment, only without CO_2 seepage through the sediment.
- D = direct chamber samples, is sediment samples taken from direct sediment chamber after the experiment where CO_2 is injected was finished.
- T–D = top-down chamber samples, is sediment samples taken from top-down sediment chamber after the experiment where CO_2 is injected was finished.
- A = after samples. Collective name for both D- and T-D-samples.

Experiment 2: The second experiment consisted of one month CO_2 leakage experiment. Sediment samples taken included:

- B = before samples, is sediment samples taken before the experiment began.
- A = after samples, is sediment samples taken after termination of the CO_2 exposure experiment. Includes samples from both chamber, D and T-D.

TESTS PERFORMED DURING THE EXPERIMENT

Tests taken during the course of the two experiments are collected from three main areas of the tank; inflowing seawater, inside the tank, and outflowing seawater. In this master thesis only sediment core samples from inside the tank are analyzed further, and the metal analyzes are used in the master theses of Gøril Aasen Slinde and Katrine Helen Sundeng, NTNU, institute of chemistry [Slinde 2011; Sundeng 2011]. Some general results from these metal analyses will be discussed in section 4.7 when primary and secondary effects of CO_2 exposure are discussed.

Inflowing seawater:
The flow of seawater passed a test station before entering the tank. Since the seawater came directly from the sea it was first filtered (Aquapure water filter AP055T, 5 µm nominal pore size). This is done to remove big particles before the seawater went into the DGT test tube. In this tube a number of DGT`s were placed. These DGT`s were later analyzed for metal content in the incoming seawater. Before the water went into the tank a number of other analysis were performed. Other test taken at this point included direct water samples and a water sample added Chelex-100 to measure metal content in the seawater. These were taken every two or three day. Also pH and total alkalinity were regularly measured.

Inside the tank:
A number of sediment core samples, sediment DGT and water DGT from inside the tank were taken before and after the experiments. This was done because sample taking for inside the tank were not possible to collect during the ongoing experiment.

Outflowing seawater:
The seawater coming out of the tank was also tested the same way as the inflowing seawater. pH, total alkalinity, and metal content in seawater (DGT, directly from seawater and Chelex-100) was analysed.

SEDIMENT SAMPLES

The sediment core samples were stored from two to ten months in the deep freezer (-22°C). Then they were transported from SINTEF Sealab to Realfagsbygget, NTNU, where the core samples were separated into four layers, each approximately 2-3 cm thick, using a plastic knife. The knife was shortly washed with acid before use and cleaned shortly with acid (3M HNO_3) in between usage to prevent contamination. The sediment core samples were all thawed until separating the layers with the plastic knife was possible. Only the last samples (from February) thawed too much, making them almost liquid so that separation of the layers became much more difficult. Each piece of the sediments were placed in a separate plastic container and numbered. The process of separating the layers was performed in quick and effective way to minimize contact with air and other surfaces. Appendix I shows all sediment samples and their designated numbers.

The sediment samples were separated into four layers, top-, second from the top-, second from the bottom- and bottom sediment layer:

- Top sediment: samples from the topmost layer, facing the seawater.
- Second from the top: samples from the layer second from the top.
- Second from the bottom: samples from the layer second from the bottom.
- Bottom: samples from the bottom most layer.

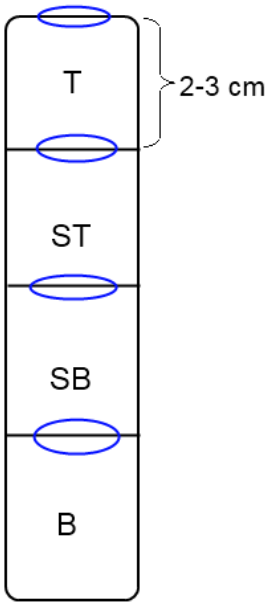

Figure 2.6: **Sediment core sample**. The sediment core sample consists of four layers, top (T), second from top (ST), second from bottom (SB) and bottom (B), each approximately 2.5 cm thick. The blue rings represent where the sediment sample was taken from each layer.

2.4 Analytical methods

2.4.1 DNA extraction and determination of concentration

Bead beating is one of many methods to lyse bacterial cells from environmental samples and with this method a high DNA yield is obtained. DNA from the sediment was extracted using UltraClean® Soil DNA Isolation Kit from MoBio. This kit involves a bead-beating step, and figure 2.7 shows the procedure for isolating DNA using this kit. The protocol provided by the manufacturer was followed, but a few steps were modified. A detailed protocol is provided in appendix V.

An article by Whitehouse & Hottel (2007) shows that compared to other commercial DNA extraction kit the UltraClean® Soil DNA Isolation Kit from MoBio outperformed the other kits. This kit is designed especially for extracting DNA from soil, by efficiently removing Taq polymerase inhibitors, which often is found in soil and sediments. All in all this kit is sensitive for different soil types and concentrations of organisms, time and cost effective, and efficiently removes PCR inhibitors.

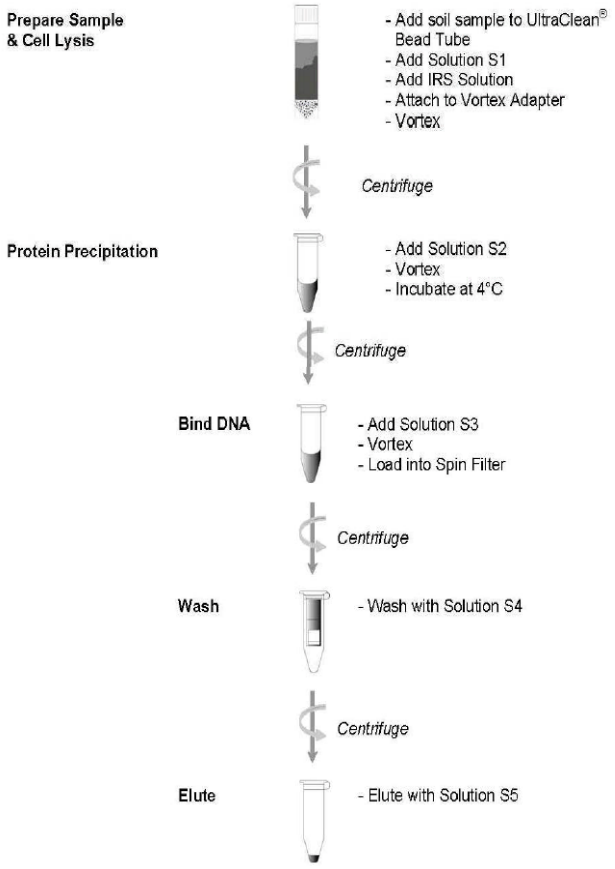

Figure 2.7: **DNA Extraction**. A general overview of DNA extraction procedure when using DNA extraction kit from MoBio. First the cell lyses, meaning that the cell walls break down. Then a combination of chemical and mechanical lysis causes the cells to break down even further. DNA can now be bound and extracted.

The concentration of the DNA extracts was determined using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific). The concentration of DNA is needed to know how much DNA template to use when amplifying the DNA using PCR.

2.4.3 PCR

Polymerase chain reaction (PCR) is a technique where DNA is amplified enzymatically through a repetitive process in vitro. Two oligonucleotide primers define the DNA region which is to be amplified, in this case a specific region (variable region 3) of the gene encoding 16S rRNA. The PCR reaction repeatedly cycles through between steps [Hurst et al. 2002].

1. Denaturation: The first step mainly sees to that the two strands of the double stranded DNA are separated, or denatured. This is done by heating the sample to approximately 95 °C.
2. Annealing: The two strands are now separated so that the two primers can bind specifically to each strand. Binding can only happen by cooling the sample to an optimal temperature. Usually the annealing temperature is between 45 and 60 °C.
3. Elongation: In the final step the temperature is increased to 72 °C so that the DNA polymerase can function optimally. DNA polymerase binds to the primers and starts the extension using the target sequence, which is copied, as a template.

PCR PROTOCOLS

The primers 338forward (F) GC and 518 reverse (R) were used to generate PCR products for the DGGE analyses. They target highly conserved regions of the 16S rRNA gene, and will amplify most bacterial taxa [Bakke et al. 2010]. The resulting PCR product of approximately 200 base pairs encompasses the variable region 3 of the 16S rRNA gene. For reamplification of DGGE bands for DNA sequencing, the primers 338F-GC-M13R and 518R were used. The M13R sequence in the 338F-GC-M13R primer specifies the sequence for the DNA sequencing primer. Primer sequences are given in Table 2.1.

PCR reactions were run using Taq Polymerase (VWR), reaction buffer (QIAGEN), 0.2mM Deoxynucleotide Triphosphate (dNTP) Mix (FINNZYMES), Bovine Serum Albumin (BSA; New England BioLabs), a total concentration of 1,5 mM $MgCl_2$ and 0.3 μM of each primer. PCR temperature cycling conditions are given in Table 2.2.

Table 2.1: **Sequences for PCR primers used in this study**. The sequence given in capital letters corresponds to the GC-clamp, and the sequence given in italics, corresponds to the DNA sequencing primer.

Primer name	**Primer sequence**
338F-GC	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGactc ctacgggaggcagcag
338F-GC-M13	*caggaaacagctatgac*CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGG CACGGGGGGactcctacgggaggcagcag
518R	attaccgcggctgctgg

Table 2.2: **PCR temperature cycling conditions.** The three main steps (denaturation, annealing and elongation) forms one cycle and are repeated, each time doubling the amount of product, a certain number of times. When dealing with sequences that are going to be analysed by DGGE, an extra long elongation step (30 min) is needed to ensure DNA polymerase has time enough to finish extension of all the products.

PCR step	**PCR for generation of DGGE fragments**	**PCR for reamplification of DGGE bands for DNA sequencing**
Initial dentaturation	95 ºC, 3 minutes	95 ºC, 3 minutes
Denturation	95 ºC, 30 seconds	95 ºC, 30 seconds
Annealing	50 ºC, 30 seconds	50 ºC, 30 seconds
Elongation	72 ºC, 60 seconds	72 ºC, 60 seconds
Number of cycles	35	38
Final elongation	72 ºC, 30 minutes	72 ºC, 10 minutes

2.4.4 Agarose gel electrophoresis

Agarose gel electrophoresis is a method where DNA fragments are separated based on length. DNA, which is negatively charged, will wander through the gel toward the positive pole. Separation is a result of small DNA fragments migrating faster through the gel compared to larger DNA fragments. This analysis functions as a quality control, for instance to check if several PCR products have been amplified when only one is the target. Another important task is to check the amount of PCR product formed in the reaction, especially when performing DGGE later, also it`s used to check for contamination during PCR by including a negative control.

Five µl templates from each PCR samples are mixed with 1 µl loading dye, which is added to keep track of the samples progress through the gel. These mixes are subjected to electrophoresis, 140 V in 45 minutes, in 1 % agarose gel with TAE (2 M Tris-HCl, 1M Acetic acid, 50 mM Ethylenediaminetetraacetic acid (EDTA)) buffer. 1 kb Plus DNA ladder (Fermentas) was used as a marker for DNA fragment size.

GelRed is a fluorescent dye which binds to the nucleic acids and gives fluorescence, is added in the agarose gel (5 µl pr 100 ml gel) so that the DNA can be visualized and photographed when exposed to UV (G:BOX, Syngene).

2.4.5 DGGE

When using conserved 16S rDNA primers to amplify DNA from a bacterial community, the PCR result in a great number of different products, similar in length but differ by variations in sequence. Different species will produce one or more products and thus the variety in products represent the bacterial diversity in the sample. Because these products are of approximately the same size they will not be separated on a typical agarose gel. Denaturing gradient gel electrophoresis (DGGE) is a fingerprinting method where PCR products of same length but different sequence composition can be separated. The principle behind this is applying the PCR products to a polyacrylamide gel with increasing chemical denaturing gradient consisting of DNA denaturants, such as urea and formamide. A high concentration of denaturants is not enough to melt the strands apart; also a high temperature is necessary. This is achieved by immersing the gel in TAE electrophoresis buffer, which is kept at 60 °C during the whole process [Muyzer & Waal, 1993].

When the PCR products migrates through the gel it will at some point reach a concentration of denaturant that will separate the two strands and migrating halts. This will occur at different point in the gel for the different PCR products, dependent on their sequence composition. If the strands had been completely denatured, the two single strands would have continued their migration through the gel, although at a lower rate than double stranded DNA. Therefore a GC-clamp, meaning a sequence of 40-60 guanine and cytosine nucleotides, is added to the 338F primer to prevent the DNA from completely denaturing. The two single strands are held partially together, resulting in a partial denaturing that halts the migration through the gel. This will make the fragment large and practically unmovable [Quinn & Keough 2002]. Where the products stop migrating is dependent on their sequence, meaning differences in A-T and C-G base pairs. A-T base pairs have a lower melting temperature compared with G-C base pairs. G-C rich sequences will therefore migrate further because a higher concentration of denaturants is required to separate the bindings, while less G-C rich sequences stop migrating relatively quick. The more G-C base pairs in the sequence, the higher concentration of denaturants are needed to melt the strands. The result is that fragments with the same length stops their migration through the gel at different positions. Numerous bands will appear on the gel and each of them will in theory represent one sequence, or bacterial population. By using this method the microbial diversity of bacterial populations present in sediment samples can be investigated [Hurst & Crawford 2002]. The pattern of bands that appear on the gel is considered the image of the bacterial community and contains the most dominant bacterial populations in the sample [Fromin et al. 2002].

The PCR products were analyzed by DGGE on 8% acrylamide gels with a denaturing gradient of 35-55%. The gel was run at 100 Volts for approximately 18 hours. A detailed DGGE protocol is found in appendix VI.

STAINING AND VISUALIZATION

The electrophorized gel was transferred to a plastic foil sheet. Staining solution (MilliQ water, SYBR Gold and 50 x TAE) are distributed across the gel and left for 1 hour in the dark. The bands are now stained and after being washed, with water, the gel visualized by UV light (G:BOX, Syngene). The gel is photographed at different exposures using the programme GeneSnap. The same applies to the agarose gel, except it is added GelRed, and therefore don't need to be stained. PCR product being separated by sequence creates multiple bands in the DGGE gel.

2.4.6 Determination of sequences for DGGE bands

Each band that appears on the DGGE gel represents a sequence type, or population of bacteria. In theory PCR fragment from a population displays identical electrophoretic mobility in the DGGE analysis, therefore forming bands [Fromin et al. 2002]. To determine which species of bacteria the DGGE bands represented, DNA from the bands were reamplified by PCR, and the resulting PCR products were used as template in DNA sequencing reactions (sent to Eurofins MWG Operon in Germany for DNA sequencing).

First, bands were eluted from the gel using a micropipette tip to remove gel material from the bands. The gel fragment was transferred to 20 µl sterile MilliQ water in eppendorf tubes and incubated at 4 °C for 24 hours, alternatively in the freezer afterwards (-20°C) for longer storage. Reamplification was performed using 1 µl of the eluate template in a PCR reaction, as described above (section 2.4.3). To evaluate quality and quantity of the PCR products (to establish if there was any product in the eluate, and which samples to send in) 5 µl PCR products were run on an agarose gel. The remaining PCR products (20 µl) were purified using QIAquick PCR Purification Kit (QIAGEN) as described by the producer. A detailed protocol is given in appendix VII. Approximately 75 ng of the purified PCR products were sent to Eurofins MWG Operon in Germany for DNA sequencing.
The returned sequences were analyzed using Ribosomal Database Project`s (RDP) Classifier tool which classifies bacterial 16S rRNA sequences. It gives accurate and rapid taxonomy [Wang et al. 2007]

2.5 Analysis and Statistics

The image exported from GeneSnap is reduced from 16bit to 8bit by using the programme ImageJ. Gel 2K software programme (Svein Norland, Department of Microbiology, University of Bergen) is used to further analyze the image. The programme gives each band in every sample on the DGGE-gel a value based on light intensity compared with the background. This means that the bands will not only be given a value based on presence or no presence, but it will give a quantitative measure of each band.

The density of the bacterial populations within a sample has been shown to be related to the intensity of the bands represented on the DGGE gel [Fromin et al. 2002]. A correlation between changes in band intensity and abundances of the corresponding population has also been shown. The reason for it not being absolutely quantifiable is because of PCR biases that compromise the quantitative interpretations. Despite being semi-quantitative at best, the band intensity can be used for comparative purposes and to follow the relative changes of a population represented by a particular band. This means that it's useful for comparing band intensity between samples, and a poor indicator of absolute abundance [Schauer et al. 2003]. The values each band is given is then exported to Excel, where the data is normalized by dividing each band value on the sum of all band values obtained from each sample.

All statistical analysis of the data is performed with the software programme PAST [Hammer & Harper 2006]

2.5.1 Cluster analysis

DGGE results from sediment samples in these two experiments represent the bacterial community. These samples can be classified into groups based on their variables (bands in the gel) where samples grouped together is more similar to each other compared to samples in other groups. Before doing the analysis the number of groups is unknown and is determined from the data. The method used is called cluster analysis. This method displays the groups in a diagram called a dendrogram. The group consists of similar samples, formed agglomerative hierarchical (bottom-up). Agglomerative approaches starts with forming a cluster, or group, between the two samples most similar. Recalculation is based on that first group, calculating the similarities between this cluster and the remaining samples. In this way more clusters are formed, eventually forming one big cluster based on the similarity or dissimilarity between samples. Links between the clusters is formed, whereas the lengths of the link represent dissimilarity between the samples. The drawbacks with this method are that the interpretation of the dendrogram is subjective, meaning two persons can draw two different conclusions from the same cluster analysis. Also the agglomerative approach means that the entire cluster

is dependent on appropriate forming of the firs cluster. If formed misleading, it affects the entire cluster. The hierarchical approach means that if a cluster is first formed, it cannot be broken, resulting in a dendrogram not representing all pair wise dissimilarities. To do this multidimensional scaling (MDS) is performed [Quinn & Keough 2002].

2.5.2 Non-metric multidimensional scaling (NMDS)

NMDS is a method where relationships between objects are based on the ranks of their dissimilarities, presented graphically. NMDS, like other multivariate analysis, aims to reduce the number of multiple variables to a new set of variables, a point in a two-dimensional space, which represents the original information, and to expose any patterns in the data.
The pattern is revealed by plotting the samples in a multidimensional space based on the new variables, in other words scaling or ordination of samples, along two axes, whereas the distance between the samples in this plot represent their dissimilarity. Distance between samples in the ordination space indicates the samples relative dissimilarity or similarity. Dissimilar samples are placed far apart and similar objects close to one another [Fromin et al. 2002]. NMDS is an ordination method where a small number of axes are chosen, in other words there are no hidden axes of variation. Also it is a numerical technique which repeatedly searches for a solution, and stops when it is found or a certain number of attempts, and there can be small differences between the results obtained [Quinn & Keough 2002].
NMDS finds a configuration which preserves rank-order dissimilarities as accurate as possible in a predefined number of dimensions. Goodness of fit is measured as "stress", which is the mismatch between the rank order of dissimilarities in the data, and the rank order of dissimilarities in the ordination. In an iterative process the ordination is adjusted until the stress appears to reach a minimum. If this value >0.3 the results are not fit for use, and the plot should in general not be interpreted unless they value is <0.2. The final configuration is the NMDS ordination solution. [Quinn & Keough 2002; Clarke 1999]

2.5.3 Bray-Curtis

Bray-Curtis similarity measure is used both in the cluster analysis and NMDS. It uses variables with a high value, ignoring zero values, since it is the high values that most likely will wary between the samples. It standardizes the sum of the variable values across samples with differences between samples across variables [Quinn & Keough 2002].

2.5.4 Oneway ANOSIM

Analysis of similarities (ANOSIM) is a hypothesis testing method for similarity data matrices. One-way ANOSIM is used to test for significant differences between chosen groups of similarity matrices [Bray & Curtis 1957]. Like NMDS, ANOSIM works on the ranks of similarities. The means of the two types of ranks are compared, yielding the R test statistic which is 1 for total separation and 0 for no separation. The R value is between -1 and 1. Positive value means that samples between groups are dissimilar, and a negative value implies the samples are more dissimilar within groups than between groups. It also produces a P-value, which if <0.05 confirms significance [Clarke 1993; Bray & Curtis 1957].

3. RESULTS

3.1 Titanium tank experiment

This master thesis focuses mainly on the sediments, all other tests described in section 2.1.3 is mainly a part of two other master thesis written as a part of this project [Slinde 2011; Sundeng 2011]. The pH was measured from the seawater coming out of the tank. In both experiments the pH were measured to be between 6.6-6.9, depending on time after sampling, for the water coming out of the tank. Experiment 2 being the successful one, had an average pH of 6.89. Lowest pH measured was 6,738 and highest was 6,963. Average total alkalinity in experiment 2 was measured as 2.34 meq/L, the lowest measured was 2.232 and highest measured was 2.392. Both pH and total alkalinity measured was stable and consistent during the whole experiment. The reason for some fluctuations in pH is probably because some of the CO_2 was transferred from the water to the air because of a change in pressure (from 10 atm inside the tank to 1 atm outside the tank). An overview of all sediment samples is found in Appendix I.

3.2 Optimization of methods

3.2.1 PCR

Not knowing the ideal PCR conditions to use on this kind of sample, a standard setup for amplifying 16S rDNA was used. First PCR was run as described in section 2.4.3, except using annealing temperature 50 °C and the cycles were repeated 30 times. The product was run on a 1% agarose gel and the results revealed little product (Figure II.A). Only one of the samples had a small amount of product, the rest had minimal amounts. Also the negative control indicated a possible contamination.

Because of the small amounts of products it was decided to increase the number of cycles to 35. This time, when the PCR products were run on an agarose gel, the amount of products formed were satisfying and also the negative control showed no contamination (Fig. II.B). Even though the amount of product was satisfying, further optimization of the PCR conditions was performed by optimizing annealing temperatures and $MgCl_2$ concentration. This is not possible to investigate just by running an agarose gel. Therefore the PCR products were to be tested on a DGGE gel to see if different PCR conditions affected the DGGE results, specifically the quality and if it affected occurrence of bands, or bacterial diversity in other words. The agarose gel, containing samples with different PCR conditions (Figure II.C), showed little difference and also here the negative control revealed no contaminations. A

DGGE gel (Figure 3.1) was run using the same samples. It appears that the bands is clearer and more numerous on the samples run with 1.5 mM $MgCl_2$ in the PCR reaction than those with 2mM. Also different annealing temperatures were used. No apparent difference between the annealing temperatures was observed, but an annealing temperature of 53°C were used because they seemed to be give slightly better results compared to 50 °C and 55 °C.

PCR optimization revealed that for these sediment samples 35 cycles with an annealing temperature of 53°C and 1.5 mM $MgCl_2$ gave the best possible DGGE results, leading to clearer and more defined bands.

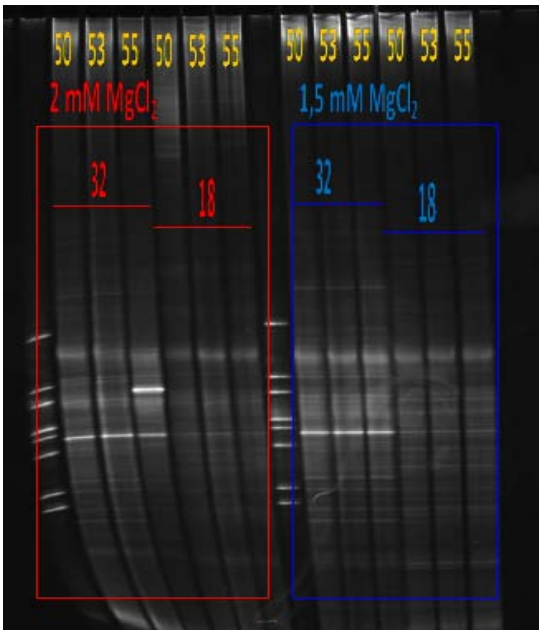

Figure 3.1: **DGGE (35-55% denaturing gradient) for gel PCR optimization.** PCR product from two samples from experiment 2 (number 32 and 18) are obtained by varying annealing temperature (yellow) and $MgCl_2$ concentration (red and blue).

3.2.2 DGGE

Not knowing what results to expect and how the bands would place themselves on the gel and which gradient to use, a broad gradient was used to get an overlook. A gel with all the samples from experiment 2 was run on a gel with denaturing gradient between 30 % and 60 %. The resultant gel appeared to be of poor quality (Fig. 3.2), but it was clear that the gradient needed to be changed since the samples migrated only partially down the gel. Therefore, a 35-55 % denaturing gradient was used for further DGGE analysis.

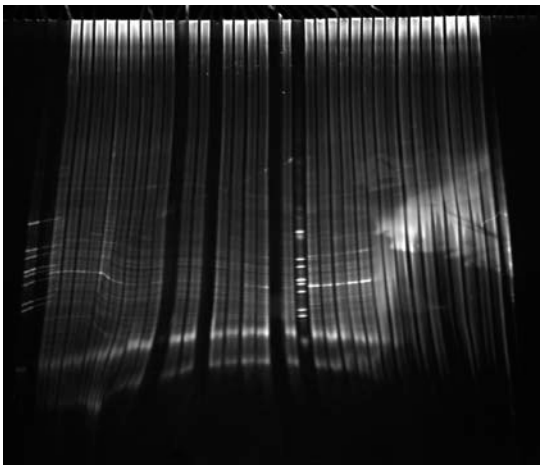

Figure 3.2: **DGGE optimization**. PCR products for all the samples from experiment 2 are used.

3.3 Experiment 1

3.3.1 PCR

The samples from experiment 1 (see figure 2.5 and appendix I, sample nr 33-64) were amplified using PCR. For most samples, the concentration of DNA was found to be around 10-20 µg/µl (concentration varied between 10.3 µg/µl and 53.84 µg/µl). For all PCR reactions, 1 µl DNA extract was used as template. Agarose gel analysis (Figure II.D) revealed that there were no correlation between DNA concentration and amounts of PCR product formed. In some samples with high DNA concentration, little product was formed, whereas in samples with "normal" amount, a high amount of product were formed, and sample 37 gave no PCR product at all. The differences in amount of product were compensated for by using various amounts in the DGGE based on results from the agarose gel.

3.3.2 DGGE

The PCR products obtained for samples from Experiment 1 was analyzed by DGGE, and the resultant gel is shown in figure 3.3. The bacterial community in the sediments appears to be quite diverse. The large number of bands indicates this. It is also clear that there are differences between the samples. For a more detailed explanation of layers, treatment and chamber see 2.1.3 section "sediment samples" and "experimental setup". The result also indicates some differences between the layers in most samples, especially in C samples. All N-samples distinguish themselves from the other samples by having weaker and also fewer bands compared to the other samples. Some differences can be seen between samples taken before CO_2 exposure(C-samples) and samples taken after CO_2 exposure (T-D and D-samples), this is especially clear in bottom layer.

Finally there is clearly a large difference between the two C-samples in each layer. They are taken from both chambers, and the difference indicates the bacterial community in the two chambers are quite different. For instance sample nr 61 are clearly different from sample 57, despite receiving the same treatment. The only difference between them is that they are taken from different chambers. If there is such a large difference between the C-samples, it is more difficult to compare them to the after samples. The alternative is to analyze samples from the two chambers separately and comparing them with the appropriate chamber samples (T-D or D).

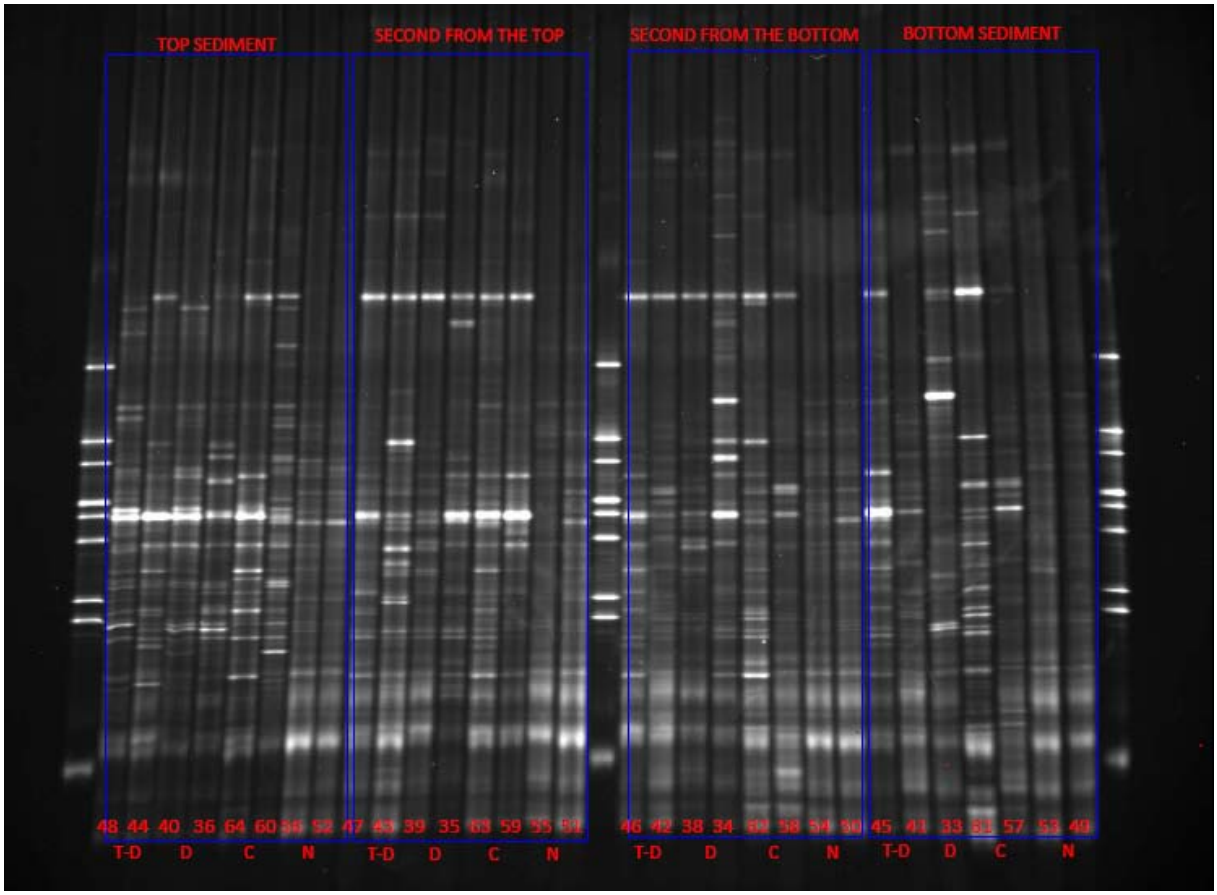

Figure 3.3: **DGGE Experiment 1.** The samples collected during experiment 1 were ordered by layers, top, second from top, second from bottom and bottom sediments. In each of these sections the sample are arranged by treatment in the order: Top-down chamber (T-D), direct chamber (D), both taken after finishing CO_2 experiment. Control samples (C) are taken after the control experiment that were run without adding CO_2 and "nature" samples (N) are taken upon gathering of sediments, before freezing. Bottom layer only has one CO_2 chamber sample. The numbers refers to the sample number found in appendix I.

3.3.3 Statistics

BEFORE FREEZING AND AFTER 1 WEEK CONTROL IN THE TANK

Cluster analysis (Figure III.A) of the DGGE gel shown in figure 3.3 clearly shows that the N-samples forms one cluster and the C-samples forms another. NMDS analysis (Figure IV.A) confirms this trend by placing the N samples in one group and C samples in another. The distance between these two groups indicates they are dissimilar. One-way ANOSIM analysis confirms that the N-samples are significantly (P=0.0001) different from the C-samples and gives R=0.7595, which also shows that the groups are dissimilar. This result confirms that the freezing and one week experiment has significantly changed the bacterial community in the sediment from its original composition. Therefore the N-samples cannot be taken into consideration when evaluating effects of CO_2, only the C-samples can be used for that purpose. N-samples are excluded from the dataset in further analysis.

LAYER DIFFERENCES

-C-SAMPLES: Cluster analysis (Fig. III.B) of C-samples shown in the DGGE gel shown in figure 3.3 revealed ST- and T-samples forms separate clusters from SB- and B- samples. It also shows that there is approximately 70 % similarity between the two ST-samples (sample nr 63 and 59 on the DGGE gel) while in the remaining layers (B, SB and T) the parallels are showing small similarities. The reason for this is that the parallels in each layer are taken from each separate sediment chamber, and apparently the bacterial community in these two chambers is not similar. NMDS analysis (Figure IV.B) of the C-samples also shows T- and ST-samples distanced them opposite from B- and SB-samples, and as with cluster analysis it is clear that the parallels, except ST layer, are showing great dissimilarity. ANOSIM testing revealed no significant differences between the layers, but at the same time there are only two parallels, and they are not similar. Only two parallels are not enough to get reliable ANOSIM analysis. It will probably be best if each layer, and parallel is separately evaluated in further analysis.

-A-SAMPLES: Cluster analysis (Figure III.C) of A-samples (both D- and T-D samples) revealed that the T-samples cluster together, indicating this layer should be considered for itself. However, one samples from the B layer is showing approximately 75 % similarity to a T layer sample. It is strange that samples from two such distant layers show such a high similarity. In general B-samples show very little similarity to all other samples therefore B-samples should also be evaluated separately. SB- and ST-samples tend to cluster together, indicating similarity between layers. NMDS analysis (Figure IV.C) of A-samples confirms that the top sediment layer forms a separate group, with samples distances not too long. B-samples have great distance from each other. Especially one sample (B-A-TD, or nr 61) is very similar to one of the top sediment samples, which is strange. This is all consistent with what was found in the cluster analysis. ANOSIM analysis (Table 3.1) confirms the top sediment layer is significantly different from the other layers, except the bottom layer. Probably because of the B-sample mentioned. The top layer needs to be evaluated separately in further analysis, so does the B layer because of the great dissimilarity between samples. It is probably the best if all layers are analyzed separately, since the results from C-samples also needs to be considered.

Table 3.1: **P-values layer comparisons**. Significant values are coloured yellow, which is the T layer. T samples are significantly different from other layers, except B layer.

Layers	T	ST	SB
ST	0,0269		
SB	0,0275	0,7733	0,7733
B	0,084	0,0897	0,3732

DIFFERENCE BETWEEN CHAMBERS

On the DGGE gel picture (Figure 3.3) it is obvious that C-samples show a significant difference between samples from the two chambers, or parallels as they are called. C-sample differences in the two chambers needs to be established before any further analysis. Cluster analysis (Figure III.B) of C-samples revealed that the similarity between the two parallels in B- and SB-layer were small (only approximately 36% similar). Similarity between T-samples was also low (about 56%). Only ST-samples showed a high degree of similarity (68 % similarity). This is confirmed by NMDS analysis (Figure IV.B), where parallels from the B-, SB- and T-samples are distancing themselves far from each other. This indicates dissimilarity between parallels. ST parallels are situated closest compared to the rest. Since only one sample is represented in each group, ANOSIM analysis cannot be performed.

In further analysis C-samples from each chamber (D or T-D) should only be compared with A-samples from the same chamber (D or T-D). Any comparison between chambers cannot be performed because of the large dissimilarity between samples taken from the two different sediment chambers.

BEFORE AND AFTER CO_2

- T-LAYER: Cluster analysis (Figure III.D) of T-samples shows little similarity between the C-sample in D-chamber (pink) and A-samples in the same chamber (red). This is also confirmed by NMDS analysis (Figure IV.D) where the three D-samples distance themselves on opposite sides of the plot. A-samples are clearly dissimilar to the C-sample in this chamber, but at the same time dissimilar to each other.

The cluster analysis of the T-D-samples show that the C-sample in this chamber (light blue) is around 73 % and 68 % similar to the A-samples (dark blue) in the same chamber. NMDS

analysis also reveals that the C-sample is dissimilar to A-samples, but at the same time the A-samples are also dissimilar from each other, the same trend shown in D-samples. The only difference is that the distance between the T-D samples are considerably shorter compared to the D-samples, meaning that T-D samples are less dissimilar compared with D-samples.

-ST-LAYER: Cluster analysis (Figure III.E) of ST-samples show that the C-samples are 75 % similar. Two A-samples from both D- and T-D chambers are also quite similar to both C-samples, but at the same time the remaining two A-samples show little similarity to the C-samples. NMDS Analysis (Figure IV.E) of the ST-samples confirms this by placing both C- samples close, which indicates similarity, while A-samples are placed far from both C-samples, displaying dissimilarity from the C-samples. At the same time the parallels in the A-samples are also very dissimilar to each other, which indicates the new bacterial communities has developed in different directions. Exposing the sediment to CO_2 in two weeks has caused the bacterial community in ST-samples to change, but it could also be caused by further recovery from previous freezing of the sediments.

-SB-LAYER: Cluster analysis (Figure III.F) of SB-samples shows that the C-sample from the T-D chamber are approximately 55 and 56 % to the A-samples. The C-sample from the D chamber shows little similarity, 60 and 47 %, to the A-samples. NMDS analysis (Figure IV.F) confirms the same trend, where A-samples from both chambers are dissimilar from each other and from the prevailing C-sample. This is similar to the pattern T-samples displayed.

-B-LAYER: Cluster analysis (Figure III.G) of B-samples reveals very little similarity between the C-sample in T-D chamber and the A-samples in the same chamber. The same applies for the D chamber samples. NMDS analysis (Figure IV.G) of B-samples also shows that the C- and A-sample in D chamber are dissimilar. In TD-samples the C-sample is dissimilar from both A-samples, but the A-samples are not that dissimilar from each other.

3.3.4 DNA Sequence determination of DGGE bands

DNA sequences were determined for selected DGGE bands (see figure 3.4) as described in 2.4.6. Sequencing was unsuccessful for most bands, probably because of the high diversity and the bands located so tight, making precise extraction difficult. In some of the samples (band 4,7,10 and 11) good enough sequences to get taxonomy results (table 3.2).

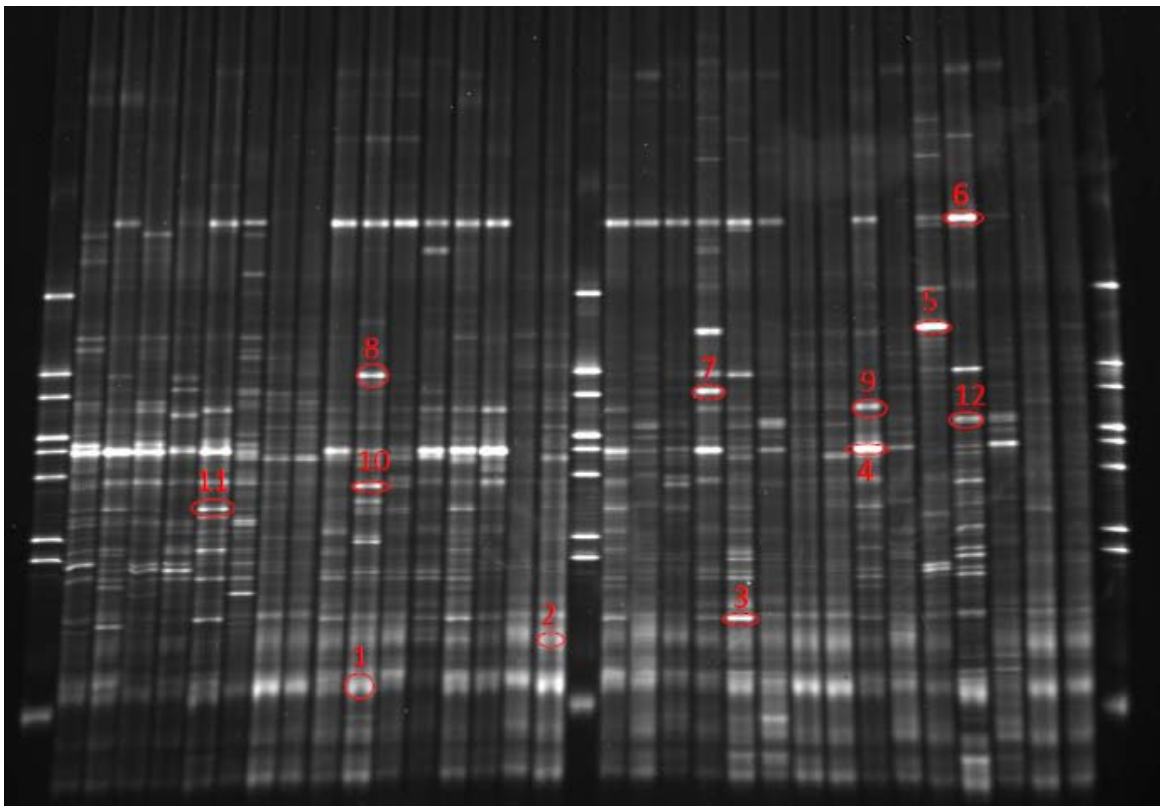

Figure 3.4: The red rings shows which bands were sent to sequencing. Bands that were bright and seemed to stand out were chosen.

Table 3.2: DNA sequencing results for selected DGGE bands (Figure 3.4) from experiment 1. Classification data was obtained by the use of classifier tool at RDP (See section 2.4.6).

Band	**Domain**	**Phylum**	**Class**	**Order**
4	Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales
7	Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales
10	Bacteria	Proteobacteria	Gammaproteobacteria	
11	Bacteria	Proteobacteria		
1, 2, 3, 5, 6, 8, 9, and 12	Bacteria			
Neg.control	Bacteria			

3.4 Experiment 2

3.4.1 PCR

Samples from the second experiment (sample nr 1-32) were amplified using PCR and the presence of products confirmed by agarose gel (Figure II.E and figure II.F). Agarose gel electrophoresis showed that the amount of PCR products was large, and the negative control gave no positive outcome. The PCR products were used further in DGGE analysis.

3.4.2 DGGE

The PCR products obtained for samples from Experiment 2 was analyzed by DGGE, and the resultant gel is shown in figure 3.5.

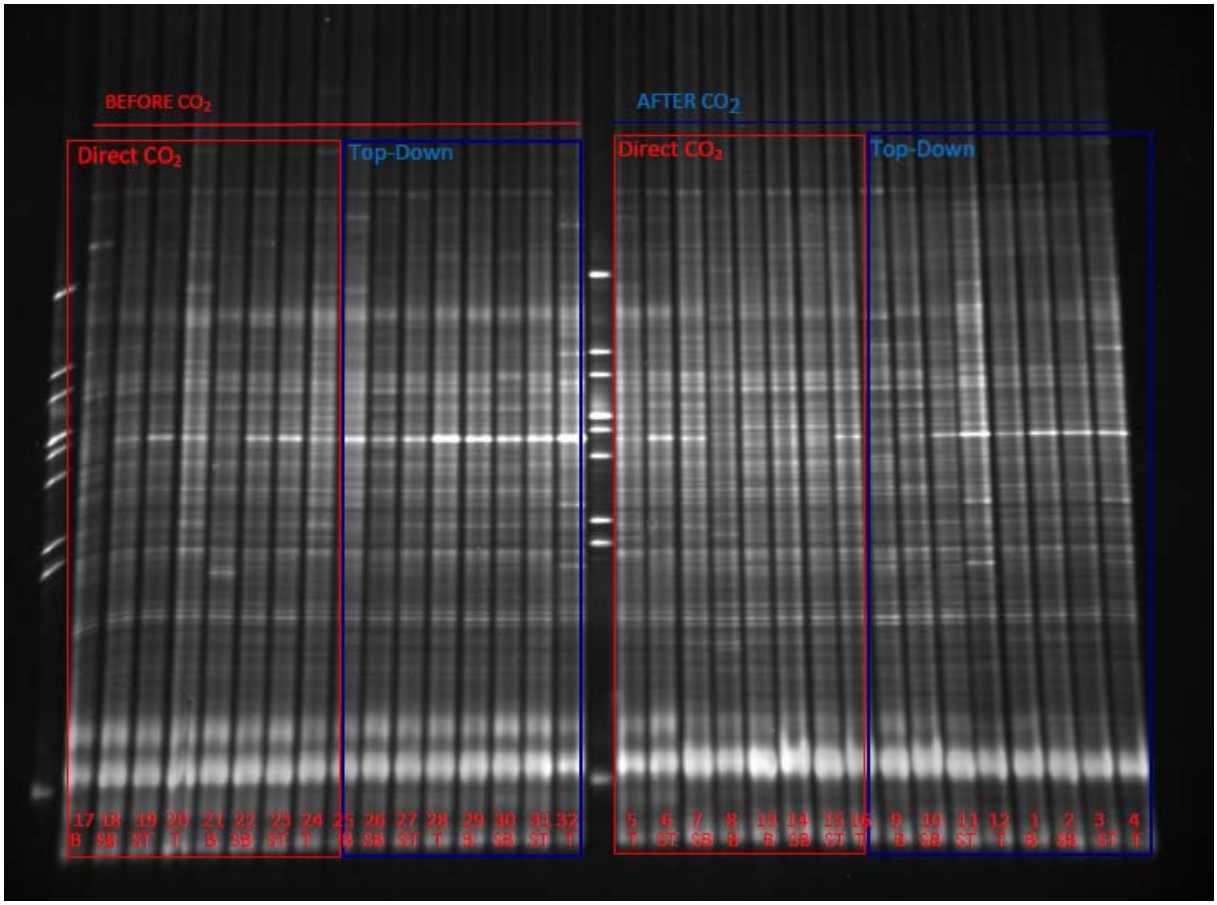

Figure 3.5: **DGGE Experiment 2**. Here the samples were ordered differently, by treatment. On the left side of the gel samples before CO_2 injection is located, while on the right side the samples from after are placed. Then the samples are divided into chambers, the sediment with CO_2 running through (red box) and the sediment next to it (blue box). In each box the samples are divided into layer, starting with bottom (B), second from bottom (SB), second from top (ST), and top (T). In each box there are two parallel samples taken.

This gel is clearly different from the first one, probably due to the use of fresh sediments instead of frozen. In general the samples from experiment 2 looks almost identical, samples seem to have little variation in band pattern.

3.4.3 Statistics

The DGGE gel results from experiment 2 are, as opposed to the gel from experiment 1, excellent, and the statistics also turned out to be credible. Sample 17 is excluded due to considerable smiling on the DGGE gel. The smiling effect was removed using Gel2K, but still the sample showed little similarity to the other samples on the gel.

LAYER DIFFERENCES

Cluster analysis (Figure III.H) of the four layers reveals that there is one layer distinguishing itself from the others. T-samples, except three samples, forms a cluster separate from the other layer samples. In general the remaining three T-samples, except one clustering with a ST-sample, show little similarity to the other layer samples. Treatment of sediment samples (before and after CO_2 exposure) is not considered in this part and it can contribute to many samples showing little similarity towards each other in the cluster analysis, since treatment with CO_2 might cause some change in the bacterial community. The samples from the other layers (B- SB- and ST-samples) do not form any pattern in the cluster, but seems to form more of a random distribution, indicating that the bacterial communities in these layers more similar than dissimilar. Vertical distribution of the bacterial community seems to be of little importance, with exception of the topmost sediment layer. This trend is also apparent in the NMDS analysis (Figure IV.H). The three layers (B- SB- and ST-samples) forming a random pattern in the cluster analysis is doing the same here, making three overlapping groups, indicating they are quite similar. Just like the cluster analysis the T-samples, except one sample which is similar to the samples in the other layers, is standing out. The top sediment layer is somewhat dissimilar to the other layers. One-way ANOSIM analysis (Table 3.3) shows that the topmost layer is significantly different from the other layers, and the three other layers do no significantly differ from each other. The top layer should therefore be treated separately, while samples from the three other layers are merged together as one, called deep sediment layer.

Table 3.3: **One-way ANOSIM analysis of layer differences**. The results show top layer is significantly different from the other layers, confirming the results from the cluster analysis and non-metric MDS. The three other layers are not significantly different from one another. Significant (P=<0,05)results are marked in yellow. R-values are included behind the P-value.

	B	SB	ST
SB	0,5539/-0,02114		
ST	0,5206/-0,01676	0,7496/-0,06194	
T	0,017/-0,2638	0,0012/0,3984	0,0418/0,1758

BEFORE AND AFTER CO_2

-TOP LAYER: Running Cluster analysis (Figure III.I) and NMDS analysis (Figure IV.I) of T-samples reveals that they are all quite similar despite the fact that half of them (A-samples) have been exposed to high concentrations of CO_2 for a month. ANOSIM analysis confirms that B-samples and A-samples are not significantly different (P=0.7697, R= -0.1458) from each other. NMDS analysis shows that one sample in the after CO_2 treatment group is placing itself nearer the B-samples than the A-samples. To check the impact of this sample, it was removed. The new cluster analysis (Figure III.J) still gave a mixed distribution of B- and A-samples. NMDS analysis (Figure IV.J) did give two distinct grouping, but all A-samples are placed very close to some of the B-samples, which mean that they are similar. The P-value was reduced to P=0.4807 and the R-value to R=-0.05556, meaning that A- samples are still not significantly different from the B-samples. The bacterial community in the top layer is therefore not severely affected by CO_2 seeping through the sediment continually for a month.

-DEEP LAYERS: The layers B, SB and ST are analyzed together as deep layer.
Cluster analysis (Figure III.K) of deep layer samples reveals that B- and A-samples, with a couple of exceptions, forms separate clusters. One A-sample (B-A-D= bottom-after-direct chamber) however, does stand out by showing little similarity to all samples, B- and A-samples. NMDS analysis (Figure IV.K) of B- and A-samples shows some of the same trend. A few of the A-samples display some similarity to a few B-samples, therefore causing the B- and A-samples to overlap on the NMDS plot, but in general the A-samples distinguish themselves from the B-samples. ANOSIM analysis confirms this to be a significant difference, with a P-value of P=0.0085, and R=0.1793.

DIRECT CO_2 CHAMBER AND TOP-DOWN CHAMBER

-TOP LAYER: Since already proven that the bacterial community in the top sediment layer was not significantly affected by CO_2 it is logical to assume that there will be no significant differences between the direct CO_2 chamber (D) and the Top-Down chamber (T-D).
Cluster analysis (Figure III.L) of T-samples, including both B-and A-samples, shows that the T-D samples forms a cluster separate from the D-samples, except one sample. It appears that both D- and T-D samples from both B-and A-samples are separated. If there already was a chamber difference between B-samples there is no point in comparing chamber differences in A-samples. NMDS analysis (Figure IV.L) visualizes this by placing D- and T-D samples opposite on the plot, for both A- and B samples. However, these are minor and uncertain

differences and not significant (Table 3.4) according to ANOSIM analysis of the samples. This means that the bacterial community in the two chambers are different already before they are exposed to CO_2.

Table 3.4: **One-way ANOSIM analysis of top layer samples from D and T-D sediment chamber**. No significant results appeared between direct chamber after samples (T-A-D) and top-down after samples (T-A-TD).

	T-A-D	T-A-TD	T-B-D
T-A-TD	0,328		
T-B-D	0,6577	0,3319	
T-B-TD	0,3368	0,6661	0,3326

-DEEP LAYER: Since there is no significant difference in the bacterial community between the deep sediment layers, they are analyzed together. A- and B-samples are evaluated separately to compare if there has been any changes in the bacterial community between the two chambers before and after exposing them to CO_2.
Cluster analysis (Figure III.M) of A-samples in deep sediment layer did show separate clustering of D- and T-D samples. The T-D samples showed similarities by clustering together, so did most D-samples also. However, two samples from the D-chamber (the two outmost samples in the cluster analysis) showed a clear dissimilarity to both D- and T-D samples. NMDS analysis (Figure IV.M) of A-samples also revealed some of the same information. Here, it is clear that one D-sample (ST-A-D) is distancing itself far from any other samples, meaning it is clearly different, causing the two chamber groups to overlap. With ANOSIM analysis giving a P-value of P=0.1048, the results are not significant, meaning that T-D samples are not significantly different from D-samples. If however, the deviant sample mentioned is removed from the dataset, ANOSIM analysis gives a P=0.0275 making the bacterial community in TD and D chambers significantly different from each other.

Although the A-samples are of interest it is important to compare the results with B-samples results to see it this not significant difference between chambers is something new. Cluster analysis (Figure III.N) of D- and T-D samples in deep sediment layer B-samples reveals that most D-chamber B-samples, except one sample (ST-B-D), show little similarity to the T-D samples. Samples from the two chambers form two separate clusters. NMDS analysis (Figure IV.N) confirms this when samples from the two groups clearly are dissimilar by appearing as two separate groups on the plot. This is a significant difference (P=0.0056, R=0.4667).
Just as with T-samples, the bacterial community in deep layer samples are already dissimilar

even before being exposed to CO_2, so any differences between the two chambers caused by CO_2 cannot be established with A-samples.

3.4.4 DNA Sequence determination of DGGE bands

DNA sequences were determined for selected DGGE bands (Figure 3.6) as described in 2.4.6. Sequencing was unsuccessful for all bands. The high diversity in this gel causes the bands to situated even more closely together compared with the DGGE gel from experiment 1. This compromises extraction, and could be causing the poor results. Also something might have happened during either the PCR or purification of PCR product that could contribute to these results being unsuccessful.

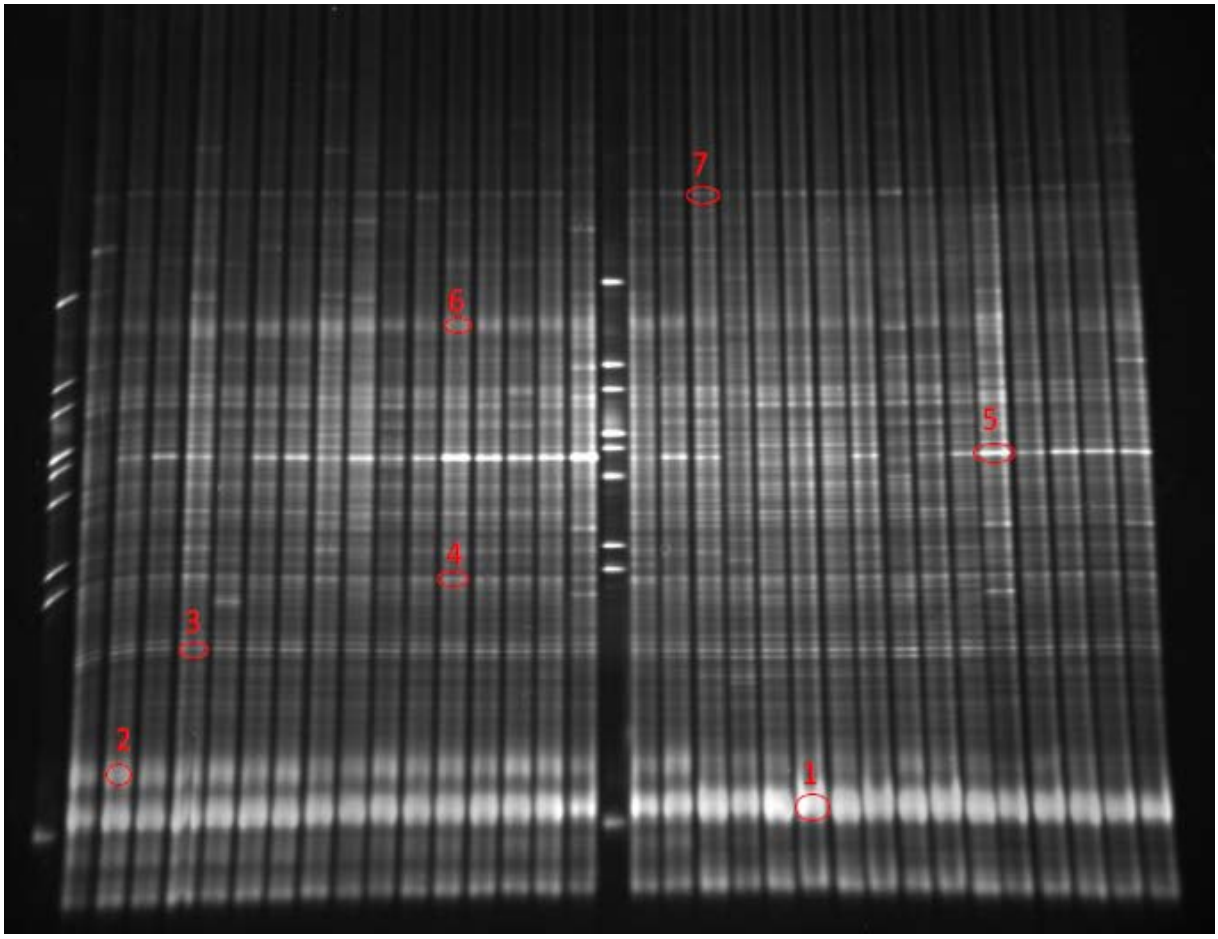

Figure 3.6: **Extraction of bands from experiment 2**. The red rings shows which bands were sent to sequencing. Bright bands and bands that seemed to stand out were chosen

4. DISCUSSION

4.1 Titanium tank experiment

This is a pilot project using an experimental setup that is unique, and nothing similar to this type of experiments has been done before. A combination of several experiment conditions makes this experiment distinguish itself from other CO_2 studies.

First of all, a titanium tank is used. The titanium tank allows the experiments conducted under pressure, simulating the conditions on the seafloor. Titanium is used because other metals will corrode when CO_2 is added, mainly because of a decrease in pH. Continuous supply of seawater from 90 m depth also provides a realistic element to this experiment. A temperature around 7 °C in the room, which is similar to the conditions on the seafloor, also contributes to keeping the conditions in the tank as similar to the seafloor as possible. The temperature of the seawater will be the same as it is at 90 m depth, and a low temperature in the room is applied to prevent it from rising. A pH between 6.6-6.9 in the tank was measured, which means that it`s probably in the worst case scenario category. Such a low pH is expected to give some effects on the bacterial community, if it were to be sensitive. How low the pH would be in the environment if an actual small leakage were to occur in the environment is unknown and will probably depend on several factors like topography and sea currents. Light conditions in the tank are also similar to the seafloor, being dark. Finally the length of the experiments spans over several weeks. When studying a bacterial community it takes time before a disturbance produces an effect. For it to be significant several dominant bacterial populations has to be changed somehow, becoming more or less dominant, or even eradicated from the community. Some populations can compensate the negative effects for a while, and some populations can even benefit from CO_2. This is a complicated process that takes time. Probably longer than a day or two, even a month might not be enough to observe the effects.

Combining all these conditions into the experiments performed makes it unique, and all together a realistic imitation of the natural conditions on the seafloor is attained. This setup is excellent for studying effects of CO_2 leakage. Most studies on CO_2 and marine environment focus on ocean acidification from the atmosphere, and with this setup it is possible to study what happens at even lower pH, when CO_2 in high concentrations enters the marine environment through a leakage from sub-seabed storages.

4.2 Sediments

The bacterial community studied in this project are natural sediment bacteria, not spiked or changed in any way. This means that most bacteria are probably uncultured, which will make them difficult to identify through sequencing. By studying a natural bacterial community and how it responds to CO_2, added to an environment similar to the sea floor, a more realistic assumption on how similar natural sediment systems responds to CO_2 is attained.

One important issue when working with coastal sediment is the importance of not assuming the sediments are homogenous. Through this thesis it is clear that the sediments definitely are heterogeneous in terms of bacterial diversity. It was obvious quite early that even in the same sediment box there could be substantial differences. Also there are differences between sediment collected at the same location at the same time. This is important to keep in mind when investigating the effects of CO_2. Being able to distinguish between natural variations in heterogeneous sediment and changes caused by CO_2 leakage is of the outmost importance. These changes are detected by using the method PCR-DGGE. This method allows monitoring of bacterial community structure and also detects any changes that might occur.

The sediment are placed in two sediment chambers, one with CO_2 running directly through, called direct chamber, or CO_2 chamber , and one without, called top-down chamber. This setup was chosen to investigate whether any changes in bacterial community occurred only in areas with CO_2 leaking directly through the sediments, or if the bacterial community in sediment in near vicinity of the leakage also suffers effects. By collecting sediment core samples, split into four layers, T, ST, SB and B, each approximately 2-3 cm thick, the effects on the bacterial community in the different layers is also studied. Layer differences are important to study because it is known that the bacterial community forms horizontal gradients in many cases. Mainly the top layer was of primary interest because this contains the highest abundance and diversity in general, but at the same time the deeper layers might also suffer effects, which is important to confirm.

Two different experiments were set up (Figure 2.5) to study the effects on the bacterial community in sediments, differing mainly length of CO_2 seepage. They are described more in detail in section 4.3 and 4.4. The experiment will hopefully reveal whether CO_2 causes the bacterial community to change or not.

4.3 Experiment 1

4.3.1 Experimental setup

Experiment 1 was conducted in from June to July 2010, using sediment stored in the freezer since February 2010. Only one sample from each sediment chamber was gathered directly after collection of sediment from the Trondheimsfjord (N-sample). The reason for not including more parallels is that these samples are not essential for the experiment. The main purpose of these samples is to check whether the bacterial community has changed from its natural state after being frozen for four months and then exposed to the experimental conditions. Using frozen sediments was not considered ideal, but was necessary since obtaining fresh sediments were impossible. Partially because of this, a "control" experiment, lasting one week, was performed before starting CO_2 injection. One week was assumed enough to allow the bacterial community to recover from any reduction due to freezing. Also it was an opportunity to investigate if the conditions in the tank itself had effects. The conditions (light, pressure, temperature, seawater) are quite similar to the natural environment of the sediments origin, so no severe changes were expected to occur in the bacterial community as a consequence of the conditions themselves. No samples were taken directly after thawing since DGGE is a method that analyzes DNA, which includes recently dead and alive bacterial. A new bacterial community needs time to establish itself so samples were taken after the control experiment to see how the community repopulated itself or if it was the same as before, and most importantly to have something to compare the A-samples with. Only one sample from each sediment chamber after the control experiment (C samples) was collected, in retrospect more parallels should have been collected at this point. Only one sample makes it statistically difficult to examine effects of CO_2.

A two week CO_2 injection experiment followed the one week "control" experiment. Two weeks are a short term experiment, and it was exciting to see whether the bacterial community was affected or not. Two weeks are probably too short to observe significant changes in the community, but this experiment also functioned as a test, to identify and fix any problems until experiment 2 was to be run. When finishing the experiment, two parallels from each chamber (D and T-D chamber samples) were collected and commonly termed as A-samples. The low number of parallels collected during all stages of the experiment is due to two reasons. The first reason is ignorance, not knowing the results, too many assumptions were made and the consequences of freezing were beyond our worst expectations. Secondly, by splitting the sediment core sample into four distinct layers it also created four times as many samples. DNA extracted from all samples had to be run on one DGGE because comparing

results from different DGGE gels is much more difficult and unpractical. Because of a limited number of wells in the DGGE gel the number of parallels had to be kept small if all were to be run on the same DGGE gel.

4.3.2 Freezing of sediments

The first experiment started in the summer 2010. Since it was impossible at the moment to get fresh sediment samples, frozen ones were used. These were collected in February, 4 months prior to the experiment. Beforehand it was assumed that the impact of freezing would be minimal on the bacteria living in the sediment. Some effects were expected, mostly minor effects like reduction in population sizes, but it was also assumed that the bacterial community would quickly recover from freezing. Literature partially agrees with this assumption. Morley et al. (1983) experimented with effects on freezing on soil bacteria. It was observed that a single freeze-thaw cycle from -27 °C to 23 °C resulted in 40-60 % mortality. But the bacteria recovered quickly and an increase in the bacterial population was observed subsequently after the experiment. The sediment samples used in this experiment did not go through such an extreme temperature change. The samples were taken during the winter, so they already were in a cold environment. Storage temperature was -22 °C, and thawed to a temperature of approximately 7 °C. Of course some mortality is expected, but it was assumed that recovery would be quick. What was not expected was that the sediment itself would change in such a large degree. Obviously a large physical change occurred, but also chemical changes could be a possible consequence. If these physical and possible chemical changes might affect the bacterial community is uncertain. However the aim of this project is to investigate the effects of CO_2, therefore the most important samples will be a comparison between C-samples and A-samples. All in all the experiment went as planned, and no events that could impact the experiment happened.

4.3.3 Analysis of sediment samples

Sediment samples from this experiment were analyzed after experiment 2 samples. Compared with samples from experiment 2, samples from experiment 1 was extremely difficult to work with. The agarose gel results (Figure II.D) revealed varying amounts of PCR product. Sediment samples, in addition to soil samples, are tough to do DNA extraction on because if inhibitors are not properly removed during the extraction, PCR might be difficult to perform. Several failed attempts might occur because something in the samples is inhibiting the reaction, probably due to improper removal during the DNA extraction. The DNA extraction kit from MoBio is effective at removing Taq polymerase inhibitors, even compared with other commercial DNA extraction kits [Whitehouse & Hottel 2007]. Problems amplifying DNA from experiment 1 samples could be caused by improper removal of inhibitors. DNA from experiment 1 and 2 samples was extracted at the same time, receiving the exact same treatment. Therefore it could be that experiment 1 samples contained a higher content of inhibitors compared with experiment 2 samples. If all inhibitors were not properly removed it could explain the troubles with amplifying the DNA from experiment 1 samples. If not the extraction method it could be caused by something else that went wrong with during the PCR reaction itself. Even though samples from experiment 1 contained varying amounts of PCR product, they were used in the DGGE but the differences in amount of PCR product were compensated for by using more/less PCR product in the DGGE depending on the band intensity on the agarose gel. The resulting DGGE gel (Figure 3.3) turned out to be of good quality. The results indicates that C-samples (samples from after control experiment) are clearly different from N-samples (samples taken upon gathering of sediments in February), which suggest that freezing and/or one week control experiment caused clear changes in the original bacterial community. Also the C-samples, taken from the two chambers, were clearly different from each other, meaning that the bacterial community can have changed in different ways from the original community in the two sediment chambers. The bacterial community in the two chambers seems to have developed differently after one week in the tank. Finally the DGGE gel results indicating layer differences. The DGGE gel was analyzed and the results treated statistically to confirm these initial results apparent on the gel.

4.3.4 Effects of freezing on the bacterial community

C-samples being different from the N-samples on the DGGE gel (Figure 3.3) indicate that storing the sediment in the freezer before the experiment started had a substantial effect on the bacterial community. It is likely that it is the freezing that caused these observed changes in the bacterial community, and not the experimental conditions. The experimental conditions are designed to be as similar as possible to the natural environment, to prevent the bacterial community to change. Experiment 2, with the same experimental conditions, did not have anything near these drastically changes. The observed change in bacterial community is confirmed statistically, using cluster analysis, NMDS and ANOSIM. Cluster analysis (Figure III.A) reveals two separate the clusters, one group of C-samples and one group of N-samples. These two groups display little similarity to each others, approximately only 28 % similarity. This is also visualized in NMDS (Figure IV.A). C- and N-samples are placed on opposite sides of the plot, indicating them being dissimilar from each other. ANOSIM confirms this dissimilarity or small similarity is significant. This means that the bacterial community structure has changed significantly between collection of the sediment and new sediment samples were taken after the control experiment, four months later.

What probably has happened is that the freezing caused a varying degree of mortality in many of the bacterial populations in the sediments. The whole community is disrupted. When placing the sediments into the tank after thawing and exposing the remaining bacteria to seafloor conditions, repopulation of the bacterial populations occurred, but the end product is a bacterial community significantly different from the original community. Alternatively the recovery of the bacterial population is a slow process, taking longer than a week. This means that the new significantly different community still might be undergoing changes in structure to recover to its original composition. The new community appears to have more intense bands, the ones that still exist, and also new bands have appeared. The CO_2 could also affect the repopulation somehow. If the bacterial community is still recovering when CO_2 is introduced it might disrupt this progress. From the DGGE gel picture (Figure 3.3) it is clear that the repopulation occurred differently in the each layer, in general more bands seems to be present in the top sediment samples and fewer in the bottom sediment samples. This indicates that there might be some differences between the layers. Any layer differences needs to be investigated and confirmed. If there are any differences between the layers, it is important that each layer is analyzed separately when investigating the effects of CO_2. If layer differences are not taken into consideration it will be much more difficult to confirm any effects of CO_2.

4.3.5 Differences between layers and chambers

Both after CO_2 exposure samples (denoted D and T-D, depending on which sediment chamber the samples are taken from, or A if samples from both chambers are included) and C-samples were tested for layer differences. This is necessary because if the bacterial community in different layers are similar, they can be treated as four parallels. C-samples and A-samples are analyzed for layer differences separately and the results compared. If there is a difference between the layers any comparison between C- and A-samples need to take layer into consideration, preferably analyze the layer samples separately.

C-samples clearly show some layer differences, both cluster analysis (Figure III.B) and NMDS analysis (Figure IV.B) from the DGGE gel (Figure 3.3) indicates that T- (see abbreviations) and ST-layer are dissimilar from B and SB layer. However, treating SB and B, ST and T as two distinct layers is probably not a good idea. The reason for this is that the difference between the layers is not significant. The reason for its insignificance might be because each group only contains two parallels, which is not enough to test for significance. The DGGE gel (Figure 3.3) clearly displays layer differences between each layer, so the layers should be treated separately. The mentioned cluster analysis and NMDS analysis also show that C-samples parallels in each layer, except ST-samples, are very dissimilar to each other. Such a high degree of dissimilarity between parallels indicates that freezing and one week experiment not only formed a bacterial community dissimilar from the original, but repopulation of the bacterial community in D and T-D chamber progressed differently. The end result is two chambers, with bacterial communities that are very little similar to one another. This is what was also observed initially on the DGGE gel. Little similarity between the bacterial community in the different layers and between parallels makes separation of each layer and chamber necessary when comparing B-samples with A-samples. It is clear that more parallels should have been taken at this point since doing this does not produce statistics of good quality.

In the case of A-samples, NMDS analysis (Figure IV.C) reveals that the T-samples differ from samples in the other layers. Also B-samples seems to be distinguishing itself from the other layers, while ST and B layer are more similar than dissimilar to each other. Only T layer are significantly different, except to B layer. The reason for such a clear change in layer difference between C- and A-samples could be caused by CO_2. This however, is not certain. It could also be the result the bacteria continuing to recover from freezing.

4.3.6 Before and after CO_2 exposure

With this setup one before sample (C-sample) from a chamber are compared with two after samples (A-samples) from the same chamber (T-D or D). Having only one C-sample to compare with two A-samples makes any results insignificant, and any results are likely to be the result of chance. It is also clear that because freezing caused such a disruption of the bacterial community, it is now difficult to distinguish between the effects of CO_2 and changes occurring due to recovery from freeze and thawing. NMDS analysis (Figure IV.D, IV.E IV.F and IV.G) and cluster analysis (Figure III.D, III.E, III.F and III.G) show results from the four layers and reveals that many C-samples, from both direct and top-down chamber, are dissimilar from the A-samples in the prevailing chambers. At the same time it is clear that the T-D and D-sample parallels in the A-samples are as much dissimilar to each others as to the C-sample it is compared with. A-samples being dissimilar to C-samples are of no importance if the A-samples are dissimilar to each others as well. Effects of CO_2 treatment itself therefore cannot be investigated with these results.

Freezing of sediments probably resulted in a reduction in many bacterial populations, causing a significant change in the bacterial community. After thawing and being exposed to the experimental conditions, the repopulation of the bacterial community did not recover into the original bacterial community. It seems like different communities were formed, causing substantial differences between the bacterial communities in the different layers and between sediment chambers. The heterogeneous nature of the sediment could also contribute to this observed effect of freezing. When comparing C-samples with A-samples it did not result in any interesting findings simply because of changes due to freezing of the sediments. Sample variation is too large, and not enough parallels were taken. The C-samples are indeed different from the A-samples, but whether this is a consequence of CO_2 or an unstable bacterial community that keeps recovering after being disturbed by freezing, is impossible to establish. What can be learned from this experience is that when studying the effects of a disturbance, like CO_2, is that the sediment should not be disturbed prior to the experiment, for instance by freezing it. Also the importance of enough sample parallels is important to keep in mind.

4.4 Experiment 2

4.4.1 Experimental setup

In the second experiment fresh sediments were used to avoid the effects of freezing. Of course, at that point it was not known how freezing would affect the bacterial community in the sediments. Starting the experiment same day as the sediment was collected was not possible, and the start of the second experiment was postponed until one week later. Meanwhile the sediments was stored inside the tank, and kept at approximately 7 °C, dark and kept moist by continually supplying seawater (1 l/min) until the experiments started. Hopefully this treatment of the sediment prevented any changes in the bacterial community until the experiment started. The water used to keep the sediments moist was the same seawater used in the experiment.

The titanium tank experiment is not to be taken lightly, since there are dangers involved. The pressure needed to be checked regularly, to make sure it was stable. Also the CO_2 needed to be controlled of leakages into the lab itself. The experiment progressed without any major obstacle. A control experiment was not included in this run. This allows a greater number of parallels to be taken, two from each chamber, four all together of both B- and A-samples. This will make the results from the statistics more credible and significance can be tested. This experiment setup is very different from the first experiment, mainly because length of the experiment is increased from two weeks to a month. Experiment 2 lasted 30 days, and the purpose of this experiment was to investigate the long-term effects of CO_2 exposure on the bacterial community. Ideally a long term experiment would last for months, but practical aspects limits the length of the experiment. An experiment lasting several months, taking samples regularly from inside the tank is preferred, but he robot arm, which was supposed to be able to do this kind of regular sample taking, lacked some of the functions necessary for collecting samples. A joint, which was to resemble the function of the human wrist, was not built, making this type of sample takings impossible. Therefore sediment samples were only taken before and after the experiment and not during the experiment. With only two years available such a long term experiment would need extensive planning beforehand, before starting the thesis even. It took almost a year for the tank to become properly finished and the people running the tank available. One month should be enough to observe the effects, the bacterial community being sensitive or not.

4.4.2 Differences between layers

Before focusing on the effects of CO_2 it is necessary to know if there are any differences between the sediment layers. Before starting the experiments it was expected that the main focus was to be on the topmost layer of the sediment samples. In general the bacterial density is highest in the topmost layer. The next layer is often also high bacterial abundance, but not necessarily as much as the topmost layer. Deeper in the sediment it is expected that the density decreases [Tholosan & Bianchi 1998]. It is not possible to measure bacterial density accurately by using the method DGGE. At best, the method is only semi-quantitative since it cannot give absolute values. If using DGGE to quantify bacterial populations it's preferred between samples on the same gel [Schauer et al. 2003]. The object of this is therefore not to estimate density, but evaluate whether there are any differences in bacterial community structure between layers. This was tested by cluster analysis, NMDS and ANOSIM, to check if the results are significant. Cluster analysis (Figure III.H) of the experiment 2 DGGE results (Figure 3.5) reveals that there is one layer distinguishing itself from the others. As expected most of the samples in the top layer is forming a cluster separate from the other samples. Every T-sample, except three, forms separate clusters from all other sediment layer samples. T-samples show similarity to each others, but at the same time some show little similarity to some of the other layer samples. That the topmost layer seems to be dissimilar to any other layer, is an important indicator of that this is the layer that should be given the separate focus and analyzed separately. The samples from the other layers do not cluster separately, but seems to form a random clustering. Also the similarity between samples in these three layers is in general high. This indicates that the bacterial communities in the layers ST, SB and B are quite similar. However it is difficult to reach a conclusion using the cluster analysis, since some of the T-samples show some similarity to samples from the other layers. Vertical distribution of the bacterial community seems to be less important in experiment 2, with exception of the topmost sediment layer. This trend is also seen on the NMDS plot (Figure IV.H), and confirms the results from the cluster analysis. NMDS analysis reveals that B, SB, and ST samples, which form a random pattern in the cluster analysis, are doing the same here, forming three overlapping groups. This distribution suggests that the bacterial community in layer B, SB and ST, not considering treatment, are quite similar. This means that the samples in these layers should be treated together as parallels in further statistical analyses. For simplicity the layers B, SB and ST are called deep sediment layers from now. The NMDS analysis also show considerable distances between samples in the same layer, especially in the top sediment layer. This indicates that the sample parallels are dissimilar, which again

confirms the heterogeneity of the sediment. As mentioned the high bacterial heterogeneity of the sediments complicated the statistical analysis by causing large sample variations, making analyses of CO_2 exposure effects on the bacterial community more difficult. Another problem with this NMDS plot is the high stress value (0.2317). A high stress value means that the distances on the maps are distorted in some degree. This is mainly a problem for samples with small distances, as longer distances are more accurate than shorter distances. This means that even though the stress value is relatively high, large pattern are still visible [Borgatti 1997]. Also the cluster analysis also confirms the large patterns shown in NMDS analysis. ANOSIM analysis confirmed that top layer samples are significantly different from the other layers, and the three other layers do no significantly differ from each other. This means that when considering effect of treatment and chamber, the topmost layer needs to be separately treated.

4.4.3 Comparing before and after CO_2 exposure samples

4.4.3.1 TOP LAYER

Cluster analysis (Figure III.I) of before (B) and after (A) CO_2 exposure samples from top sediment layer provided mixed clusters with both B-samples and A-samples grouping together. This implies that T-samples were not affected by the CO_2 treatment. The only trend visible is that samples from direct chambers (D) have a tendency to cluster together, and the same applies for top-down samples (T-D). Even though there are no effects of the CO_2 treatment on the bacterial community in T-samples based solely on cluster analysis, it gives an indication that there might be some differences between the two chambers. This will be investigated in section 4.4.4.

NMDS analysis (Figure IV.I) of the same samples revealed that one A-sample distanced itself far from the other A-samples on the plot, showing great dissimilarity. At the same time it is obvious that among B-samples there are also variations in dissimilarity among samples. Two of the B-samples are found on opposite sides of each other on the plot, their large dissimilarity confirming that large variations in the bacterial community exists already before CO_2 exposure. The ANOMSIM analysis showed no significant difference in before and after CO_2 treatment samples in the top sediment layer. With a P-value of P=0.7697, which is very high, it is clear that B- and A-samples are more similar than dissimilar. An R-value of -0.1458 also shows this.

To test whether the one deviant A-sample mentioned was making the results insignificant, it was removed from the dataset. The new cluster analysis (Figure III.J) is still the same, B-samples clustering together with A-samples. NMDS analysis (IV.J) now shows how similar B- and A-samples really are. T-A-D sample is placed close to T-B-D, indicating there is no change between before and after CO_2 treatment in that D-chamber sample. Same applies for the T-D chamber samples. The ANOSIM analysis confirms that the bacterial community in top sediment samples has not been significantly changed by a month with CO_2 treatment. This gives a strong indication on that the bacterial community in the top sediments is resistant to CO_2. However, the small sample size suggests that any final conclusion should not be drawn. More research is needed before confirming the bacterial community in the top sediment layer is resistant to CO_2 exposure. The results are a good indication on it being resistant, and it`s apparent that the bacterial community can withstand large inputs of CO_2. The reason for this is unknown, but in general disturbances causing mortality can be counteracted by quick recovery. Several factors described in the introduction favour quick recovery among bacteria; abundance, widespread dispersal, diversity, high growth potential, high physiological flexibility, rapid evolutionary adaption and short generation time favours recovery.
It is possible that the bacterial diversity stays the same although different aspects of the bacterial community, like function or activity, can be changed. When investigating such a complex community it is difficult to go into details. Some species might be eradicated, only to be replaces by a genetically similar species. The DGGE method will not detect this; only give a quick overall estimation of the bacterial community structure.

The consequences of this layer being resistant are probably positive for the marine ecosystem. Bacteria are important for the marine environment and affect many aspects of the sedimentary microenvironment directly [Thiyagarajani et al. 2010]. The bacterial community being resistant probably means that it performs as normal despite it being exposed to such a high concentration of CO_2. This doesn't mean that should be exempted from further studies. The results are not a final conclusion. The structure of the bacterial community doesn't change, but that doesn't mean that other aspect of the bacterial community changes with CO_2. Come bacterial populations might be more or less affected than other, and this is not visible through a DGGE gel. If the focus was narrowed down from the entire community to certain populations, or groups of bacterial, effects might be seen.

4.4.3.2 DEEP LAYER

Cluster analysis of B, SB, and ST layer samples (Figure III.K) shows clearly that several clusters form, but in general it seems like B- and A-samples are separated. Some similarity between a few B- and A-samples is obvious. In general all samples, before and after CO_2 exposure, have a high degree of similarity to each other. NMDS analysis (Figure IV.K) of A- and B-samples in deep sediment layers show some overlap between B- and A-samples, but in general it seems like the A-samples distinguish themselves from the B-samples. The stress value however is 0.2501, which is high. Since only the major trend is interpreted from this plot, a high stress value is not very important to the big picture. ANOSIM analysis confirms that there is a significant difference, with a P-value= 0.0085 and a positive R-value=0.1793, between B- and A-samples. This means that the bacterial community in deep sediment layer samples is affected by CO_2, confirmed by the change in community structure. This is the opposite result of the bacterial community in top sediment layer samples, which resisted CO_2. Unfortunately there is no way to establish during this master thesis the reasons for the observed effects. Deep sediment layers being sensitive to CO_2 might be due to several reasons.

As mentioned, it is assumed that the changed bacterial community might be caused directly or indirectly by CO_2. Direct causes, or primary effects are caused by the CO_2 itself, the bacterial not being able to live in an acidified and CO_2 rich environment. Indirectly causes, or secondary effects, can cause the observed changes in bacterial community structure because CO_2 is changing the metal chemistry of the seawater and sediment. If it is either one, or a combination of both, is impossible to establish now, but will be discussed in section 4.7. One reason to why the deep sediment layers experience effects and not the top sediments could be because of the fact that they are located deeper in the sediment; therefore they are not receiving the buffering capacity the seawater provides to the top layer. The conditions deeper in the sediments might be harsher compared to the surface. Also the top sediments in general have a higher bacterial abundance and diversity compared with deeper sediments, which favours quick recovery. These reasons are only speculations, but it is logical to assume some of these proposed reasons is valid and contributes in some degree to the observed effect.

4.4.4 Top-down and direct chamber

During the previous analyses some indications of there being a difference between the two chambers has been shown. Cluster analysis of top layer sediment samples (Figure III.L) also reveals indications of there being some differences between samples in the two chambers. This is also shown in NMDS analysis (Figure IV.L) of the same samples. Direct chamber samples (denoted D) distance themselves from most top-down chamber samples (denoted T-D). However at this applies to both A- and B samples. Since B-samples shows the same dissimilarity between the two chambers as the A-samples, then this dissimilarity is probably not a result of CO_2, but more a result of natural variation in bacterial community from heterogeneous sediment samples. Same applies for deep sediment layer samples. NMDS analysis (Figure IV.N) of B-samples in deep sediment layer samples shows that the D-samples are dissimilar from T-D samples. This means that in deep sediment layer samples, as with top layer samples, differences between the bacterial communities in the two sediment chambers already exists, even before the being exposed to CO_2. This is a significant difference, P=0.0056, which means that any effects of CO_2 exposure on the two chambers cannot be detected since there already is a difference. When comparing chamber differences in A-samples the cluster analysis (Figure III.M) seems to differentiate between the two chambers, but in general the samples are showing similarity towards each other. NMDS analysis (Figure IV.M) shows that the chambers form two groups, except for one D-samples that clearly deviates. Unless this sample is removed the difference between the bacterial communities in the two chambers is insignificant according to ANOSIM analysis. It seems like something has changed since this dissimilarity is not as clear as before. This could of course be one consequence of CO_2 causing significant changes in the bacterial community in deep layer A-samples. In both top and deep layer sediment samples the difference between direct and top-down chambers seems to be unimportant. The reason for this is, again, probably the heterogeneity of the sediments. If there is any differences in how these two chambers affects the bacterial community, more homogenous sediment must be used to confirm it. Alternatively samples must be collected from the exact same spot in future experiments. This is another confirmation of how diverse this community and sediment is, which makes it tough to examine and analyze the results.

4.5 Sequencing

Sequencing of a few selected bands was performed to identify some of the bacteria present on the DGGE gel. By identifying some of the clearest and the most frequent occurring bands, more detailed information about the bacterial community can be obtained. This information can again be used in further experiments, for instance if one or several of the bacteria is of interest. More bands from experiment 1 were sequenced compared with experiment 2 because the number of bands present in this gel was higher and placed further apart, therefore making it easier to extract them. DGGE gel from experiment 2 had fewer bands, but they were situated closed together, resulting in greater difficulties with extracting the bands from the gel. This probably contributed to the poor quality of results from experiment 2. Not a single band sampled from experiment 2 DGGE gel contained an applicable sequence. Another reason for the poor results could be the appearance of double bands visualized in the agarose gel (Figure II.H). It was clear that samples with double bands gave lower quality of sequences than samples containing a single band. At the same time some bands in experiment 1 had double bands, but the sequence obtained could still be used. Not being able to obtain sequence data of reasonable quality is a common problem when working with DGGE. An important reason for these troubles is the possibility of one band containing different sequences [Schauer et al. 2003]. Even though some agarose gel samples from experiment 1(Figure II.G) contained double bands, come information were obtained. Most samples from this gel were only classified as bacteria, which is not very useful since the primers used only targets bacteria. At best the order of the bacteria were decided, which also tells very little. For instance Alteromonadales is a marine bacterium, present almost everywhere. In general the best sequences came from samples with single bands on the agarose gel.

The possibility of poor quality results when double banded samples were sent in was a known possibility, but there was no time or resources to solve this problem. By not being able to identify the bacteria present in the samples, an opportunity to confirm what type of bacteria present in the samples is lost. If some of the bacteria species is known, it is also possible to assume something about activity. This can again be investigated more in depth when future experiments are to be performed in this project. Then more focus should be given sequencing and maybe also forming cloning libraries. Sequencing is not the primary focus of this master thesis, but it would have been interesting to find some information about species present in the samples.

4.6 Differences between the experiments

The experimental setup of the two experiments are very different, and the results equally different. Experiment 1had an experimental setup that lead to results of poor quality. Experiment 2 on the other hand was a success. In general sediment samples from experiment 1 that was the most difficult to analyze. It was almost impossible to amplify DNA using PCR, while the second experiment had no such difficulties with PCR. The most probable explanation was as mentioned insufficient removal of inhibitors during DNA extraction. This, and the consequences of freezing the sediments prior to the experiment, was probably the most obvious differences between the two experiments.

If the bacterial community in N-samples from experiment 1 (Figure 3.3) are compared with B-samples from experiment 2 (Figure 3.5) they should display some resemblance since they are both taken upon gathering of the sediments. The sediments were taken on the exact same location but at different times of the year. It is clear that the bacterial community in N-samples are less diverse, containing fewer and weaker bands, compared with B-samples from experiment 2. The possibility that seasonal differences could be playing an important role is important to consider. Experiment 1 sediments were collected in February, during the winter, and experiment 2 in September, during the autumn. The conditions on the seabed are probably not very fluctuant, but it is likely that the organic content in the sediments wary between seasons. Experiment 1 sediment taken during the winter, in a period when the primary production is small, could have a small organic content compared to experiment 2 sediments, which probably have a higher content or organic matter due to a high primary production during the summer. Organic content in sediments are important to bacteria because that is their source of energy. This means that there could be a difference in the nutritional status of the bacteria in the two experiments. It would be interesting to investigate if seasonal differences influence the susceptibility of the bacterial community towards CO_2 exposure. It could be that starved bacteria (winter) tolerate less compared to well fed bacteria (autumn). All this is important to keep in mind in further experiments.

4.7 Primary and secondary effects

The bacterial community in deep sediment layer did go through a significant change as a result of the conditions in the tank. Whether this is a result of CO_2 directly (primary effects) or because of a change in metal chemistry (secondary effects), or a combination of both, is impossible to conclude with based on these experiments alone. During these experiments there was no method to distinguish between the two effects, as it is difficult separate them. The alternative is to acidify the seawater using some sort of acid instead of CO_2, or add dissolved metals to investigate the effects separately. However none of these alternatives is really an option considering it are the effects of the CO_2 leakage as a whole that is being investigated. During the course of this project not only the bacterial community was investigated. Also different analysis of metal concentration in sediment and seawater was performed. These results are important since metals play many critical roles to the bacteria in sediment. Among these roles is utilization of organic matter, by using the electron acceptors Fe(III) and Mn(IV). Oxygen is the preferred electron acceptor but its general small concentrations, especially deeper down in the sediment profile, in marine sediments make other electron acceptors, like Fe and Mn, important for the survival of the bacterial community [Nealson 1997]. The metals Fe and Mn functions as electron acceptors and if they are disrupted somehow, the bacterial community probably will be affected.

During these CO_2 exposure experiments Fe concentration was significantly increased. Different methods of collecting the metals were used (section 2.1.3) and most analysis showed an increase in iron during experiment 2. Manganese and Cobalt concentration also increased during this experiment [Slinde 2011]. Metals becoming more mobile as a result of the conditions in the tank will probably have some consequences. Whether this change is large enough to affect the bacterial community is uncertain, but this leakage were to last several months, the change in iron and manganese chemistry would probably cause some sort of change, significant or insignificant, in the bacterial community. If the iron and manganese becomes more mobile, the established horizontal gradient of electron acceptors in the sediment might be disrupted, and this will assuredly have some consequences for the bacterial community. At the top sediment layer, which did not suffer any effects of CO_2, the use of oxygen and nitrate (NO_3^-) as electron acceptors is the most important way of oxidizing organic matter, while further down the sediment profile, Mn and Fe becomes more important. It was the bacterial community in deep sediment layer that were affected by CO_2. The mobilization of Fe and Mn from sediments might therefore be an important reason to why only the deep sediment layer suffered effects from CO_2.

Although Fe and Mn being mobilized is an important indication of secondary effects playing an important role, CO_2 primary effects cannot be eliminated as a contributing cause. As mentioned the top layer might not suffer the same consequences as deep sediment layer because the overlaying seawater functions as a buffer, preventing the pH in this layer to become too low. The deeper sediment layer could by not being in contact with seawater have a lower pH in this layer compared with the topmost layer. At the same time, if this theory is right there should be some differences between the two sediment chambers, as one is exposed to CO_2 all the way through and the other not.

Other metals than iron and manganese were also investigated during this project. Metals that showed significant differences in water DGTs are Cerium (Ce), Lanthanum (La), Lead (Pb), aluminium (Al), Chromium (Cr), Arsenic (As), Uranium (U). Those that did not show any difference include copper (Cu), cadmium (Cd), zinc (Zi), and nickel (Ni) [Sundeng 2011]. Although some of the metals showing significant differences it is still a difference in seawater and not sediment. Also if these changes results in high concentrations of these metals, it is still uncertain whether this is enough to cause disturbances in the bacterial community or not. It was the bacterial community in the deep sediment layers that was affected, and a higher concentration in the seawater of these metals would probably cause more of a disturbance in the top sediment layer than to the deeper sediment layers.

Regardless of whether primary or secondary effects is causing the observed changes in community structure in deeper sediment layers, or if it is a combination of both, in the case of a real leakage in the environment it is more important to find the consequences of the observed effect that the cause of it. When more knowledge about the consequences of CO_2 exposure through leakages from subsea storages exists, more focus on what is causing the effects can be prioritized. The goal of this project is after all to investigating the consequences of CO_2 leakage on chemical and biological parameters.

4.8 Experimental summary and improvements

All in all the bacterial community in experiment 2 was more resistant than anticipated. Top sediment layer did not change at all after one month of CO_2 being added into the system. Deep sediment layer however being more sensitive. What consequences this implies is uncertain. The top sediment layer is the most important in terms of bacterial abundance, diversity and function. The fact that this layer is not affected is positive for the marine ecosystem. Much research remains before any conclusions can be made. By using the method PCR-DGGE a quick overview of the bacterial community is obtained, and it gives information about bacterial populations being present or not. This method is ideal when investigating something this new and with such a short time span at disposal. The result is a fingerprint of the dominant bacterial populations in the sediment community, and when it changes, the bacterial community displays sensitivity. What this sensitivity implies and what the consequences are is an important next step to figure out. It is also important to not exclude the top sediment layer from further work since other aspects than the bacterial community structure might be affected by CO_2. The bacterial community might keep its structure while the activity of important functions is affected. Further research is needed, whether the bacterial community is changed or not is of no use if it is not investigated further.

If the experiment were to be run again or improved, a few things would have been done differently:

- First of all, be consistent, and not switch between frozen and fresh sediments. Using frozen sediments was of course a necessity at the moment, but the results clearly imply how disastrous this is to the bacterial community. It is clear that a week in titanium tank was not enough to stabilize the new bacterial community, and the results are invalid as a result of this. The effect of freezing on the bacterial community was clearly much more disrupting compared to CO_2 exposure alone, since observing that the thawed sediment clearly caused a change in community structure. As fresh sediments as possible should be used when studying a natural bacterial community. In experiment 2 it is obvious that fresh sediments produced much more reliable results.

- Better planning before each experiment is necessary. If experiment 1 was a success, it would still be questionable because of the lack of parallels. Only having one sample to do comparisons with are not enough statistically, and the chance of the data being the result of coincidence is high. By better planning, enough parallels to run proper statistics can be obtained. Experiment 2 barely had enough samples, but if the layers are reduced to only two next experiments (top layer and deep layer) more samples can be taken and thereby more credible results obtained. It is important to keep in mind the limited number of wells on the DGGE gel. It is easiest to compare samples from the same DGGE gel. Alternatively several gels can be run, but this requires even better planning beforehand. What samples to put on each gel needs to be carefully planned.

- Length of the experiment also seems to be important. As mentioned, the robotic armed that was supposed to be used did not function properly. If samples were taken regularly from inside the tank one could in theory see how, and if, the bacterial community responds to CO_2 as a function of time. By monitoring the bacterial community it`s easier to detect gradual changes. This way more information about the response of the bacterial community is obtained, and not only change or no change, as this thesis focuses on. If this regularly collection of sample are combined with a long term experiment, lasing several months, it would confirms whether the top sediment layer really is resistant, or if it`s just able to compensate for a short period of time.

- Samples should be taken from the exact same spot. The results show indications of there being considerable variation in the bacterial community even in the same sediment chamber, and also between sediment chambers. If samples are taken at the same spot in each sediment chamber, or in near vicinity, massive differences between parallels can hopefully be avoided. Differences between sediment chambers however are not easily solved. All of these problems can be reduced by using more homogenous sediments. If taking sediment samples from the same spot it is important to keep in mind that one cannot take a core samples using this method since the spot then will disappear. The most practical is to focus only on the top sediment. Only a small amount of sediment is needed for each sampling, and this can be done easily once the robotic arm functions properly. The deep sediment layer is more difficult to sample with a robotic arm. It definitely should not be sampled at the same spot the top sediment is sampled, as this would disrupt the top sediment layer. Finding or developing a sampling method for deep sediment sampling would be important if deep

sediment layers are to be studied further. It is important to keep in mind that it was the deep layer that suffered significant changes due to CO_2, so it is probably here the CO_2 signature on the bacterial community can be found. The top sediment layer did not change, but it is the layer easiest to sample during an ongoing experiment.

- If using DGGE in further studies then sequencing should receive more attention, to try to identify the bacterial population that clearly are affected. Then, instead of focusing on the entire bacterial community, a more narrow approach is obtained by targeting a limited number of bacterial populations in the sediment samples. By identifying and narrowing down to certain bacterial populations more specific primers that targets specific groups of bacteria, for instance Fe(III) reducing bacteria, can be designed. What aspect of the bacterial community that should receive this focus needs to be decided based on results, abundance, function or activity of the bacteria present in the sediment.

- Other methods than DGGE is relevant when studying the bacterial community response to CO_2. DGGE has many benefits; it is especially a fast and economical analysis when an overview of changes in community structure is needed. Another method to assess microbial communities includes terminal restriction length polymorphism (T-RFLP) and has several benefits. Among the benefits includes its being a rapid and sensitive analysis, making it ideal technique for comparative community analysis [Marsh 1999]. When combined with constructing a 16S rDNA clone library it provides a powerful tool to track population dynamics and individual populations that changes during the experiment. Of course combining a clone library with DGGE is also possible, making it easier to identify the bands present and changing in the gel. Both DGGE and T-RFLP has several benefits and limitations so which method to use depends on what is needed from the analysis [Thiyagarajani et al. 2010]. Of course availability of the two methods also needs to be considered. In this thesis DGGE was the only alternative if the samples were to be analysed at NTNU (Norwegian university of science and technology). Combining these methods with others, for instance fluorescent in situ hybridisation (FISH) makes it possible to track and monitor specific populations during the experiment[Richardson et al. 2002]. This method can also prove to be useful in this project. If continuing to use DGGE, a cloning library should be considered since this opens for more detailed studies. The only problem is that it costs time and money.

- Coastal sediments from the Trondheimsfjord are used and it becomes quite clear that the high heterogeneity of the sediment causes problems. It might be an idea to use more homogenous sediments, or preferably from the location where CO_2 is stored, for instance the Utsira formation. Of course this might be logistically difficult. Collecting and transporting sediments from such a distance is expensive and time consuming. If this is to be done, as much information as possible about the sediment should be known beforehand. And of course it requires extensive planning. By using sediment from locations where CO_2 is actually stored a more relevant assumption on effects of leakages can be obtained.

- Unfortunately no real control experiment is possible to perform using this experiment setup. There are only one titanium tank, and no way to separate the experimental conditions from a negative control. Alternatively a control experiment should be run prior to the experiment each time. However it is not possible to run long term control experiments. The control experiment that was run prior to the first experiment was a failure due to the freezing of sediments. During the second experiment a control was not necessary because the sediments were put in the tank almost directly after collection. Until the experiment started it was kept moist, cold and dark, in that way not too different from its original environment. The experimental conditions in the lab are so similar to what the seafloor normally is that no significant effects on the bacterial community are anticipated. However, a short control experiment should be performed prior to each experiment in the future, just to confirm that the conditions in the tank have no significant effects on the bacterial community.

5. CONCLUSIONS

In retrospect experiment 1 should have been better planned. The consequences of freezing the sediments before the experiment started were underestimated. Freezing caused a significant change in the bacterial community, disrupting the bacterial community to such an extent that the effect of CO_2 became impossible to investigate. The recovery time during the control experiment was too short, so that when comparing before and after CO_2 exposure samples, the observed effects were most likely caused by a combination of recovery from freezing and CO_2 effects. Therefore in future experiments frozen sediments should be avoided.

Experiment 2, using fresh sediments, was a success. The sediment core samples were split into four layers, but only top sediment layer was significantly different from the others, reducing the numbers of layers to two in further analyses; top layer and deep layers.
One month of injecting CO_2 resulted in a significant change of the bacterial community in the deep sediment layers, indicating sensitivity. In contrast, the bacterial community in the top sediment layer did not change which indicates resistance. However, the low sample size suggests that no final conclusion can be made with respect to the top sediment layer.
Experiment 2 samples also demonstrated the heterogeneity of the sediments, with before CO_2 exposure samples varying to a large extent, making comparisons between before and after CO_2 exposure samples much more difficult. Because of this heterogeneity of the sediments there already was a difference between the bacterial communities in the two sediment chambers, even before the experiment started. Therefore the effect of CO_2 exposure on the bacterial community in the two chambers could not be determined.
Whether CO_2 directly, primary effects, or a change in metal chemistry, secondary effects, was discussed, and both possibilities seems likely to contribute to the observed change in the bacterial community in deep sediment layers.

Determination of DNA sequence was performed on several DGGE bands to identify the bacterial population present, but no useful information was obtained.

This thesis has contributed to increasing the knowledge about effects on the bacterial community structure in sediments when exposed to CO_2 leakage through the sediments. Now more studies on the bacterial community can be performed with a more limiting approach. Further studies needs to focus on a longer lasting experiment where samples are taken from inside the tank, during the ongoing experiment. In this way the bacterial community is monitored and changes in the community observed more easily. Several other improvements

have been suggested, including taking samples from the exact same spot, considering using more homogenous sediments, and many more. Also more focus on DNA sequencing of samples should be given, to identify and narrow the search down to the populations most important. It is recommended that at least some of these improvements should be followed.

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APPENDIX I: SEDIMENT SAMPLES + NANODROP

					Nanodrop (µg/µl)			
Number	Sediment	Date	Info/chamber	Weight	1 parallel	2 parallel	3 parallel	Average
1	bottom	22.10.2010	top-down	0,311	13,7	12,4		13,05
2			B	0,347	12,9	13,3		13,1
3			outer	0,321	7,9	8,8		8,35
4	top			0,315	9,6	9,4		9,5
5	top	22.10.2010	CO2	0,306	11,8	11,8		11,8
6			A	0,298	8,9	9,2		9,05
7			middle	0,395	8,6	8,7		8,65
8	bottom			0,438	11,6	12		11,8
9	bottom	22.10.2010	top-down	0,35	8,6	8,5		8,55
10			B	0,353	9,9	9,3		9,6
11			middle	0,423	8,7	8,8		8,75
12	top			0,45	12,3	12,1		12,2
13	bottom	22.10.2010	CO2	0,273	9	8,4		8,7
14			A	0,275	10,6	9,9		10,25
15			outer	0,325	11,5	10,3		10,9
16	top			0,44	7,9	8,1		8
17	bottom	14.09.2010	CO2	0,296	6,8	7,2		7
18			A	0,369	7,6	7,7		7,65
19			1	0,3	7,3	7,5		7,4
20	top			0,341	8,3	9,5		8,9
21	bottom	14.09.2010	CO2	0,53	11,9	11,5		11,7
22			A	0,34	9,9	9,1		9,5
23			2	0,314	11,2	10,5		10,85
24	top			0,382	9,5	8,3		8,9
25	bottom	14.09.2010	top-down	0,497	10,1	9,5		9,8
26			B	0,458	8	6,9		7,45
27			1	0,423	10,9	10,6		10,75
28	top			0,362	9,6	9,4		9,5

29	bottom	14.09.2010	top-down		0,634	10,2	9,1		9,65
30			B		0,517	6,8	7,1		6,95
31				2	0,433	11,5	11,5		11,5
32	top				0,482	12,2	11		11,6
33	bottom	05.07.2010	CO2		0,347	18,4	21,9		20,15
34			A		0,39	43,8	25,6	30	33,1333333
35			sample B		0,321	39,9	42,2		41,05
36	top				0,314	20,5	20,2		20,35
37	bottom	05.07.2010	CO2		0,365	24,2	24,5		24,35
38			A		0,288	23,5	27,5		25,5
39			sample A		0,419	32,6	22,5	22	25,7
40	top				0,374	15,1	25,6		20,35
41	bottom	05.07.2010	top-down		0,343	22,1	22,6		22,35
42			B		0,435	10,8	9,8		10,3
43			sample A		0,473	30,2	28,3		29,25
44	top				0,354	13,6	14,6		14,1
45	bottom	05.07.2010	top-down		0,536	17,2	48,6	18,1	27,9666667
46			B		0,354	21	16		18,5
47			sample B		0,425	31,3	22,7		27
48	top				0,483	18,4	18,5		18,45
49	bottom	18.02.2010	nr 1		0,409	33,4	14,4	15,5	21,1
50					0,332	11,9	11,8		11,85
51					0,283	30,5	16,3	16	20,9333333
52	top				0,282	20	26,6		23,3
53	bottom	18.02.2010	nr 2		0,422	15,8	16,1		15,95
54					0,373	10,7	10,2		10,45
55					0,328	17,6	17		17,3
56	top				0,424	28,2	19,4		23,8
57	bottom	23.06.2010	CO2		0,334	28	47,9	37,6	37,8333333
58			A		0,412	63,9	29,7	67,9	53,8333333
59					0,311	57,1	47,7		52,4
60	top				0,363	19	28,7		23,85

61	bottom	23.06.2010	top-down	0,36	69,8	21,3	47,1	46,0666667
62			B	0,36	19,1	17,5		18,3
63				0,323	17,5	23,5		20,5
64	top			0,365	16,6	12,4		14,5

APPENDIX II: AGAROSE GEL PHOTOGRAPHS

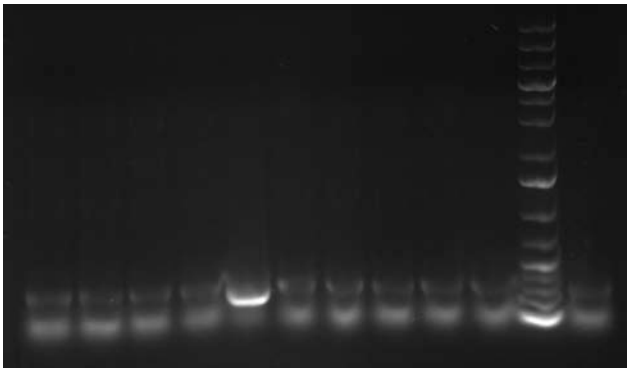

Figure II.A: **30 cycles**. First PCR run revealed 30 cycles resulted in little product in the agarose gel analysis.
Sample 1-10, DNA ladder and negative control.

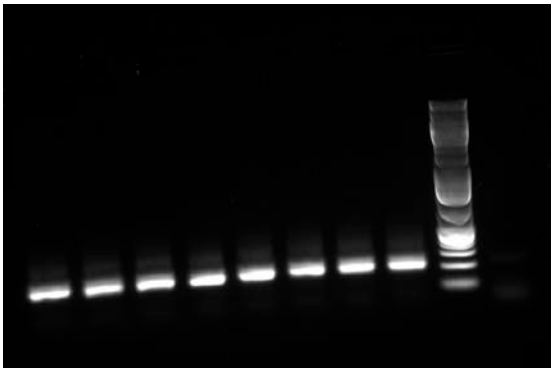

Figure II.B: **35 cycles.** Second sun revealed satisfying amount of PCR product when the number of cycles is increased to 35.
Sample 1-5 (1µl template), 1-5 (2µl template), DNA ladder and negative control.

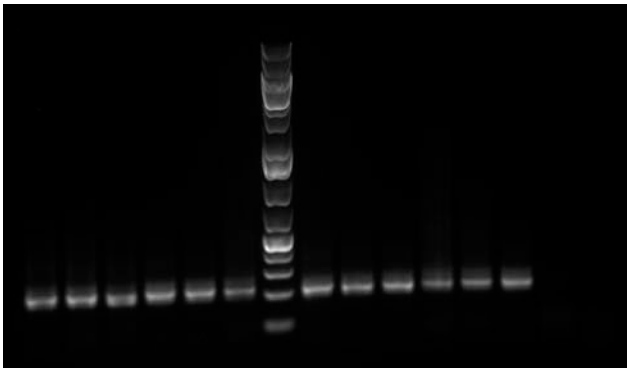

Figure II.C: **PCR optimization.**
Extra $MgCl_2$ on left side of the ladder, without extra $MgCl_2$ on right side of the ladder and varying annealing temperatures seems, based on the agarose gel, to have no substantial differences.
Same order of samples as in figure 3.1.

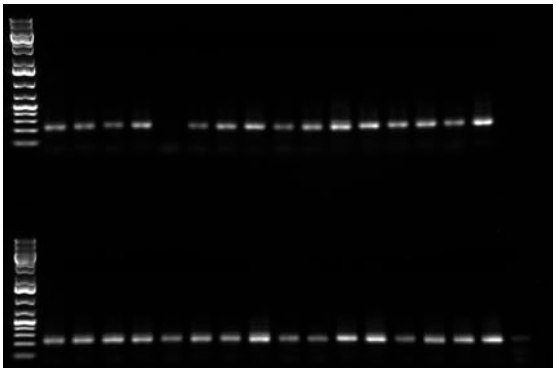

Figure II.D: **Experiment 1.** PCR products that are used when running DGGE has varying amounts of product on the agarose gel.
First row: sample 33-48
Second row: sample 49-64

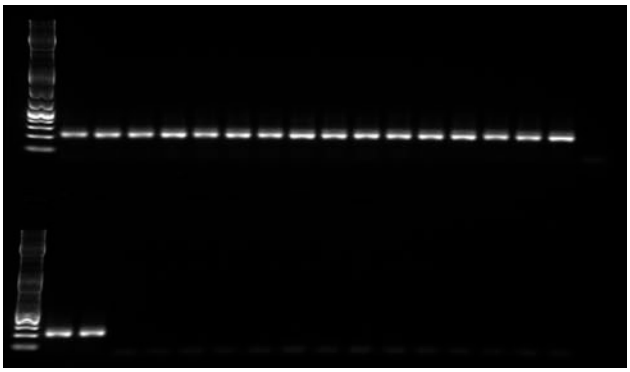

Figure II.E: **Experiment 2.** PCR product from experiment 2 used further to run DGGE have a satisfying and uniform amount in each sample.
First row: sample 17-32
Second row: sample 5 and 6

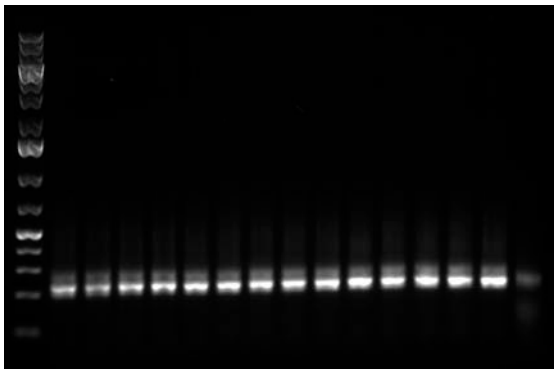

Figure II.F: **Experiment 2.** PCR product from experiment 2 used further to run DGGE have a satisfying and uniform amount in each sample.
Sample: 7, 8, 13-16, 9-12, 1-4

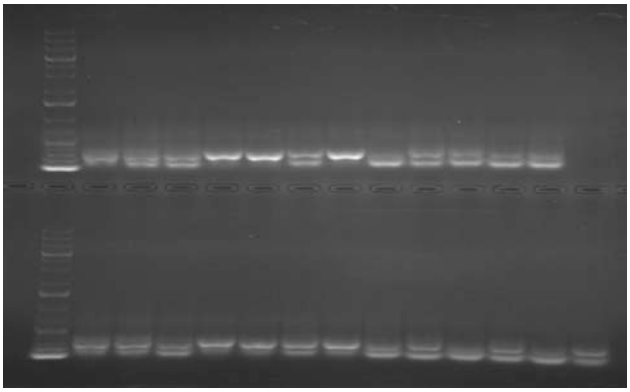

Figure II.G: **Sequencing experiment 1**.
First row: DGGE band 1-12
Second row: DGGE band 1-12 + negative control.
Only first row samples and negative control sample on second row was sent to sequencing since some of these samples had one band and not two.

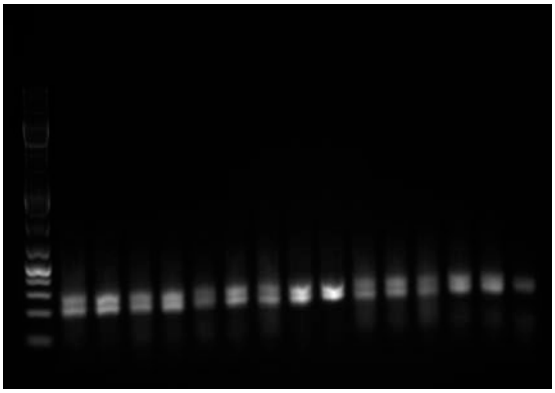

Figure II.H: **Sequencing experiment 2**.
DGGE band 1-7, 1-7 and negative control.
It was the second 1-7 bands that were sent to sequencing. All samples contain double bands.

APPENDIX III: CLUSTER ANALYSIS

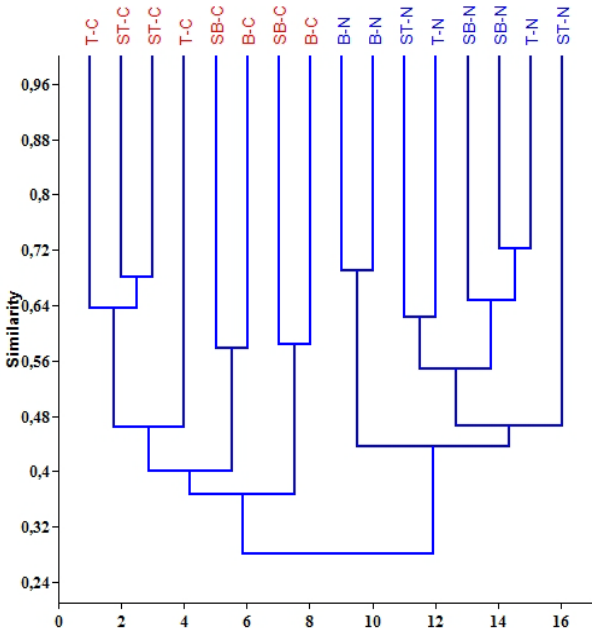

Figure III.A: **Before freezing and after one week experiment**. Before freezing (X-N = blue) samples are compared with samples taken after one week CO_2 free experiment (X-C =red) and the two groups clearly forms separate clusters. N samples show little similarity to C samples.

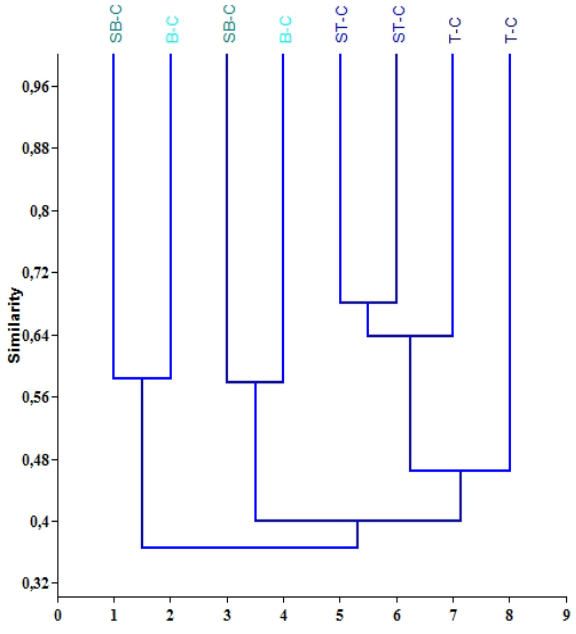

Figure III.B: **Layer and chamber differences C samples**.
The different layers (B, SB, ST and T) took after one week experiment (X-C). The similarity between different layer samples is small. Chamber differences are apparent since the similarity between parallels in the same layer are very small, except ST parallels.

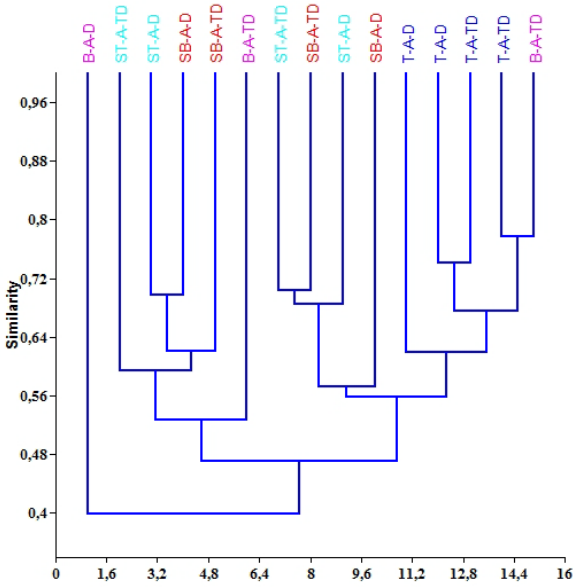

Figure III.C: **Layer differences after CO_2**. The different layers (T=blue, ST=turquoise, SB=red, B=pink) does not form separate clusters, but the T layer does distinguish itself from the others. It is clear that many samples from different layers show some similarity, making

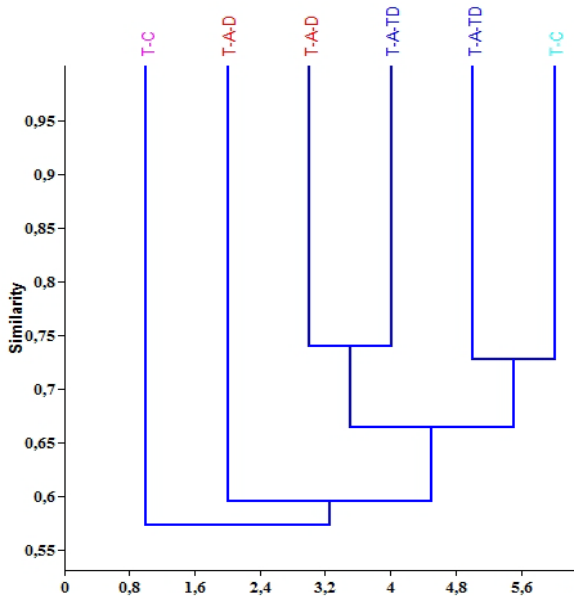

Figure III.D: **T-layer before and after CO_2**. Before sample direct chamber (pink) is 58 % similar to the after samples (red) in direct chamber. Top-down chamber before sample (turquoise) are 73 % similar to one after sample and 58 % similar to the other after sample.

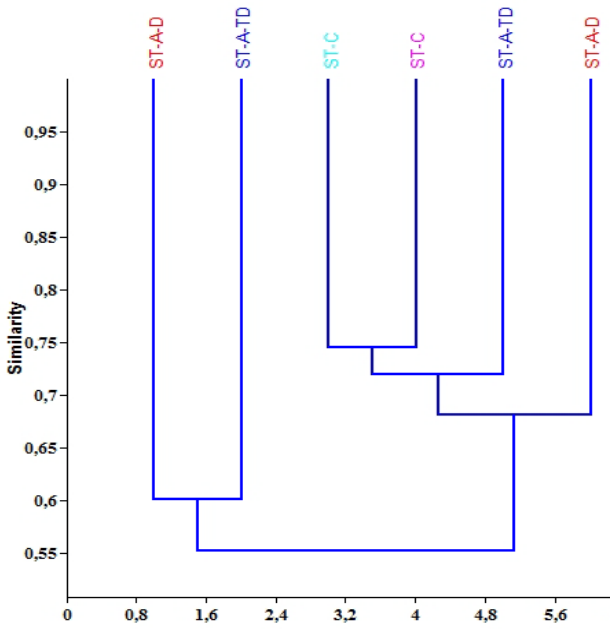

Figure III.E: **ST- before and after CO_2.** Before sample direct chamber (pink) is 70 and 55 % similar to the after samples (red) in direct chamber. Top-down chamber before sample (turquoise) are approximately 73 and 55 % similar to the other after sample in top-down chamber (blue).

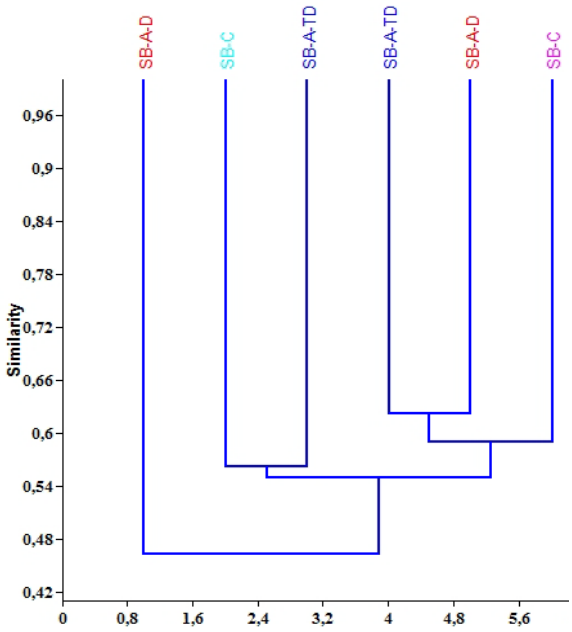

Figure III.F: **SB-before and after CO_2.** Before sample direct chamber (pink) is 60 and 47 % similar to the direct chamber after samples (red). Before sample top-down chamber (turquoise) is approximately 56 and 55 % similar to the after sample in top-down chamber (blue).

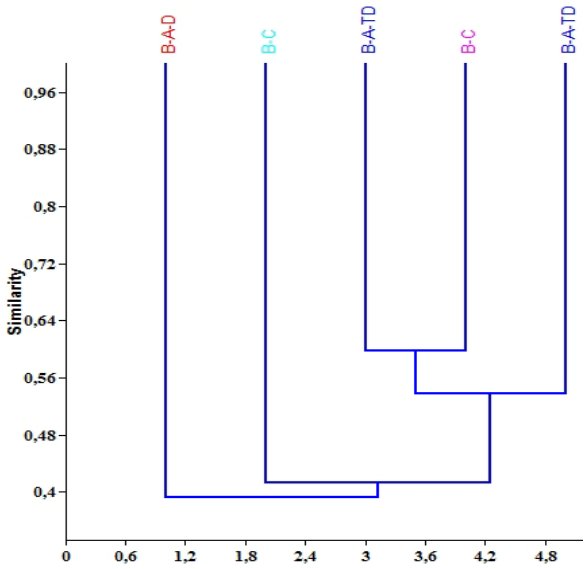

Figure III.G: **B-before and after CO_2.** Before sample direct chamber (pink) is only 40 % similar to the direct chamber after sample (red). Before sample top-down chamber (turquoise) is approximately 41 % similar to the after sample in top-down chamber (blue).

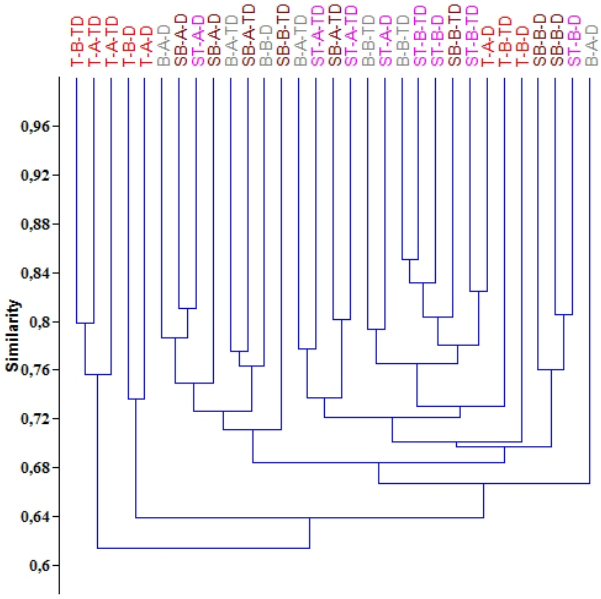

Figure III.H: **Sediment layers experiment 2**. The top sediment (samples) show clustering. This indicates they are dissimilar to the other layers, which seems to have random clustering. The bacterial community in the other layers seems (ST=pink, SB=brown and B=grey) to be quite similar to each other, and dissimilar to the top sediment layer.

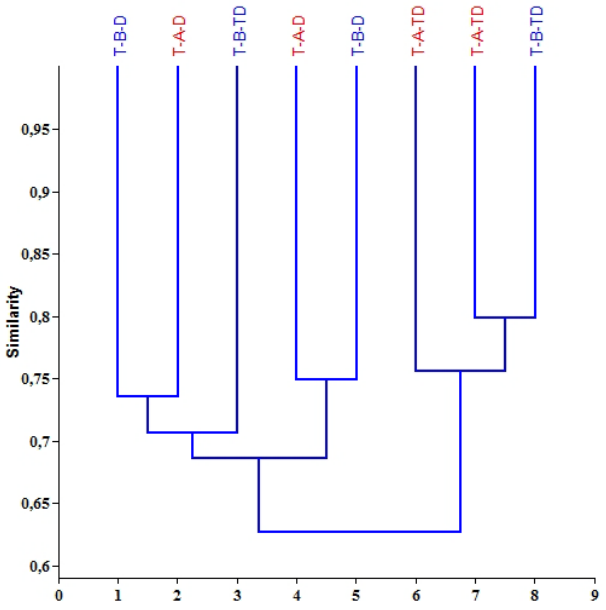

Figure III.I: **Before and after CO_2 top sediment layer**. Before CO_2 samples (blue) in top sediment layer are forming clusters with after CO_2 samples (red) in top sediment layer, indicating that they are similar.

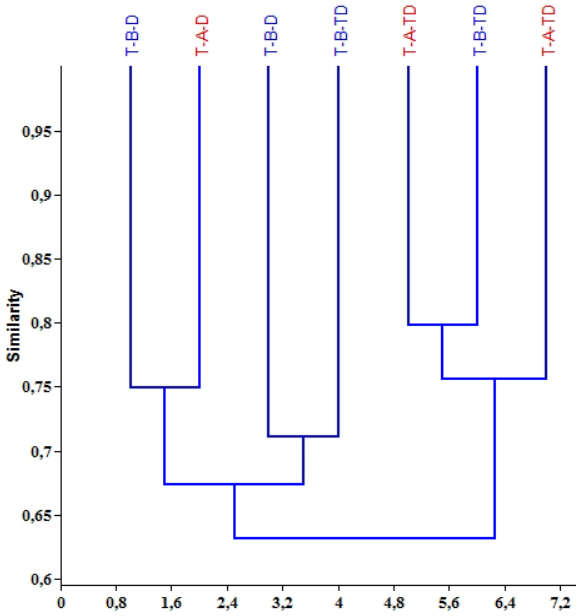

Figure III.J: **Before and after CO_2 top sediment layer**. Removing the deviant sample did not result in a better cluster analysis. The before CO_2 samples (blue) are still clustering together with after CO_2samples (red). This confirms that samples before and after is similar, approximately 63 % similarity.

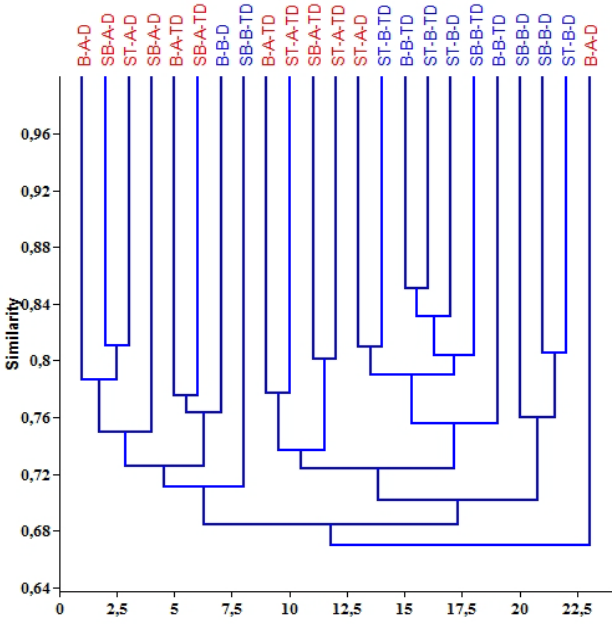

Figure III.K: **Before and after CO_2 deep sediment layer**. Before CO_2 samples (blue) are clustering together, as do the after CO_2 samples (red). A few exceptions in both groups forms clusters with the other group. In general samples from both groups are displaying great similarity towards each other, but still there seems to be differences between the two groups.

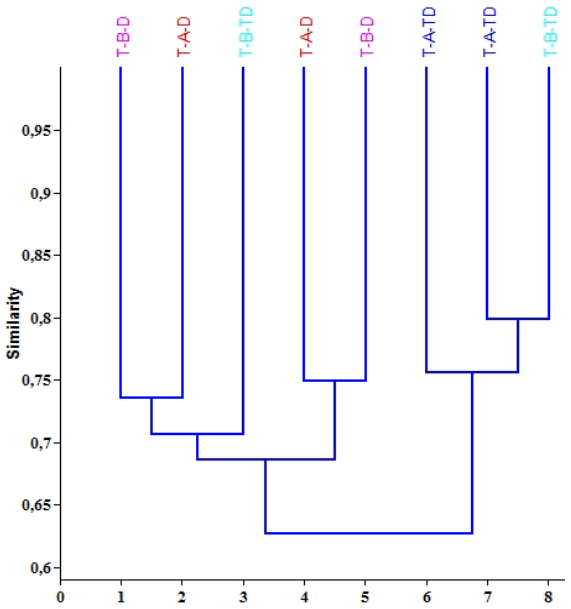

Figure III.L: **D vs. TD top sediment layer**. Before CO_2 samples in direct chamber (pink) shows similarity to the after samples same chamber (red). No apparent differences between the two chambers in other words. Before samples in top-down chamber (turquoise) also show great similarity to the after samples in top-down chamber (blue). However, one of the parallels does is not included in the cluster, indicating some differences between parallels in the two before samples.

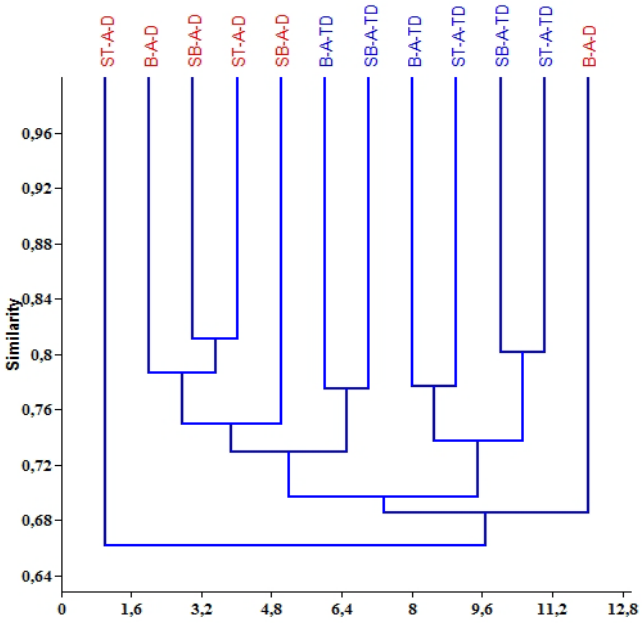

Figure III.M: **D vs. TD deep sediment layer after samples**. Top-down chamber samples (blue) are forming separate clusters away from the direct sediment chamber samples (red), which indicates that there probably are some differences between the two chambers after they have been treated with CO_2.

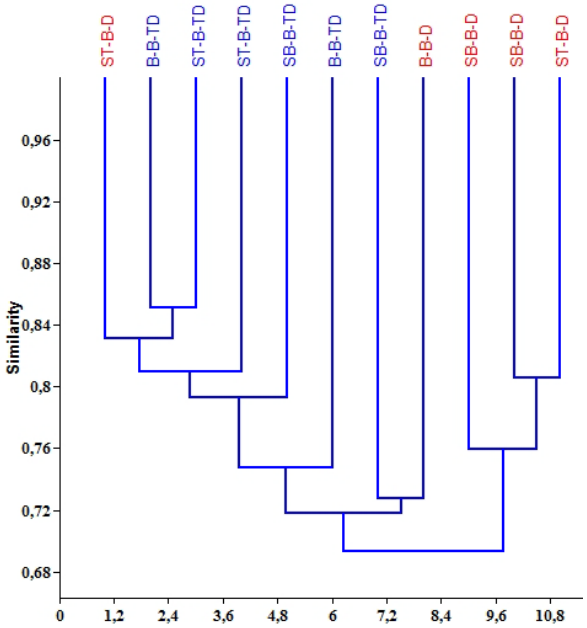

Figure III.N: **D vs. TD deep sediment layer before samples**. Top-down chamber samples (blue) are forming separate clusters away from the direct sediment chamber samples (red), which indicates that there probably are some differences between the two chambers even before they were treated with CO_2.

APPENDIX IV: NMDS

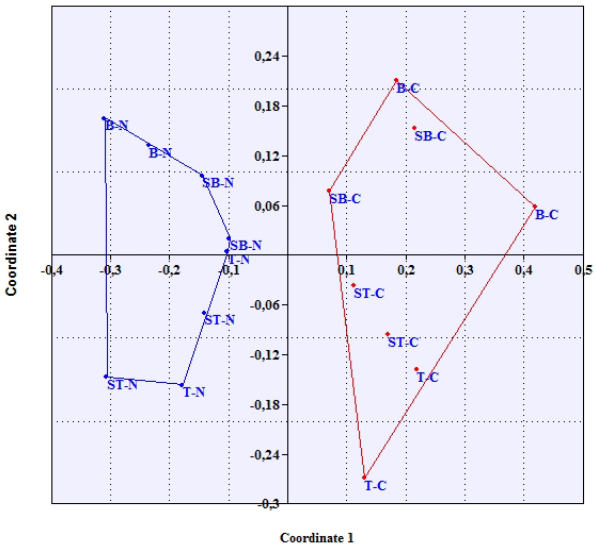

Figure IV.A: **1) Before freezing and after one week experiment**. Sediment samples directly after gathering the sediments (N-samples (blue)) from the Trondheimsfjord are clearly dissimilar from sediment samples taken after thawing and one week of control experiment (C-samples (red)).
The analysis has a stress value of 0.1344.

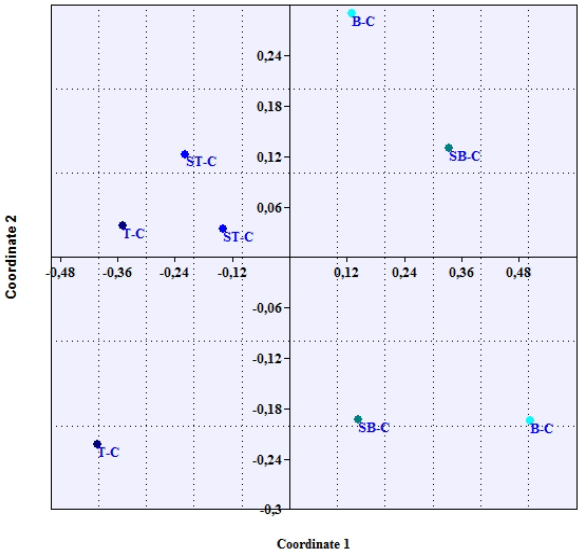

Figure IV.B: **Layer and chamber differences C samples.** ST (blue) and T (dark blue) layer samples are apparently distancing themselves from B (turquoise) and SB (blue green) layer samples. However the parallels (samples taken from different sediment chambers) are also dissimilar from each other.
The analysis has a stress value of 0.1056.

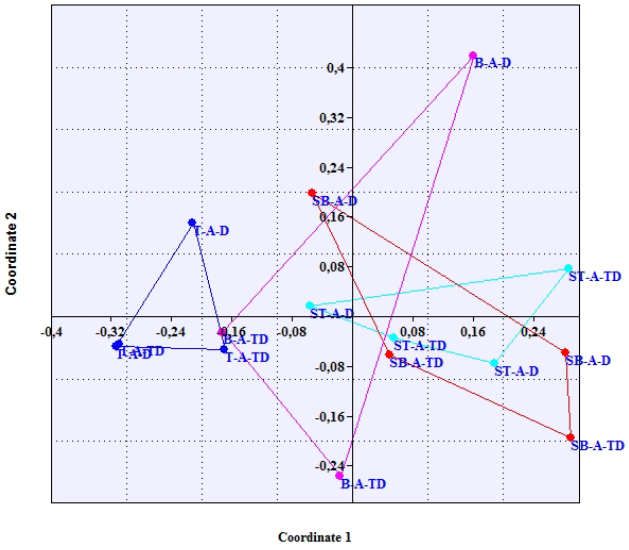

Figure IV.C: **Layer differences after CO_2.** T layer samples (Blue) seems to be placed away from the other layers. ST (turquoise) SB (red) and B (pink) layers overlap each others, but at the same time it is difficult to draw any conclusions.
The analysis has a stress value of 0.1583.

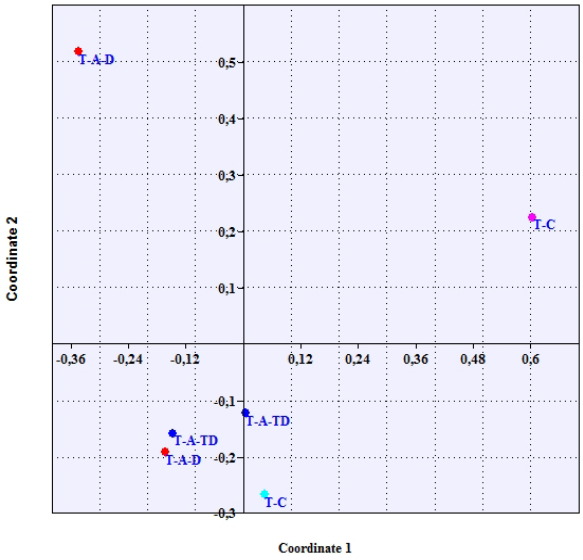

Figure IV.D: **T- before and after CO_2.** Direct chamber C sample (pink) are clearly dissimilar to both direct chamber after samples (red), which also are dissimilar to each others. The same applies for top down chamber before (turquoise) and after (blue) CO_2. Only difference is that the dissimilarity between before and after samples, and after samples parallels is smaller.
The analysis has a stress value of 0.

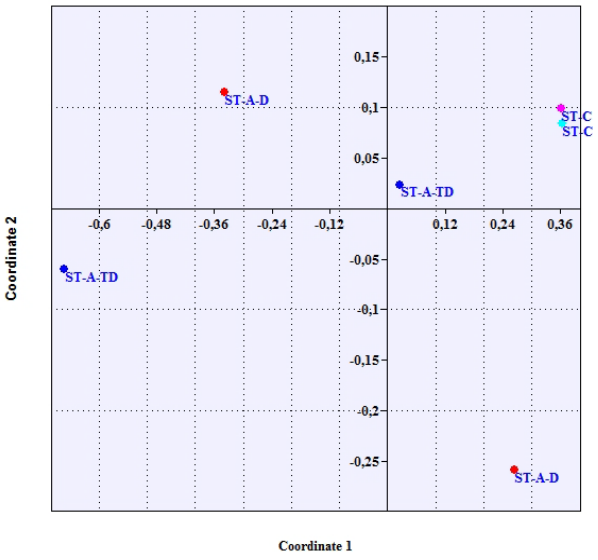

Figure IV.E: **ST- before and after CO_2.** Both direct chamber before sample (pink) and top down chamber before sample (turquoise) are very dissimilar to the after direct (red) and top-down (blue) samples. But the parallels in the after samples in both chambers are at the same time also very dissimilar from each other.
The analysis has a stress value of 0,04016.

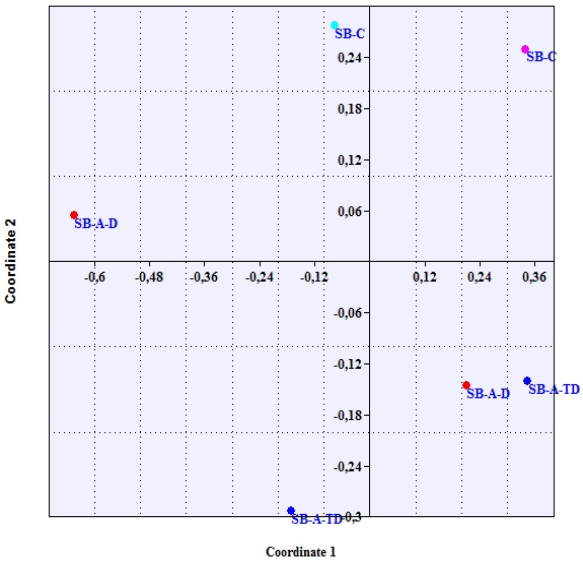

Figure IV.F: **SB- before and after CO_2.**
Direct chamber before sample (pink) and top down chamber before sample (turquoise) are dissimilar to the after direct (red) and top-down (blue) samples. Just like the other layers the parallels in the after samples are dissimilar from each other.
The analysis has a stress value of 0.

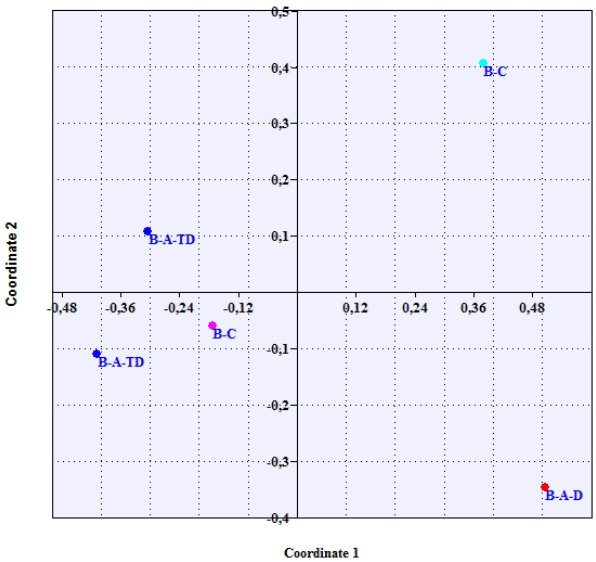

Figure IV.G: **B- before and after CO_2.** Direct chamber before sample (pink) are dissimilar to the after direct chamber after sample (red). Top down chamber before sample (turquoise) are dissimilar to the after top-down chamber samples (blue). The two after samples in the top-down chamber are placed relatively close together, indicating they are not very dissimilar.
The analysis has a stress value of 0.

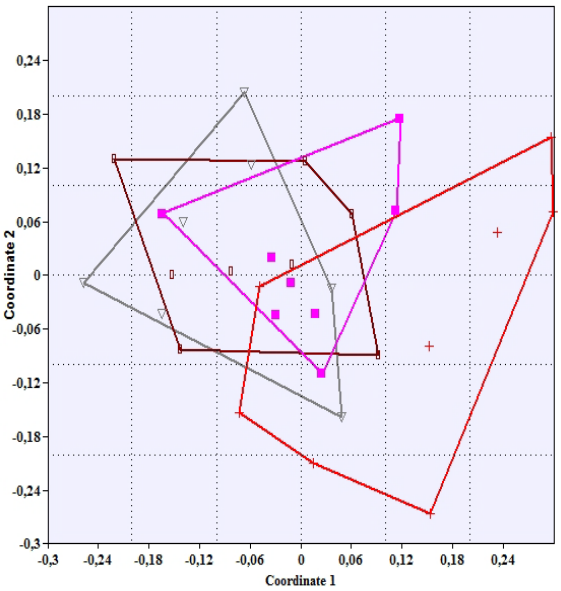

Figure IV.H: **Sediment layers experiment 2.** The top layer (red) is grouping itself away from the other layers, which is almost on top of each other. Again the dissimilarity of the top sediment is apparent. The other layers (ST (pink), SB (brown) and B (gray) show the opposite trend, indicating little dissimilarity.
The analysis has a stress value of 0,2317

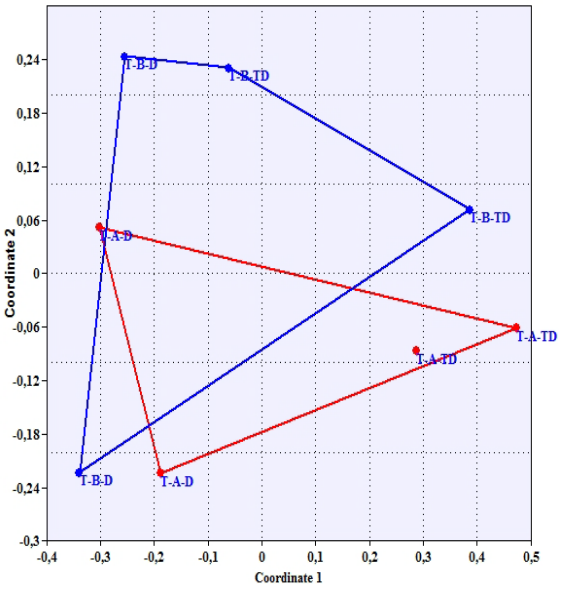

Figure IV.I: **Before and after CO_2 top sediment layer**. After CO_2 samples (red) show a great deal of overlap with before CO_2 samples (blue). At the same time it is only one after sample which causes this overlap.
The analysis has a stress value of 0,08965.

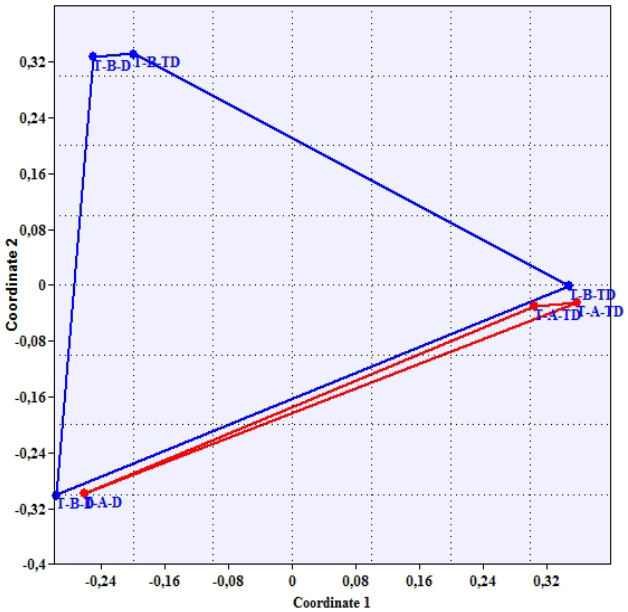

Figure IV.J: **Before and after CO_2 top sediment layer.**
After removing the deviant sample the after CO_2 samples (red) did not overlap with the before CO_2 samples (blue), but the distance between some of the before and after samples are very small. This indicates little dissimilarity between some of the before and after CO_2 samples.
The analysis has a stress value of 0,1535

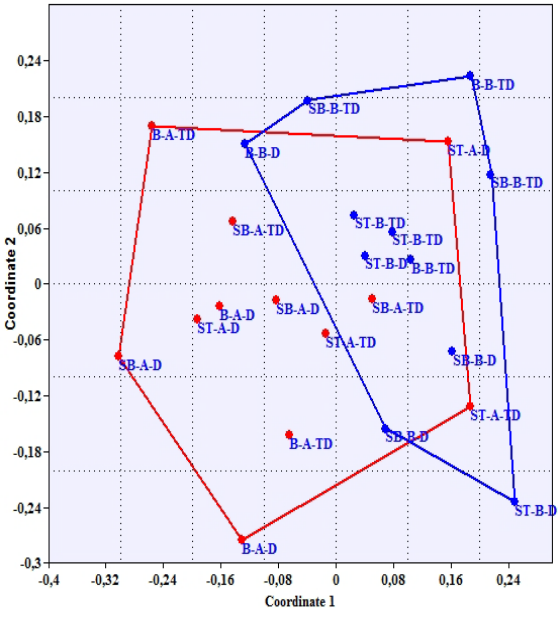

Figure IV.K: **Before and after CO_2 deep sediment layer**. After CO_2 samples (red) appear to be dissimilar to before CO_2 samples (blue). A few (three) after samples do overlap with before samples. The analysis has a stress value of 0,2501.

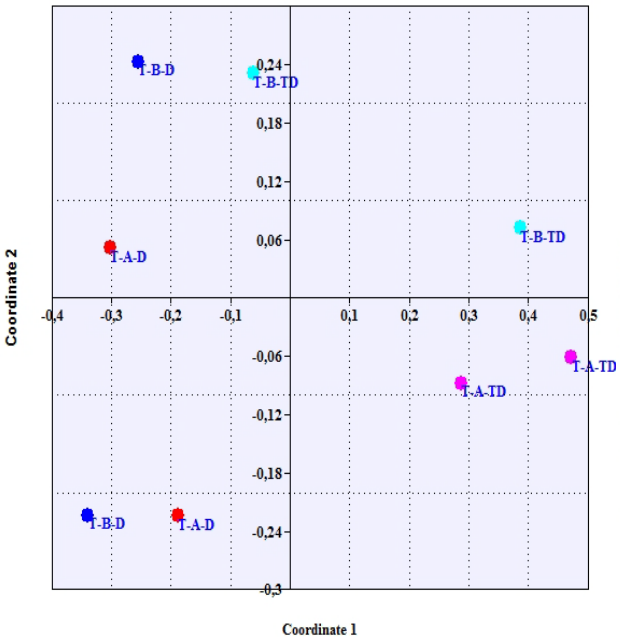

Figure IV.L: **D vs. TD top sediment layer.** Top down before (turquoise) and after (pink) samples are clearly dissimilar to direct before (blue) and after (red) samples, except one before TD sample. TD and D after samples are dissimilar. At the same time there are also great dissimilarity between the before samples, which complicates any conclusion. The analysis has a stress value of 0,08965

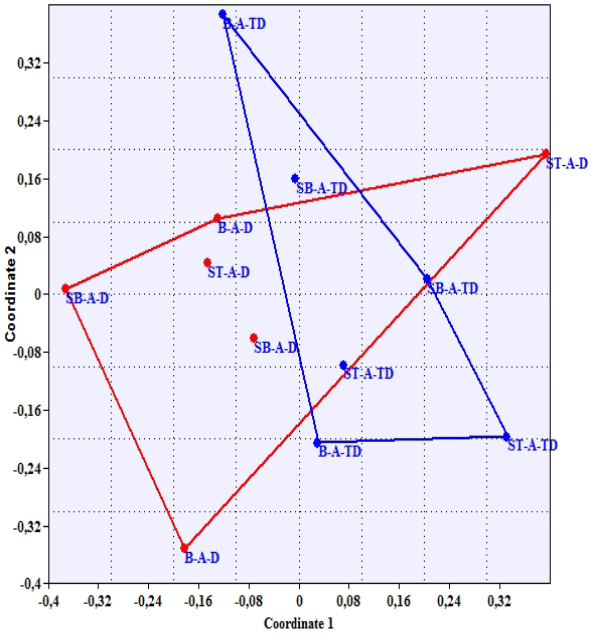

Figure IV.M: **D vs. TD deep sediment layer after samples.** Direct chamber samples (red) are dissimilar from top-down chamber samples (blue) if not for one deviant D sample.
The analysis has a stress value of 0,1569.

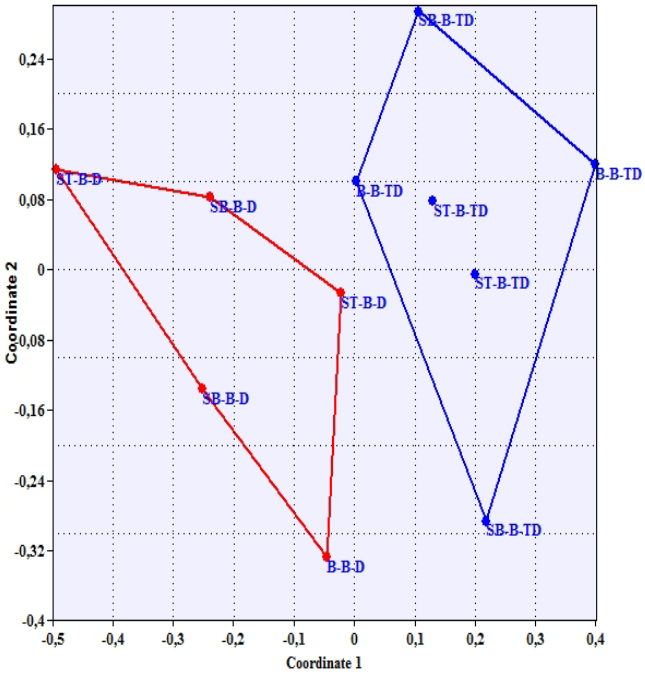

Figure IV.N: **D vs. TD top layer deep sediment layer before samples.** Direct chamber samples (red) are dissimilar from top-down chamber samples (blue).
The analysis has a stress value of 0,1618.

APPENDIX V: DNA EXTRACTION PROTOCOL

MoBio UltraClean® Soil DNA Isolation Kit

1. Approximately 0, 3 gram of sediment sample was added to the 2 ml Bead Solution Tubes provided. Vortex gently.
2. 60 µl of Solution S1 were added. Vortex before 200 µl of IRS Solution (Inhibitor Removal Solution) was added.
3. The bead beating step was supposed to be done in the Vortex Adapter from Mobio, but since this apparatus was too expensive to buy just for this single step, instead another apparatus which did the same job (shaking the tubes) were used.
4. Centrifuge tubes at 10,000 x *g* for 30 seconds before transferring the supernatant to a clean 2 ml Collection Tube.
5. Add 250 µl of Solution S2 and vortex for 5 seconds. Incubate at 4°C for 5 minutes.
6. Centrifuge the tubes for 1 minute at 10,000 x *g* before transferring the supernatant for a clean 2 ml Collection Tube.
7. Add 1 ml of Solution S3 to the supernatant and vortex for 5 seconds.
8. Load approximately 700 µl onto a Spin Filter and centrifuge at 10,000 x *g* for 1 minute.
9. Discard the flow through, add the remaining supernatant to the Spin Filter, and centrifuge at 10,000 x *g* for 1 minute.
10. Add 0, 3 ml of Solution S3 and vortex for 5 seconds.
11. Step 11 is repeated until all supernatant has passed through the Spin Filter.
12. Add 300 µl of Solution S4 and centrifuge for 30 seconds at 10,000 x *g*.
13. Discard the flow through.
14. Centrifuge again at 10,000 x *g* for 1 minute.
15. Carefully place Spin Filter in a new clean 2 ml Collection Tube.
16. Add 50 µl of Solution S5 to the centre of the white filter membrane.
17. Centrifuge at 10,000 x *g* for 30 seconds.
18. . Discard the Spin Filter. DNA in the tube is now ready for any downstream application. No further steps are required. We recommend storing DNA frozen (-20°C to -80°C).

APPENDIX VI: DGGE PROTOCOL

- Mounting of glass plates and equipment:
  The glass plates, spacer and comb was washed with deconex and 96 % ethanol before everything was assembled and placed in the gel box, and fastened.

- Preparation of DGGE solutions:
  The solutions needed to cast a gel with 35 %-55 % denaturing gradient are prepared and mixed. Also a stacking gel (0 %) is prepared:

Denaturing %	0%	80%	TEMED+10 % APS	Total volume
35	13,5	10,5	16µl + 87µl	24 ml
55	7,5 ml	16,5 ml	16µl + 87µl	24 ml
0	8 ml	0 ml	16µl + 87µl	8 ml

  0 % and 80 % acrylamide solution, Tetramethylethylenediamine (TEMED), ammonium persulfate (APS) is mixed before it`s added to a gradient mixer.

- Casting the gel:
  When casting the gel the gradient mixer will make the linear denaturing gradient necessary in DGGE. At the bottom the highest concentration (55 %) is found, before it decreases linearly to 35 %. 0 %, called “stacking gel” are added to make the top of the gel. The gel is left to polymerize for two hours before it is placed in the buffer tank with buffer solution (20 l 0.5 x TAE) kept at 60° C.

- Preparations and addition of samples:
  Wells are made by removing the comp from the gel. Samples (+/- 4µl loading dye and +/- 15µl PCR template) and the DNA ladder are prepared and applied to the wells after the wells are washed with buffer.

- Running the gel:
  Low voltage is applied for 10 minutes before turning on high voltage and buffer recirculation. The DGGE is left to run at 60 °C in 0.5 x TAE for approximately 18-19 hours using Ingeny DGGE system.

APPENDIX VII: PCR PURIFICATION PROTOCOL

QIAquick PCR Purification Kit (Qiagen)

5 volumes (100µl) of Buffer PB is added to 1 volume (20 µl) of the PCR sample and mixed.
1 µl 3M sodium acetate (pH 5,2) is added and the sample is mixed.
The sample is added in a QIAquick spin column, which is placed in a collection tube, and centrifuged for 30-60 seconds.
The flow-through is discarded and spin column is placed back in the collection tube.
0,75 ml Buffer PE is added to the spin column, and centrifuged for 30-60 seconds.
The flow-through is again discarded and after placing the spin column back in the collection tube, it is centrifuged an additional 1 minute.
The spin column is transferred to a new tube, and 25 µl MilliQ water is added. The sample is centrifuged and the purified DNA is now in the column, ready to sequencing.