Investigation of the putative iron reducing capabilities of Lokiarchaeota

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Master thesis in geomicrobiology

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Abstract

Marine subsurface sediments are large and ecologically significant microbial habitats. The archaeal phylum Lokiarchaeota is a group of organisms commonly found in these sediments. Their metabolism is unknown, but based on several indirect lines of evidence, it has been suggested that they are dissimilatory iron and/or manganese reducers, oxidising organic carbon using ferric iron [Fe(III)] and manganese [Mn(IV)] as electron acceptors. This study aims to further investigate these claims using a two-pronged approach: Firstly, to attempt to enrich Lokiarchaeota *in vitro*, and monitor the growth using molecular methods; secondly, to correlate Lokiarchaeota abundance data from quantitative polymerase chain reaction (qPCR) with data on iron and manganese concentration in the porewater of a long sediment core from the Arctic Mid-Ocean Ridge.

The results of the study are largely inconclusive, but some evidence that support the hypothesis was found. 16S rRNA gene community profiles suggest that Lokiarchaeota might have grown in one of the enrichments containing amorphous Fe(III)-oxide and pyruvate, and a possible correlation between dissolved Fe(II) and Lokiarchaeota abundance was found in the sediment core.

Acknowledgements

This study was conducted at the Center for Geobiology (CGB) at the University of Bergen. The samples studied were collected by scientists and crew on R/V G.O. Sars on CGB's summer research cruises to the Arctic Mid-Ocean Ridge in 2010 and 2014.

I would like to thank my supervisor – Dr. Steffen Leth Jørgensen – for letting me brave the deep waters, both figuratively and literally, for his invaluable guidance and assistance (and patience), and for being a Generally Great Guy. I also want to thank all the other wonderful people at CGB who have assisted me in some way or other. Thank you to everyone who participated in 2014 research cruise on the G.O. Sars for a great experience that I will not soon forget. And thanks to my good friend Håvard Falck for last minute proofreading and typography discussions.

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Contents

1 Introduction

1.1 Deep sea sediments

Marine sediments are one of the largest ecosystems on the planet, covering more than two thirds of the earth's surface (Teske & Sørensen 2008). Until relatively recently, the deep sea sediments remained almost unexplored, and they were assumed to be biologically inactive (Jannasch & Wirsen 1973). However, in the past decades, new studies have afforded us a greater understanding of their significance and the microbial activity they play host to (Parkes et al. 2000).

The sediments are primarily composed of organic matter deposited from the photic surface layers of the ocean. Most of this is removed by microbial processes at the sediment-water interface, but some of it is buried and accumulates over time, and as a result these sediments store more organic carbon than any other environment in the world (Jørgensen 1983, Parkes et al. 2000). It is also one of the habitats with the highest total number of microbial cells (Kallmeyer et al. 2012, Whitman et al. 1998).

Prokaryotic cell numbers vary, but are typically on the order of 10^8–10^9 cells/cm^3 in the topmost layers of coastal and continental margin sediments, and lower for deep sea sediments in the open ocean. They normally decline logarithmically with increasing depth, and can be on the order of 10^5–10^6 cells/cm^3 at 1000 m below the seafloor (Jørgensen et al. 2012, Parkes et al. 1994). The lower limit of life in deeply buried sediments is likely determined by temperature to be around 2–4 km below seafloor, as the geothermal gradient is 30–50 °C/km (Jørgensen & Boetius 2007).

A diverse range of metabolic processes occur in this deep-sea environment, such as carbon oxidation, ammonification, methanogenesis, methanotrophy and reduction of sulfate, manganese and iron (D'Hondt et al. 2004). There appears to be a clear link between the abundance of microbes and the availability of organic carbon (Kallmeyer et al. 2012), and also between the distribution of certain groups of organisms and the availability of terminal electron acceptors (such as sulfate, manganese and iron) (Cragg et al. 1992).

The prokaryotic populations in deep-sea sediments contain a diverse range of both Bacteria and Archaea. However, only a very small portion of these have been isolated and cultured (Fry et al. 2008). It is likely that some of these organisms – of which we still know very little about – play an important role in the major cycles of elements and energy in the ocean and, by extension, the planet as a whole (Jørgensen & Boetius 2007).

1.2 Dissimilatory iron reduction

The geochemical composition of the interstitial water in marine sediments is divided into distinct zones containing products from different redox reactions (Figure 1.1). This is believed to be because different metabolisms are competing, and as electron acceptors with higher reduction potential are depleted in the topmost layers, successively less favourable electron acceptors take over (Froelich et al. 1979).

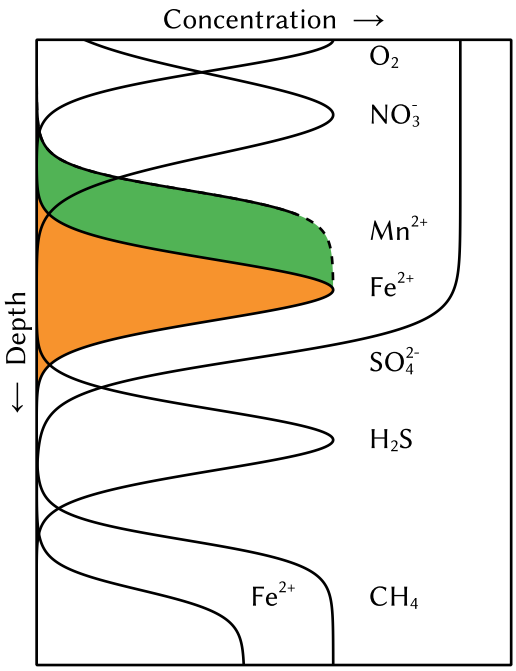


Figure 1.1: Geobiochemical zonation in sediments.

The reduction of ferric iron [Fe(III)] to ferrous iron [Fe(II)] is one of the more energy-yielding of these redox reactions. A range of Bacteria and Archaea in anoxic environments are capable of using external Fe(III) as a terminal electron acceptor. This is called dissimilatory iron reduction, as the iron is not assimilated by the microorganism (Lovley 2013). It predominantly occurs in stratified environments such as stagnant water or sediments (Nealson & Myers 1992). It is recognised as an important and ubiquitous metabolic pathway, and can in some sediments be the dominant process in the oxidation of organic matter (Canfield et al. 1993). It has been theorised that it is one of the first forms of respiration to have evolved (Lovley 2013, Vargas et al. 1998).

As Fe(III) is normally encountered in the relevant environments as Fe(III) oxides – which are insoluble in circumneutral pH – it is not readily available for utilisation by microorganisms. The insoluble Fe(III) is unable to diffuse over cell membranes, and so the reduction reaction has to take place outside the cell. The electron transfer mechanisms employed by iron reducing microorganisms (FRM) are not well understood, although a few strategies have been proposed. A terminal iron reductase – the enzyme responsible for catalysing the reaction – has not yet been identified (Weber et al. 2006).

Most FRM are also capable of reducing Mn(IV) to Mn(II) (Lovley 2013). Mn(IV) is likewise usually found as insoluble oxides in nature, and the challenges inherent to Fe(III) reduction also apply to Mn(IV). The zone where Fe(III) and Mn(IV) reduction occurs in sediments is normally between the nitrate and sulfate reduction zones (Lovley & Chapelle 1995, Figure 1.1). The reduced Fe(II) and Mn(II) are more soluble in water than their oxidised counterparts, and so increased levels of iron and manganese in the porewater from sediments samples are often indicative of Fe(II) and Mn(IV) reduction.

1.3 Lokiarchaeota

The Lokiarchaeota is a group of Archaea previously classified in the Crenarchaota phylum. It was first described in 1999 by Takai and Horikoshi under the name Deep-Sea Hydrothermal Vent Crenarchaeotal Group (DHVC) (Takai & Horikoshi 1999) and Vetriani and colleagues under the name Marine Benthic Group B (MBG-B) (Vetriani et al. 1999). It was later renamed by Takai and colleagues to Deep Sea Archaeal Group (Takai et al. 2001). Recently it has been proposed as a distinct phylum under the name Lokiarchaeota (Spang et al. 2015).

Lokiarchaeota has been found in a range of marine locations and other habitats (Jørgensen et al. 2013), and are very commonly found in deep sea sediments, where they sometimes can constitute up to 100 % of the archaeal community (Fry et al. 2008, Jørgensen et al. 2013). They appear to only thrive in anoxic environments. No members of Lokiarchaeota have been cultured, and their metabolism is unknown. It has been suggested that they are involved in sulfate reduction and methane oxidation (Inagaki et al. 2006), but other authors have disputed this (Jiang et al. 2008), suggesting instead that they are heterotrophs (Biddle et al. 2006).

Jørgensen and colleagues found a tight link between abundance of Lokiarchaeota and iron in several sediment cores, both solid iron oxide and dissolved iron in the porewater. They also found a correlation between Lokiarchaeota and dissolved manganese, and between Lokiarchaeota and organic carbon. They suggest that Lokiarchaeota could oxidate organic carbon using Fe(III) and possibly Mn(IV) as terminal electron acceptors (Jørgensen et al. 2013, Jørgensen et al. 2012).

1.4 About the study

1.4.1 Study site and sampling

The sediment cores described in this study were all retrieved from the relative vicinity of the Loki's Castle Vent Field described by Pedersen and colleagues (Pedersen et al. 2010, Figure 1.2). This is the area where Jørgensen and colleagues previously found a correlation between Lokiarchaeota and iron (Jørgensen et al. 2013, Jørgensen et al. 2012). It is located on the Arctic Mid-Ocean Ridge, in the sharp bend in the transition between the Mohns Ridge and the Knipovich Ridge. The area has been the destination for several research cruises on R/V G.O. Sars conducted by the Center for Geobiology (CGB) at the University of Bergen the last decade. Two of the cores studied were retrieved in 2010 and one during the 2014 cruise, on which the author of this thesis participated.

1.4.2 Aims

The aim for this study was to explore the relationship between Lokiarchaeota and iron. To do this, a two-pronged approach was adopted: Firstly, to attempt to create an enrichment of Lokiarchaeota *in vitro*, working under the assumption that they are indeed iron reducing organisms. The enrichments were based on two sediment cores (GS10-GC14 and GS14-GC12) col-

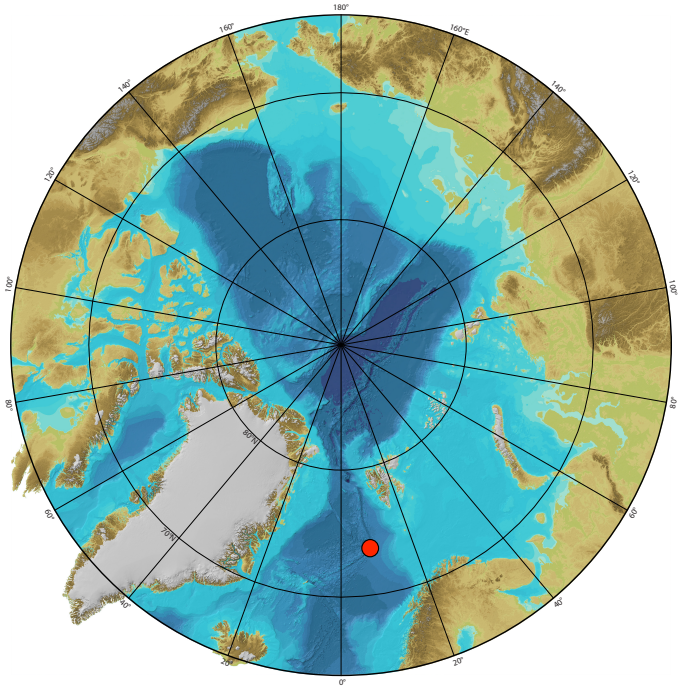


Figure 1.2: Approximate location of Loki's Castle and the sampling area in the arctic region. Image reproduced from the International Bathymetric Chart of the Arctic Ocean (IBCAO), https://www.ngdc.noaa.gov (Jakobsson et al. 2012).

lected along the Arctic Mid-Ocean Ridge and were monitored using molecular methods such as quantitative polymerase chain reaction (qPCR) and Ion Torrent 16S rRNA gene tag sequencing.

Secondly, the study aimed to further investigate the validity of the assumption that Lokiarchaeota are iron reducers by studying the depth profile of a third sediment core (GS10-PC12) from the same area, specifically looking at the concentration of Fe(II) in the porewater and the abundance of Lokiarchaeota as measured by qPCR.

2 Materials and methods

2.1 Samples and experimental setup

All of the samples that were studied in this project were collected during two of CGB's annual research cruises on the G.O. Sars. Two sediment cores – GC14 and PC12 – were collected during the 2010 cruise and one core – GC12 – was collected during the 2014 cruise. The following describes the workflow for the various samples.

2.1.1 GS10-GC14

This sample is a gravity core retrieved on June 30, 2010 in the Mohns Ridge valley (73°45′47′′N 8°27′50′′E), less than 30 km north-northeast of the Loki's Castle Vent Field at 3283 metres below sea level. It has previously been described by Jørgensen and colleagues (Jørgensen et al. 2013, Spang et al. 2015). After retrieval the core was split longitudinally into two halves – a working half and an archive – before subsampling. The core was later stored at 10 °C in the core repository of the University of Bergen.

The samples studied in this project were collected from the working half in May 2014 using a sterilised spatula. The workflow is described in Figure 2.1. Three different horizons were subsampled – 70 cm, 154 cm and 165 cm. These horizons were chosen for their proximity to other horizons with peak Lokiarchaeota abundance, as revealed by previous investigations of the core (Jørgensen et al. 2013). Approximately 0.5 g was used for DNA-extraction (see Section 2.2.2.1) and a few grams were mixed with sterile artificial seawater to make a slurry for later use (Section 2.2.1). The slurry was kept at 4 °C.

The relative abundance of Archaea, Bacteria and Lokiarchaeota was estimated using qPCR on 16S rRNA genes (Section 2.2.2.3) and the 70 cm horizon was chosen as the best candidate for enrichment due to it having the highest abundance of Lokiarchaeota. The enrichment was done as described in Section 2.2.1 and Table 2.1, using a slurry containing ~0.15 gram sediment per milliliter slurry. DNA was extracted from the enrichments after two days and again after two weeks. The DNA was screened for Lokiarchaeota and bacterial 16S rRNA genes using conventional PCR and electrophoresis (Section 2.2.2.2). Based on visual inspection of the gel, enrichments 5-8 (Table 2.1) were selected for sequencing using Ion Torrent (Section 2.2.2.4). All the Lokiarchaeota bands were fairly weak, but these enrichments were among the strongest. They also had similar enrichment conditions – they were all the enrichments with both antibiotics and H_2 – which facilitates comparison between them.

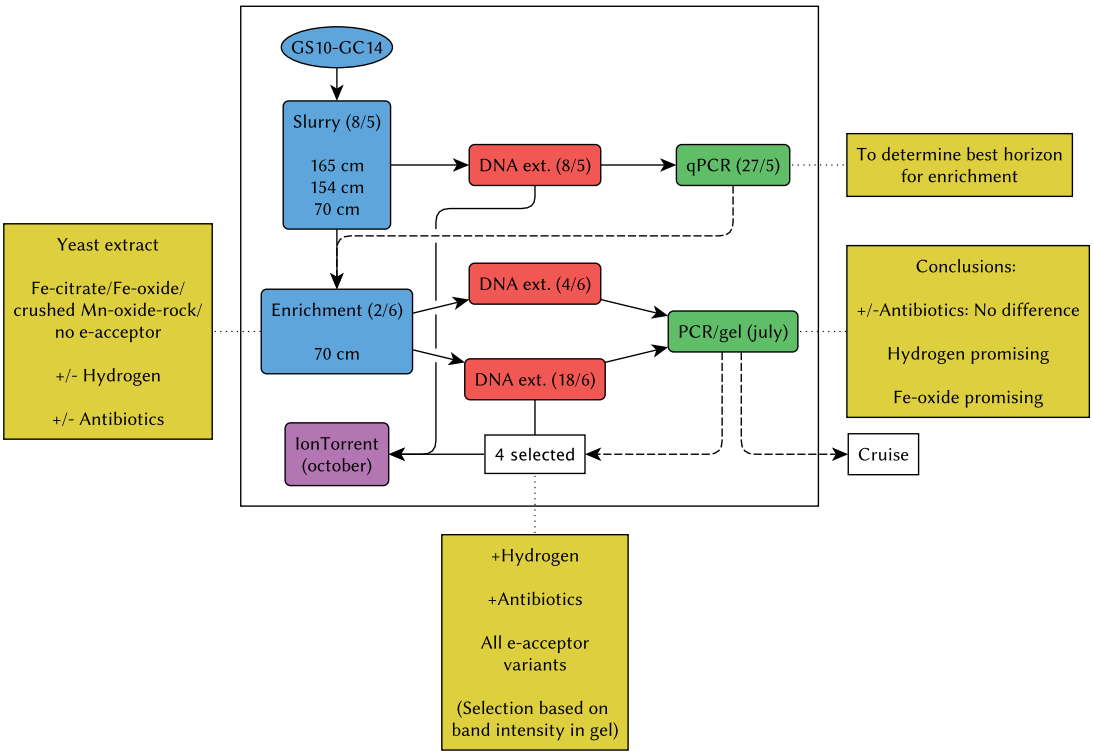


Figure 2.1: Workflow for the gravity core GS10-GC14. Solid lines represent transfer of material, dashed lines represent information that informed decisions in the process and dotted lines represent additional information in connection to various parts of the chart.

Table 2.1: Enrichment conditions for the gravity core GS10-GC14. All the enrichments were incubated anaerobically at 10 °C.

Antibiotics	Electron donor	Electron acceptor	Enrichment №
Yes	Yeast extract	Fe-citrate	1
		Amorphous iron oxide	2
		Manganese oxide	3
		No e^- acceptor	4
	Yeast ext. and H_2	Fe-citrate	5
		Amorphous iron oxide	6
		Manganese oxide	7
		No e^- acceptor	8
No	Yeast extract	Fe-citrate	9
		Amorphous iron oxide	10
		Manganese oxide	11
		No e^- acceptor	12
	Yeast ext. and H_2	Fe-citrate	13
		Amorphous iron oxide	14
		Manganese oxide	15
		No e^- acceptor	16

2.1.2 GS14-GC12

This gravity core was retrieved on July 31, 2014 in the Mohns Ridge valley (73°21′24′′N 7°33′22′′E) – around 30 km southwest of Loki's Castle – at 3280 m depth. The recovered length was 3.7 m. The core was subsampled at specific intervals (Table 2.2) using sterile plastic syringes with the tip cut off. For microbiology, two samples were taken from each horizon – one for DNA-extraction and one for potential enrichments. At the same time, pore water was collected for chemical analysis using Rhizon samplers.

Table 2.2: Subsampled horizons in the gravity core GS14-GC12.

Depth (cm)	Microbiology	Pore water	Depth (cm)	Microbiology	Pore water
3	×		200	×	
10		×	216		×
22	×		220	×	
26		×	240	×	
36		×	248		×
40	×		260	×	
47		×	276		×
60	×		280	×	
73		×	297	×	
83	×		307		×
97		×	310	×	
100	×		322		×
122	×		330	×	
127		×	337		×
143	×		350	×	
158		×	352		×
160	×		358		×
180	×		360	×	
186		×			

The workflow for the study of the core is described in Figure 2.2. DNA was extracted using the procedure described in Section 2.2.2.1. The DNA was amplified by PCR and then screened for Lokiarchaeota, Archaea and Bacteria using electrophoresis (Section 2.2.2.2). Based on a visual inspection of the gel, the 190 cm horizon was chosen for enrichment, as it had the band with highest intensity. This procedure was performed aboard the G.O. Sars.

A slurry was prepared by mixing 5 ml sediment with filter-sterilised seawater to make a total volume of 30 ml. 2 ml of this was used for each enrichment, which were prepared as described in Section 2.2.1 – with a few exeptions. Based on preliminary results from the GS10-GC14-enrichments, these enrichments were only done *with* antibiotics (Table 2.3). In addition, the amount of yeast extract in the basal medium was changed to 0.05 g and 0.9 g of pyruvate was added.

The enrichments were prepared on board the ship and stored for 3-5 days at 4 °C. Then they were flown back to Bergen in a portable cooler where they were incubated at 10 °C. After ~3 weeks, DNA was extracted again and the relative abundance of Lokiarchaeota, Archaea and

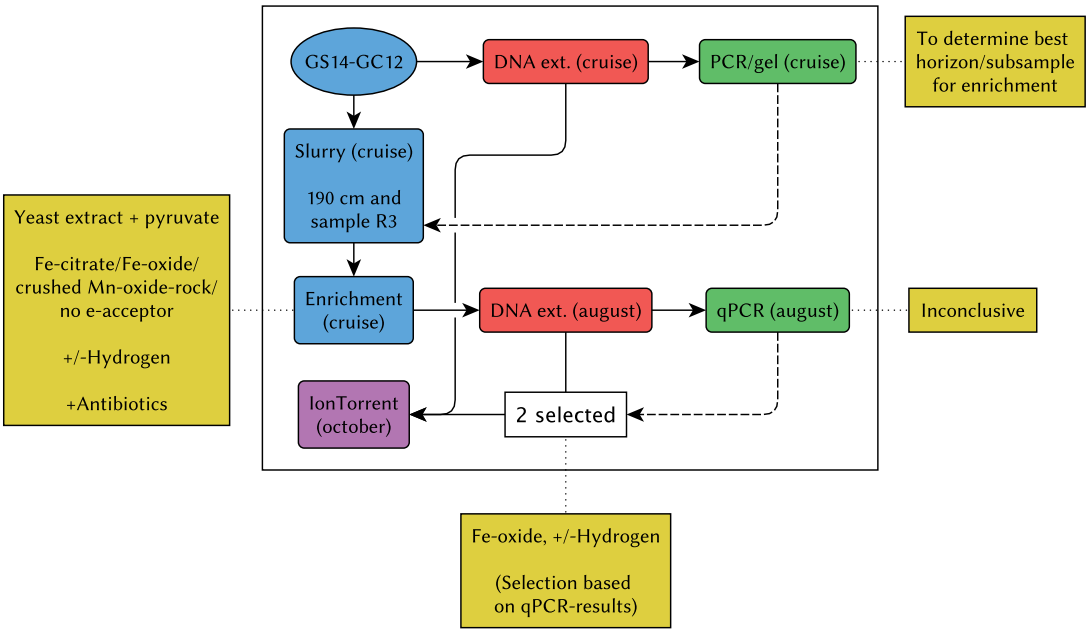


Figure 2.2: Workflow for the gravity core GS14-GC12. Solid lines represent transfer of material, dashed lines represent information that informed decisions in the process and dotted lines represent additional information in connection to various parts of the chart.

Bacteria was estimated using qPCR as described in Sections 2.2.2.1 and 2.2.2.3. Based on the qPCR results, two of the enrichments (2 and 6, Table 2.3) – as well as the slurry – were selected for sequencing using Ion Torrent (Section 2.2.2.4).

Table 2.3: Enrichment conditions for GS14-GC12. *Organic compounds* refers to yeast extract and pyruvate. All the enrichments recieved antibiotics and were incubated anaerobically at 10 °C.

Electron donor	Electron acceptor	Enrichment №
Only organic compounds	Fe-citrate	1
	Amorphous iron oxide	2
	Manganese oxide	3
	No e^- acceptor	4
Org. comp. and H_2	Fe-citrate	5
	Amorphous iron oxide	6
	Manganese oxide	7
	No e^- acceptor	8

2.1.3 GS10-PC12

This is a piston core collected using a Calypso corer. It was recovered on June 29, 2010 at 73°42′1″N 8°1′48″E, less than 20 km north-northwest of the Loki's Castle Vent Field at 2375 meters below sea level. It is 1776 cm long.

The core was subsampled at 30 cm intervals from depths 176 cm through 1766 cm. The subsampling was done using sterile plastic syringes with the tip cut off. DNA was extracted as described in Section 2.2.2.1.

16S rRNA genes for Lokiarchaeota, Archaea and Bacteria from selected horizons were quantified using qPCR as described in Section 2.2.2.3. A few samples were amplified in 1:10 and 1:50 dilutions in addition to undiluted to check for inhibiting factors. No difference was observed, so all the samples were quantified undiluted.

The concentration of dissolved iron and manganese in the porewater of the core was determined by Ole Tumyr using inductively coupled plasma mass spectrometry (ICP-MS).

2.2 Methods

2.2.1 Enrichment

Slurries were prepared by mixing sediment with sterile artificial seawater or sterilised seawater in sterile 50 ml serum bottles. The bottles were capped with thick rubber stoppers and crimps and then flushed with N_2 for several minutes to remove oxygen. The amount of sediment in the different slurries is specified above.

All glassware was rinsed thoroughly in sterile water and rubber stoppers were boiled to remove soap residues and other possible contaminants.

2.2.1.1 Preparation of media

Basic medium Modified from Zhang et al. (1999). The following was dissolved in 1 l of sterile water:

HEPES buffer	2.4 g
NaCl	30.0 g
$MgCl_2 \cdot 6H_2O$	5.0 g
$NaHCO_3$	2.5 g
$CaCl_2 \cdot 2H_2O$	0.1 g
NH_4Cl	1.0 g
Yeast extract	0.5 g
KH_2PO_4	0.01 g
Resazurin (0.2 %)	0.5 ml

The pH was adjusted to 7.7 before autoclaving the medium and storing it at 4 °C.

Basic medium with Fe(III)-citrate The ferric citrate was prepared as described in Lovley (2013). 800 ml of sterile water was heated to near boiling temperature on a stirring hot-plate. 13.7 g Fe(III)-citrate was added for a final concentration of ~50 mM. When the ferric citrate was dissolved the solution was cooled to room temperature in an ice bath. Then the pH was adjusted

to 6.0 using NaOH before adding medium constituents as described for the basic medium and bringing the volume to 1 l with sterile water. The media was the autoclaved and stored at 4 °C. Care was taken to not expose the medium to direct sunlight as to avoid photoreduction of the Fe(III).

Poorly crystalline Fe(III)-oxide The iron oxide was also prepared as described in Lovley (2013). $FeCl_3 \cdot 6H_2O$ was dissolved in water for a concentration of 0.4 M before adjusting the pH slowly to 7.0 using NaOH. The dissolved chloride was removed by centrifuging for 15 min at 5000 rpm, removing the supernatant and resuspending the iron oxide in water. This was repeated six times.

Manganese oxide A rock obtained from manganese nodules in the Norwegian Sea was crushed to a fine powder in a sterile mortar and kept in a sterile Falcon tube prior to adding it to the enrichments.

Mineral solution From Christa Schleper, personal communication. The following was dissolved in 987 ml of sterile water:

HCl (~12.5 M)	8 ml	(100 mM)
H_3BO_3	30 mg	(0.5 mM)
$MnCl_2 \cdot 4H_2O$	100 mg	(0.5 mM)
$CoCl_2 \cdot 6H_2O$	190 mg	(0.8 mM)
$NiCl_2 \cdot 6H_2O$	24 mg	(0.1 mM)
$CuCl_2 \cdot 2H_2O$	2 mg	(0.01 mM)
$ZnSO_4 \cdot 7H_2O$	144 mg	(0.5 mM)
$Na_2MoO_4 \cdot 2H_2O$	36 mg	(0.15 mM)
$FeCl_2 \cdot 4H_2O$	100 mg	(0.5 mM)

The solution was autoclaved and stored at 4 °C. Before use the solution was diluted 1:10 in sterile water, transferred to sterile serum bottles, flushed with N_2 for 1 min and capped using thick rubber stoppers and crimps.

Vitamin solution From Christa Schleper, personal communication. The following was dissolved in 1 l of sterile water:

Biotin	0.02 g
Folic acid	0.02 g
Pyridoxine HCl	0.10 g
Thiamine HCl	0.05 g
Riboflavin	0.05 g
Nicotinic acid	0.05 g
DL Pantothenic acid	0.05 g
P Aminobenzoic acid	0.05 g
Vitamin B12	0.01 g
Choline chloride	2.00 g

The pH was adjusted to 7.7 using KOH before filter-sterilising the solution, transferring it to sterile Falcon tubes and storing it at 4 °C. Before use the solution was diluted 1:10 in sterile water, transferred to sterile serum bottles, flushed with N_2 for 1 min and capped using thick rubber stoppers and crimps.

Other stock solutions A Cysteine-HCl solution (50 mg/l) and two antibiotic solutions (ampicillin and kanamycin, 5 mg/ml each) were also prepared by dissolving in filter-sterilised water in sterile serum bottles before flushing and capping as described above.

2.2.1.2 Preparing the enrichments

The different enrichment conditions for the experiments are outlined in Tables 2.1 and 2.3. 30 ml of basic medium with or without iron citrate were added to 50 ml serum bottles (27 ml for bottles with iron oxide), and ~3 g of the crushed manganese rock was added to the relevant bottles. The bottles were flushed with N_2 for ~1 min before adding thick rubber stoppers and crimps and then autoclaved. After the bottles were cooled 0.3 ml each of the vitamin and mineral solutions were added using sterile syringes. The medium was reduced using Cysteine-HCl (~0.5 ml, enough to turn the resazurin from pink to blank). Finally, antibiotics (0.3 ml of each type) and iron oxide (3 ml) was added the appropriate bottles.

The bottles were inoculated with 2.0 ml of sediment slurry and incubated at 10 °C.

2.2.2 Molecular methods

2.2.2.1 DNA extraction

DNA was extracted from all the samples using MO BIO Laboratories' PowerLyzer® PowerSoil® DNA Isolation Kit according to the manufacturer's protocol (MO BIO Laboratories, Inc. 2014) with some minor modifications which are outlined below:

- Approximately 1 ml of sample was added to the bead beating tubes. The protocol calls for 750 µl bead solution, but 20 µl polyadenylic acid (poly-A) was substituted for 20 µl of the bead solution (Hugenholtz et al. 1998). The bead beating was done in a FastPrep®-24 homogeniser from MP Biomedicals for 45 seconds at speed setting 6.
- The DNA was eluted using 100 µl sterile water.

- All samples from enrichments were washed with an oxalate buffer (Lovley & Phillips 1988b) before DNA extraction in order to remove iron that would otherwise inhibit the extraction. This was done by mixing the samples with ~1 ml of oxalate buffer in the bead tubes, centrifuging at 10 000 g for 10 min and discarding the supernatant.

2.2.2.2 PCR and electrophoresis 16S rRNA genes were amplified by polymerase chain reaction (PCR) in a Veriti® Thermal Cycler from Applied Biosystems®. Each reaction contained 10 µl HotStarTaq® Plus Master Mix from QIAGEN® at 2x concentration, 8.8 µl sterile water, 0.1 µl of each primer from 100 µM stock solutions and 1 µl template for a total reaction volume of 20 µl. The thermal conditions for the PCR were as follows:

- Polymerase activation – 5 min at 95 °C
- 25-35 cycles of:
  - Denaturation – 45 s at 95 °C
  - Annealing – 45 s at temperature determined by primers (Table 2.4)
  - Elongation – 60 s at 72 °C
- Final elongation – 7 min at 72 °C
- Cooling at 4 °C

Table 2.4: Primers used in PCR and qPCR.

Target	Forward primer	Reverse primer	Annealing temperature
Bacteria	bac341f [1]	518r [2]	58 °C
Archaea	Uni519f [3]	Arc908r [4]	60 °C
DSAG	DSAG 535f [5]	Arc908r [4]	59 °C

[1] 5′CCTACGGG(A/T)GGC(A/T)GCA3′ (Jørgensen et al. 2012).
[2] 5′ATTACCGCGGCTGCTGG3′ (Muyzer et al. 1993).
[3] 5′CAGC(A/C)GCCGCGGTAA3′ (Øvreås et al. 1997).
[4] 5′CCCGCCAATTCCTTTAAGTT3′ (Jørgensen et al. 2012).
[5] 5′ACCAGCTCTTCAAGTGG3′ (Jørgensen et al. 2013).

Electrophoresis was performed using a 1.5 % agarose gel stained with GelRed™ from Biotum in 1×TAE. The gel was run for 30 min at 50 V.

2.2.2.3 qPCR 16S rRNA genes were quantified using quantitative polymerase chain reaction (qPCR) using a StepOnePlus™ Real-Time PCR System from Applied Biosystems®. The samples were run in either duplicates or triplicates. Each reaction contained 10 µl SYBR® Green Mastermix from QIAGEN, 8.8 µl sterile water, 0.1 µl of each primer from 100 µM stock solutions and 1 µl template for a total reaction volume of 20 µl. The thermal conditions were:

- Activation – 15 min at 95 °C
- 30-35 cycles of:

  - Denaturation – 30 s at 95 °C.
  - Annealing – 30 s at temperature determined by primers (Table 2.4).
  - Elongation – 45 s at 72 °C.
  - Fluorescence detection.
- Melt curve:
  - Denaturation – 15 s at 95 °C.
  - 60 s at 60 °C, after which the temperature is increased in 0.5 °C increments to 95 °C, with fluorescence detection after each increment.

If a standard curve is not used the qPCR-program returns the threshold cycle (C_t), which is the number of cycles required for the fluorescent signal to exceed the background level. The lower the C_t is, the higher the concentration of target DNA in the sample. This number can be compared to other samples using

$$\text{Difference in concentration} = 2^{\Delta C_t}. \tag{2.1}$$

If a standard curve is created it is possible to get absolute values for the target DNA concentration. The concentration in the original sample can then be calculated using

$$\text{Target DNA-concentration in sample (copies/ml)} = \frac{C \cdot V_e}{V_t \cdot V_s}, \tag{2.2}$$

where C is the number of copies reported by the qPCR, V_e is the elution volume, V_t is the volume of qPRC-template and V_s is the volume of the sample from which the DNA was extracted.

In this case $V_e = 100\,\mu l$ and $V_t = 1\,\mu l$, so the equation simplifies to

$$\text{Target DNA-concentration in sample (copies/ml)} = \frac{C \cdot 100}{V_s}. \tag{2.3}$$

Standard curves were made using 10^2–10^7 dilution series of DNA from *Escherichia coli* for Bacteria, archaeal 16S rRNA genes in fosmid 54d9 (Treusch et al. 2005) for Archaea and a PCR amplicon from a Lokiarchaeota clone (Jørgensen et al. 2013) for Lokiarchaeota.

2.2.2.4 16S rRNA gene library preparation and Ion Torrent sequencing To investigate the community compositions before and after the attempted enrichments, 16s rRNA gene amplicons were sequenced using the Ion Torrent method by Life Technologies™. The samples were first amplified by PCR using primers 519f_Amp1 (5′CAGC(A/C)GCCGCGGTAA3′) and 805r_Amp1 (5′GACTAC(A/C/T)(A/C/G)GGGTATCTAATCC3′). These primers target both Archaea and Bacteria and thus gives a more complete picture of the community composition than primers targeting only one of the groups. Each sample was amplified in triplicate and each reaction contained 12.5 µl HotStarTaq® Plus Master Mix at 2x concentration, 9.75 µl sterile water,

0.125 µl of each primer from 100 µM stock solutions and 2.5 µl template for a total reaction volume of 25 µl. The thermal conditions were 5 min activation at 95 °C before 30 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 1.5 min. After amplification, the triplicates were pooled and the length of the PCR products were verified using electrophoresis as described in Section 2.2.2.2. Then the product was cleaned using the GenElute™ PCR Clean-Up Kit from Sigma-Aldrich® and the concentration of dsDNA was measured using a Quantus™ Fluorometer from Promega.

The products were then equipped with multiplex identifiers (MIDs), which are *barcode sequences* that allow identification of the individual samples after pooling them in the amplicon library. This is done by PCR using primers 519f_Amp2 (5′CCATCTCATCCCTGCGTGTCTCCGACTCAGAA<MID><519f_Amp1>3′, where <MID> is a five nuclebase sequence that is unique to each sample) and 805r_Amp2 (5′CCTCTCTATGGGCAGTCGGTGAT<805r_Amp1>3′). The thermal conditions were 5 min activation at 95 °C before 7 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 1.5 min. The product was verified by electrophoresis like above.

Primers were removed from the samples using Agencourt AMPure XP from Beckman Coulter. AMPure XP beads were mixed with the samples in a 7:10 ratio and incubated at room temperature for 5 min to allow the amplicons to bind to the magnetic beads. The solution was then placed on a magnet stand for 5 min before removing the supernatant. The beads were washed for 1 min with 500 µl 70 % ethanol twice while still on the magnet before being allowed to air-dry for 5 min. Finally the amplicons were eluted from the magnetic beads using 25 µl 10 mM Tris-Cl, vortexed for 20 s and placed back on the magnet for 5 min to separate the beads from the amplicons. The cleared liquid was then transferred to clean tubes without disturbing the beads.

The amplicons were checked with electrophoresis to verify removal of primers, and the dsDNA concentration was measured by Quantus Fluorometer. They were then diluted to 26 pM and added to the amplicon library in equal amounts.

The amplicon library was sequenced on the Ion Torrent PGM platform in the core facility at the University of Bergen.

2.2.2.5 Taxonomic assignment of reads. The sequence reads obtained from the Ion Torrent sequencing were quality filtered, chimera checked and operational taxonomic units (OTUs) were clustered (with 97 % similarity) using UPARSE/USEARCH (Edgar 2010, 2013) with a 0.5 quality cut-off. The resulting OTUs were taxonomically assigned using the CREST software implementing the SilvaMod reference database (Lanzén et al. 2012). This outputs the relative abundance of 16S rRNA genes from the different taxonomic groups detected in the samples.

3 Results

3.1 GS10-GC14

3.1.1 Abundance of 16S rRNA genes

The qPCR reported that the 70 cm horizon had ~40 times higher 16S rRNA gene concentration for Lokiarchaeota than the 154 and 165 cm horizons and that ~66 % of the archaea in the 70 cm horizon was Lokiarchaeota, compared to ~40 % for the other two. The bacteria to archaea ratios were approximately 37, 676 and 60 to one (Table 3.1).

Table 3.1: Threshold cycle (C_t) for amplification of 16S rRNA genes by qPCR in gravity core GS10-GC14.

Horizon depth (cm)	Lokiarchaeota	Archaea	Bacteria
70	27.0	26.4	21.2
154	32.3	31.1	21.7
165	32.5	31.1	25.2

The initial screening of the enrichments from core GS10-GC12 using PCR and electrophoresis showed that the enrichments with H_2 were positive for Lokiarchaeota, as were the ones with Fe-oxide without H_2. However, all the bands were fairly weak. All of the enrichments were strongly positive for Bacteria.

3.1.2 Community composition

The concentration of dsDNA after amplification in the samples sent for Ion Torrent sequencing were in the range of 3000–9000 pM except for the Fe-citrate enrichment which had a concentration of ~200 pM. The sequencing yielded between 11 977 and 18 032 reads in all the samples, and all the enrichments had around 100 different OTUs, whereas the slurry had 346 (Table 3.2).

Table 3.2: Concentration of dsDNA in samples from sediment core GS10-GC14 that were sent for Ion Torrent sequencing, and the number of sequence reads and operational taxonomic units (OTUs) obtained from the sequencing.

Sample	dsDNA concentration before dilution (pM)	Sequence reads	OTUs
Slurry	3155	18032	346
Fe-citrate	209	14117	128
Fe-oxide	3417	14567	109
Mn- and Fe-oxide	3046	13157	95
No e^- acceptor	8422	11977	129

Of the sequence reads obtained from the slurry, 54.8 % were bacterial and 43.7 % were archaeal. Proteobacteria (26.1 %) and Planctomycetes (16.5 %) were the most abundant bacterial phyla, but

a range of others were also present (Figure 3.1). The most abundant archaeal phyla were Lokiarchaeota (24.6 %) and Thaumarchaeota (16.3 %). All the enrichments were dominated by Bacteria, particularly Proteobacteria (68.0–90.6 %, but also to a lesser degree Bacteriodetes, Firmicutes and other phyla. Archaea was poorly represented in the enrichments, but the Fe-citrate enrichment contained some Lokiarchaeota (1.1 %) and Thaumarchaeota (1.1 %).

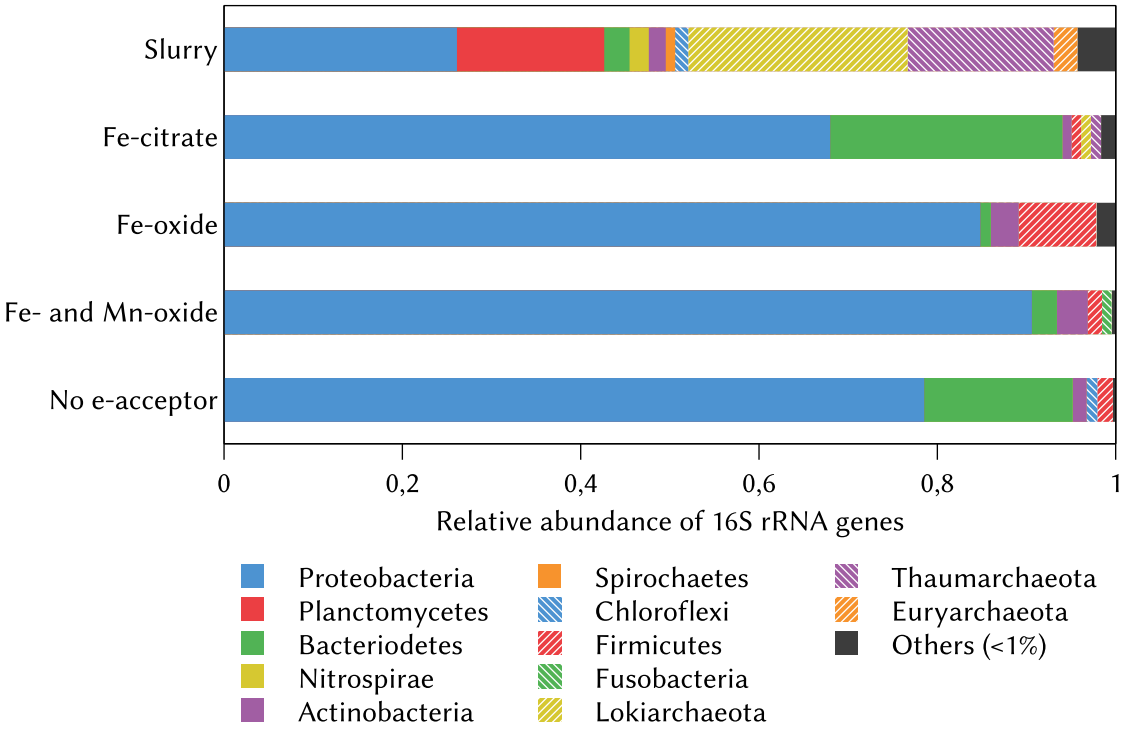


Figure 3.1: Phylum level community composition of gravity core GS10-GC14 based on 16S rRNA gene sequences.

Most of the Proteobacteria in all the samples were either Alphaproteobacteria (all of which were from the order Sphingomonadales) or Gammaproteobacteria (Figure 3.2). A large portion of the Gammaproteobacteria in the Fe-citrate enrichment were *Halomonas* (8.3 %), and *Pseudomonas* was detected at 2–5 % in all the enrichments except the one without electron acceptors. One particular OTU from and unidentified Gammaproteobacteria was dominant in all the enrichments (27.1 %, 49.5 %, 59.4 % and 14.8 % respectively, but only 0.7 % in the slurry). Other bacterial classes of note were Phycisphaerae in the slurry (12.0 %), Flavobacteria in the Fe-citrate enrichment (25.7 %, of which 24.2 % were *Maribacter*) and Bacilli (8.7 %, of which 7.2 % were *Bacillus*). The enrichment without electron acceptors had 27.1 % of the Alphaproteobacteria genus *Paracoccus* and 15.6 % of the Bacteriodetes genus *Flavisolibacter*. Apart from Lokiarchaeota, the most prominent archaeal class was Marine Group I in the slurry (15.6 %).

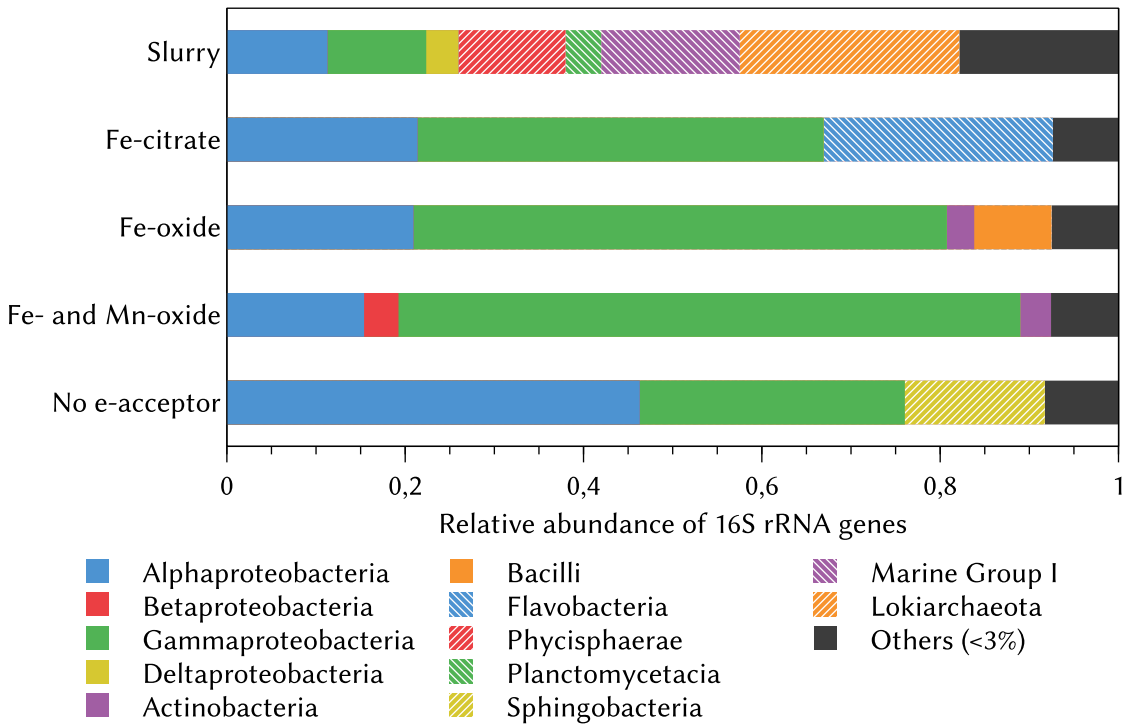


Figure 3.2: Class level community composition of gravity core GS10-GC14 based on 16S rRNA gene sequences.

3.2 GS14-GC12

3.2.1 Abundance of 16S rRNA genes

In the qPCR of GC12, Lokiarchaeota was undetectable or barely detectable in all the enrichments. The Bacteria to Archaea ratio ranged from 7:1 to 32:1 (Table 3.3).

Table 3.3: Threshold cycle (C_t) for amplification of 16S rRNA genes by qPCR in gravity core GS14-GC12.

	Lokiarchaeota	Archaea	Bacteria
No H_2, Fe-citrate	32.8	30.1	27.4
No H_2, Fe-oxide	32.8	28.5	25.3
No H_2, Mn- and Fe-oxide	—	34.4	29.4
No H_2, no e^- acceptor	—	30.1	25.8
H_2, Fe-citrate	33.0	30.6	26.9
H_2, Fe-oxide	—	28.0	24.7
H_2, Mn- and Fe-oxide	—	34.6	29.7
H_2, no e^- acceptor	32.6	28.9	24.9

3.2.2 Community composition

The dsDNA concentrations after amplification in the samples sent for Ion Torrent sequencing ranged from 3500–12 500 pM. The sequencing yielded between 10 841 and 14 604 reads for each

sample. The slurry and the enrichment with H_2 had 232 and 357 OTUs and the enrichment without H_2 had 721 (Table 3.4).

Table 3.4: Concentration of dsDNA in samples from sediment core GS14-GC12 that were sent for Ion Torrent sequencing, and the number of sequence reads and operational taxonomic units (OTUs) obtained from the sequencing.

Sample	dsDNA concentration before dilution (pM)	Sequence reads	OTUs
Slurry	12 350	13953	232
Fe-oxide, H_2	3626	10841	357
Fe-oxide, no H_2	6284	14604	721

The sequence reads from the slurry were vastly dominated by Bacteria (99.6 %), most of which were Proteobacteria (95.2 %) (Figure 3.3). Only 1 read of Lokiarchaeota was detected in the slurry sample. The Fe-oxide enrichment with H_2 was also dominated by Proteobacteria (70.5 %), but other phyla were also represented, such as Actinobacteria (2.5 %), Chloroflexi (6.8 %) and Planctomycetes (6.9 %). Some Archaea (5.8 %) was detected, mainly Euryarchaeota (4.9 %). A small amount of Lokiarchaeota reads (0.2 %) were also obtained. The Fe-oxide enrichment without H_2 exhibited more diversity. The dominant bacterial phyla were Proteobacteria (29.2 %) and Actinobacteria (26.7 %), but Chloroflexi (6.6 %), Firmicutes (7.3 %) and Planctomycetes (3.1 %) were also prominent. Archaea constituted 10.8 %, of which Euryarchaeota (3.4 %) and – notably – Lokiarchaeota (6.1 %) were the most abundant.

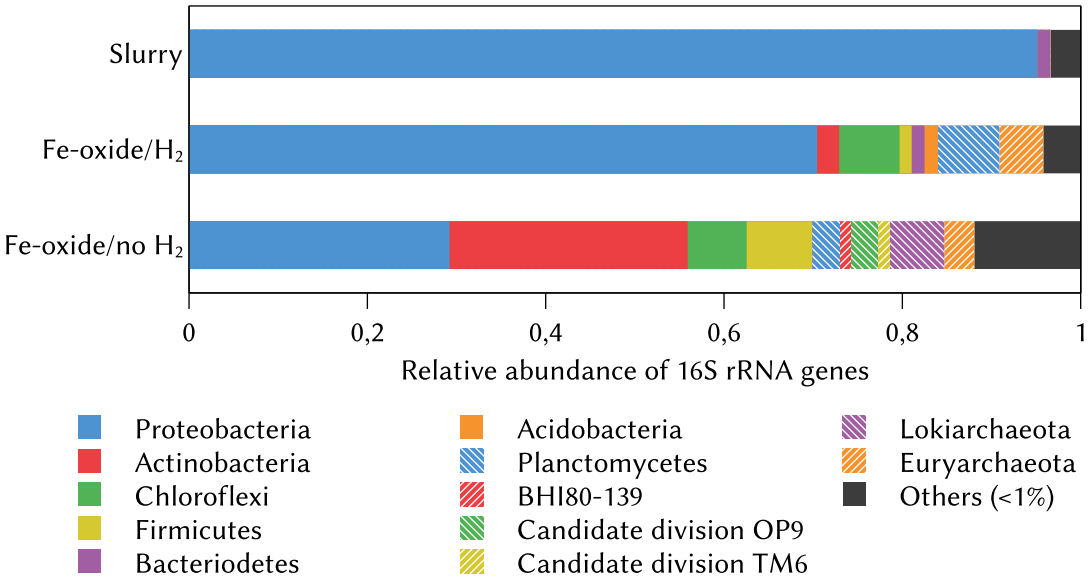


Figure 3.3: Phylum level community composition of gravity core GS14-GC12 based on 16S rRNA gene sequences.

Most of the Proteobacteria reads in the slurry were from Gammaproteobacteria (93.0 %, Figure 3.4), as were the ones from the Fe-oxide enrichment with H_2 (59.5 %). However, in the slurry they were mostly Oceanospirillales (85.8 %), whereas in the enrichment that order had been almost completely replaced by others groups, mainly *Shigella* (31.5 %) and Stenotrophomonas

(10.2 %). Other notable bacterial classes were Alphaproteobacteria (6.5 %), Deltaproteobacteria (3.2 %), Chloroflexi subdivision 6 (5.4 %). All of the Euryarchaeota detected in the enrichment with H_2 were Halobacteria. In the Fe-enrichment without H_2, most of the Actinobacteria were either OPB41 (14.4 %) or Thermoleophilia (specifically Solirubrobacterales, 9.3 %), the Chloroflexi were mainly Anaerolineae (3 %) and Chloroflexi subdivision 6 (2.3 %) and the Firmicutes were mainly Clostridia (6.9 %).

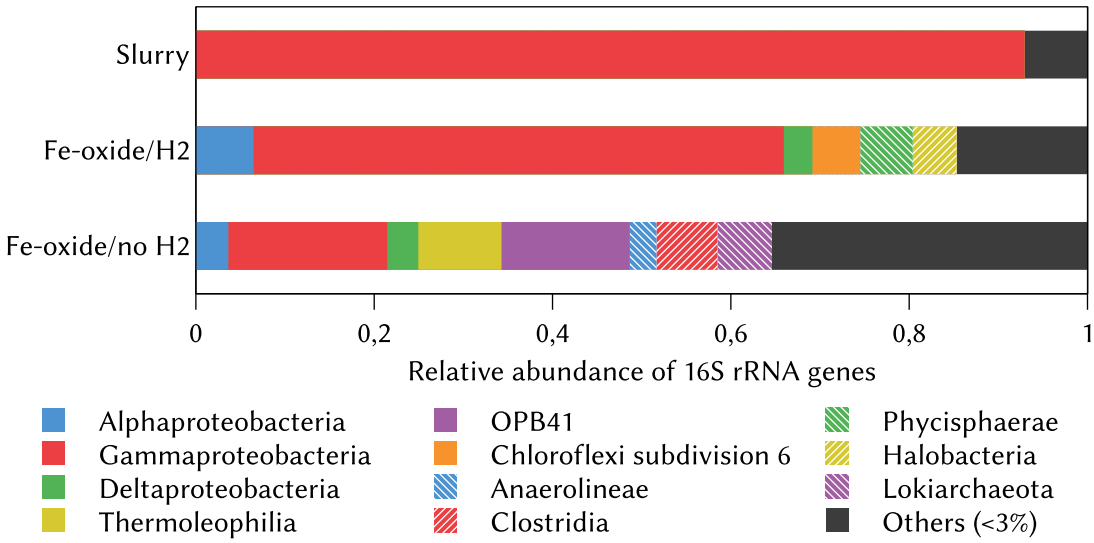


Figure 3.4: Class level community composition of gravity core GS14-GC14 based on 16S rRNA gene sequences.

3.3 GS10-PC12

3.3.1 Iron and manganese profiles

The manganese concentration in PC12 increased steadily from zero at around 400 cm depth to almost 150 µM at 750 cm, declined to 100 µM at around 1200 cm, increased to 150 µM again at around 1400 cm before finally declining to 70 µM at the bottom of the core (Figure 3.5).

The iron concentration stayed close to zero until around 750 cm when it increased to 80 µM at around 1050 cm. It stayed at that level until decreasing all the way back to zero between 1250 and 1350 cm, and then increased steadily to 120 µM at the bottom of the core, except for a large peak to 360 µM at 1676 cm.

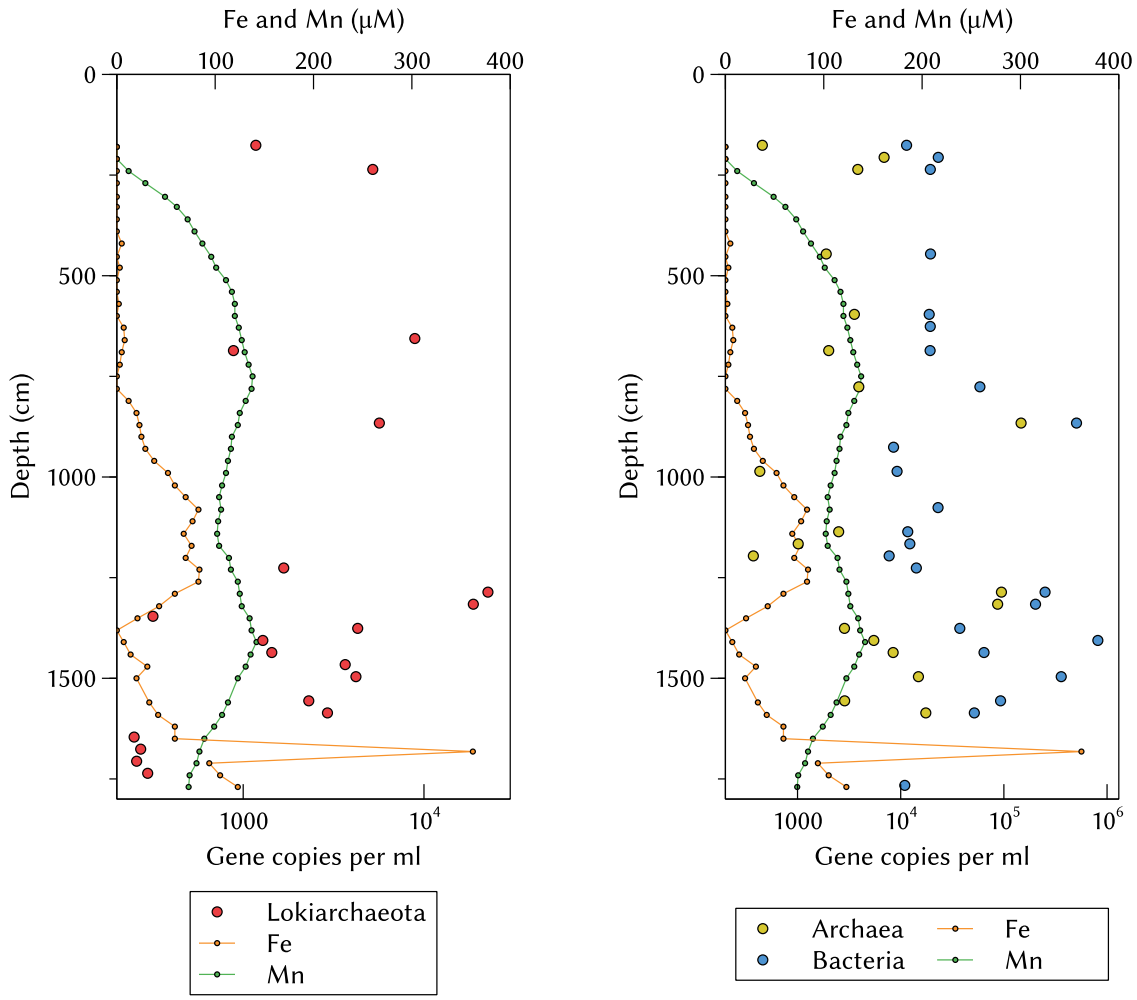
3.3.2 Quantification of 16S rRNA genes

The Lokiarchaeota 16S rRNA gene count varied between 800 and 8000 copies per ml in the top 12.5 m of the core. At 1286 and 1316 cm the count increased to 2.3×10^4 and 1.9×10^4 copies per ml respectively, then decreased to the order of 10^3 again before dissappearing almost completely

in the lower two metres (Figure 3.5a). There were also several horizons throughout the core were Lokiarchaeota could not be detected (not shown).

The archaeal gene count was on the order of 10^3 copies per ml in the top 12.5 m, except for a few horizons were is was zero or close to zero, and a peak of 1.5×10^5 copies per ml at 866 cm. The 1286 and 1316 cm horizons had around 9×10^4 copies per ml, and the lower 4 m had between 2×10^3 and 2×10^4 copies per ml (Figure 3.5b).

The top 12.5 m had on the order of 10^4 bacterial 16S rRNA copies per ml except for the 866 cm horizon, which had 5×10^5 copies per ml. The next 5 m varied between 4×10^4 and 8×10^5 copies per ml (Figure 3.5b).

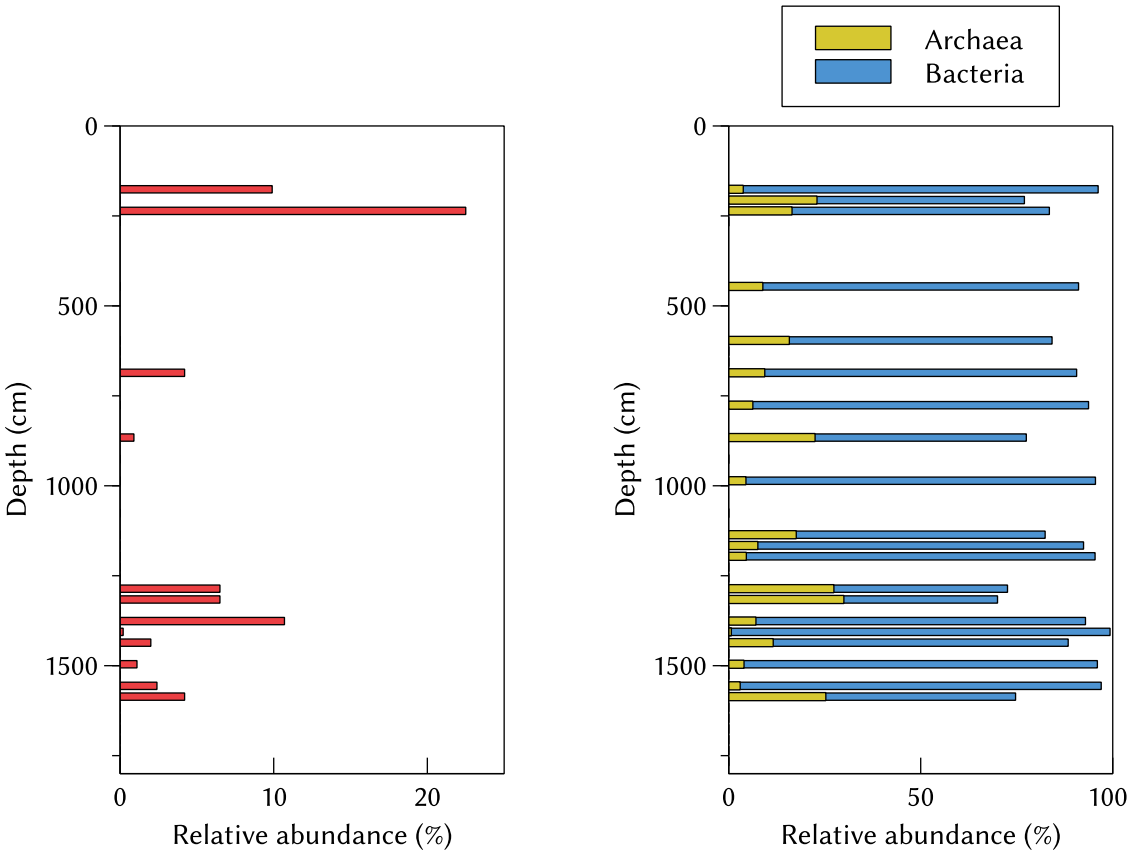


(a) Lokiarchaeota

(b) Archaea and Bacteria

Figure 3.5: Semilogarithmic plots of the absolute abundance of 16S rRNA gene copies in the piston core GS10-PC12 based on qPCR, and a linear plots of the concentration of dissolved iron and manganese in the porewater of said core.

The ratio between Archaea and Bacteria varied between 0.01 and 0.43 in the horizons were both were detected. The abundance of Lokiarchaeota 16S rRNA genes relative to the total number of 16S rRNA genes (Archaea and Bacteria) was in the range of 0–23 % (Figure 3.6).



(a) Lokiarchaeota

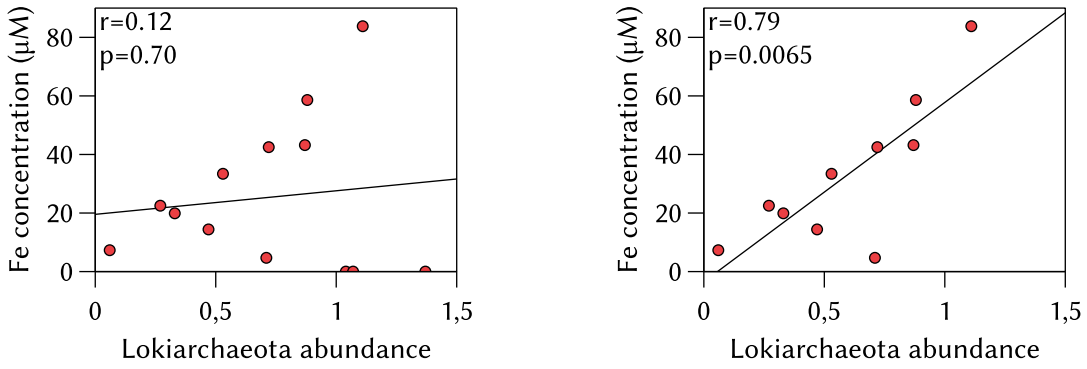
(b) Archaea and Bacteria

Figure 3.6: Relative abundance of 16S rRNA gene copies in sediment core GS10-PC12 based on qPCR.

3.3.3 Iron concentration and abundance of Lokiarchaeota

To investigate the proposed link between Lokiarchaeota and iron, the abundance data were log-transformed and the data points for the iron concentration was matched with the closest data points for the Lokiarchaeota abundance (all geochemistry sampling horizons were offset by 4 cm from the microbial samples). A Pearson correlation coefficient analysis was performed, but did not reveal a significant correlation ($r = 0.12$, $p = 0.70$, see Figure 3.7a). However, disregarding the horizons where no iron was detected at all yielded a significant correlation ($r = 0.79$, $p = 0.0065$, see Figure 3.7b). These were the top two horizons – before the iron zone, but where manganese is present – and the one at 1376 cm (Figure 3.5). However, this leaves a sample size of only 10.

No correlation between manganese concentration and Lokiarchaeota abundance was found.



(a) All datapoints.

(b) Outliers removed.

Figure 3.7: Pearson correlation between the relative abundance of Lokiarchaeota (based on qPCR of 16S rRNA genes) and the concentration of dissolved iron in the porewater of piston core GS10-PC12. Lokiarchaeota abundance is given as the logarithm of the relative abundance in %.

4 Discussion

4.1 Enrichments

Jørgensen et al. (2013) suggest that the metabolism of Lokiarchaeota involves oxidation of organic matter coupled with reduction of iron and/or manganese. Working under that assumption, this study made several attempts to enrich Lokiarchaeota *in vitro*. The first attempt used an inoculum from a 4 year old sediment sample (GS10-GC14) in which Lokiarchaeota had previously been studied (Jørgensen et al. 2013). A few promising horizons were screened using qPCR, and the one with the highest abundence of Lokiarchaeota (with a Lokiarchaeota:Archaea:Bacteria ratio of approximately 2:3:110) was chosen. The enrichment media used yeast extract as a source of complex carbon and various forms of Fe(III) and Mn(IV) as electron acceptors. H_2 was also supplied to some enrichments based on preliminary genome information, and as it has been shown to be a possible electron donor for iron reducers (Caccavo et al. 1992, Lovley et al. 1989, Ohmura et al. 2002, Slobodkin & Wiegel 1997). The enrichments were performed anaerobically, and no nitrate or sulfate was supplied. Thus, dissimilatory Fe(III) and Mn(IV) reduction should theoretically be the most energetically favourable form of respiration possible in the enrichments.

After approximately two weeks of incubation, conventional PCR was performed on DNA extractions from the enrichments. In order to evaluate which enrichment approach was most viable when fresh sample material from the 2014 cruise became available, the PCR products were screened by visual inspection of electrophoresis gel. Based on band intensity, the enrichments with H_2 were the most promising. No difference was observed between the enrichments with and without antibiotics. The decision was made to use antibiotics in subsequent enrichments, as it may provide a competitive advantage to Archaea over Bacteria. Therefore, the enrichments with both H_2 and antibiotics were regarded as the most interesting for further study.

When performing 16S rRNA gene tag sequencing on these enrichments, the results indicated that Lokiarchaeota constituted almost a quarter of the community in the slurry; however, they had disappeared almost completely in all the enrichments. A number of common sedimentary groups of microorganisms were detected, such as Alpha-, Beta-, Delta- and especially Gammaproteobacteria (e.g. *Halomonas*), Planctomycetes and Marine Group I (Fry et al. 2008). A few possible iron reducers were detected as well, such as *Bacillus spp.* and *Pseudomonas spp.* (Lovley 2013).

In the control enrichment that was not given any iron or manganese, there should in theory not be any high energy yielding electron acceptors. Therefore, the most probable form of metabolism to have occurred here is fermentation. However, the most dominant groups of organisms in the enrichment were *Paracoccus* (27.1 %) and *Flavisolibacter* 15.6 %. *Paracoccus* are either aerobes or facultative anaerobes capable of nitrate reduction (Kelly et al. 2006), and *Flavisolibacter* are aerobic (Baik et al. 2014, Yoon & Im 2007). Therefore, it is unlikely that conditions in the enrichment were completely anoxic. A third organism that was detected in large numbers (14.8 %) in the control enrichment – an unidentified Gammaproteobacterium – was also dominant in all the other enrichments (27.1 %, 49.5 % and 59.4 % respectively in Fe-citrate, Fe-oxide and Mn-oxide),

but not in the slurry (0.7 %). All of this might indicate that there were trace amounts of oxygen in the enrichments. Unfortunately, these data were not available at the time of the cruise, or else stricter precautions could have been taken to ensure the complete removal of oxygen from the media.

A second enrichment series was prepared on board R/V G.O. SARS while on cruise in the Norwegian Sea based on newly recovered sediment samples (from sediment core GS14-GC12). The choice of inoculum was less informed in this case, as a qPCR could not be performed due to limitations to what equipment could be brought on the ship. Several cores where screened in the same manner as GC12 (Table 2.2); in sum, around 10 cores of varying length – a total of approximately 100 horizons – were screened for selected groups of microorganisms, including Lokiarchaeota. This was all done shipboard using conventional PCR and electrophoresis. A horizon from GC12 was chosen for enrichment based on the intensity of the Lokiarchaeota bands in the electrophoresis gel.

These enrichments used pyruvate as the primary electron donor, although a small amount of yeast extract was added in this case as well. This change was founded on preliminary genomic information from a Lokiarchaeota metagenome study. For logistical reasons, the number of parallell enrichments had to be kept relatively small; hence, other possible electron donors could not be evaluated on the cruise.

After incubating for approximately three weeks, the enrichments from GC12 were screened using qPCR, but no Lokiarchaeota was detected. For pragmatic reasons, only three samples from these enrichments could be sent for gene tag sequencing at the time. In addition to the slurry, the two enrichments that the qPCR reported to have the highest abundance of Archaea were selected; these were both the enrichments with Fe-oxide – with and without H_2. The DNA sent for sequencing were from the same extraction as the DNA used as qPCR template. Surprisingly, Lokiarchaeota constituted around 6 % of the community in the enrichment without hydrogen. This marked the only time in the study where there was an indication that Lokiarchaeota could have been enriched. The sequencing of the selected enrichments also revealed a number of groups commonly found in sediments, including Alpha-, Gamma- and Deltaproteobacteria, Chloroflexi, Planctomycetes and Actinobacteria (Fry et al. 2008). Other possible iron reducers detected included Clostridia (Lovley 2013) and Bacteriodetes (Beal et al. 2009).

The discrepancy between results from the gene tag sequencing and the qPCR could be due to a number of issues regarding the use of PCR-based techniques in community analysis – such as inhibition, primer mismatch and molecular sampling error due to low template concentration (discussed in Section 4.2).

Oceanospirillales, Shigella and Stenotrophomonas were also detected in the sequenced samples from GC12; these are organisms not commonly found in sediments (Fry et al. 2008). In particular, Oceanospirillales completely dominated the slurry. This could possibly be an indication of contamination. If so, this likely occured aboard the ship – possibly from the filtered seawater used to create the slurry – as Oceanospirillales is typically found in the ocean (Garrity et al. 2005).

Spang et al. (2015) suggest that Lokiarchaeota is capable of phagocytosis. Based on this information, an attempt was made at enriching for Lokiarchaeota using cells from a pure culture of *Lutibacter sp.* Samples from these enrichments were gene sequenced using Ion Torrent, but preliminary results from this experiment were negative, and the line of enquiry was not pursued further due to time constraints. An attempt was also made with iron sulfide as electron acceptor, but this did not yield any results either.

Had time allowed, the next attempts would have involved trying different combinations of electron donors and forms of iron and manganese. Various fermentation acids - and acetate in particular – are commonly used by dissimilatory iron reducers as electron donors (Lovley 2013).

As for the various forms of iron, poorly crystalline (or amorphous) Fe(III) oxides – as used in this study – is the most environmentally relevant form. Fe(III)-citrate – the other form of ferric iron used – is often used for culturing iron reducers. As it is soluble, it is easier for the organisms to utilise, which makes culturing easier; however, it is not commonly found in the environment. It may also be toxic to some iron reducers. Fe(III) chelated with nitrilotriacetic acid (Fe(III)-NTA) is another soluble alternative; however, it may also be toxic, and it tends to precipitate if exposed to high salinity or high temperature. More highly crystalline Fe(III) oxides – which are more common in the environment than the soluble forms – could also have been evaluated, but they tend to be very difficult for the microorganisms to utilise (Lovley 2013).

Finally, the manganese used in this study was from an undefined environmental sample. It may very well have contained iron oxide and other minerals in addition to manganese oxide. For a more controlled experiment, birnesitte – a form of Mn(IV) oxide – could have been prepared much in the same way that the poorly crystalline iron oxide was prepared for this study (Lovley 2013).

4.2 Correlation between iron and Lokiarchaeota

There seemed to be a correlation between the relative abundance of Lokiarchaeota and the concentration of dissolved iron in the porewater of the long sediment core studied in this project. However, the correlation was only significant when including just the horizons where both iron and Lokiarchaeota was detected (Figure 3.7). The results seem to conform to the data from previously studied sediment cores presented in Jørgensen et al. (2013). Interestingly, these data also have outliers where Lokiarchaeota was detected in horizons with little or no iron in the porewater (Figure 4.1).

The ability of microorganisms to reduce iron [Fe(III)] often go hand in hand with the ability to reduce manganese [Mn(IV)] (Lovley 2013). Thus, assuming that Lokiarchaeota are iron reducers, their continued presence in horizons without iron could be explained by them still having access to Mn(IV). Indeed, the data show that manganese is present in the porewater in the horizons where iron is not, suggesting that manganese reduction takes place in those horizons.

Furthermore, chemical interaction between iron and manganese could complicate the picture. Mn(IV) has been shown to oxidize Fe(II) in anoxic sediments, thus removing iron from the pore-

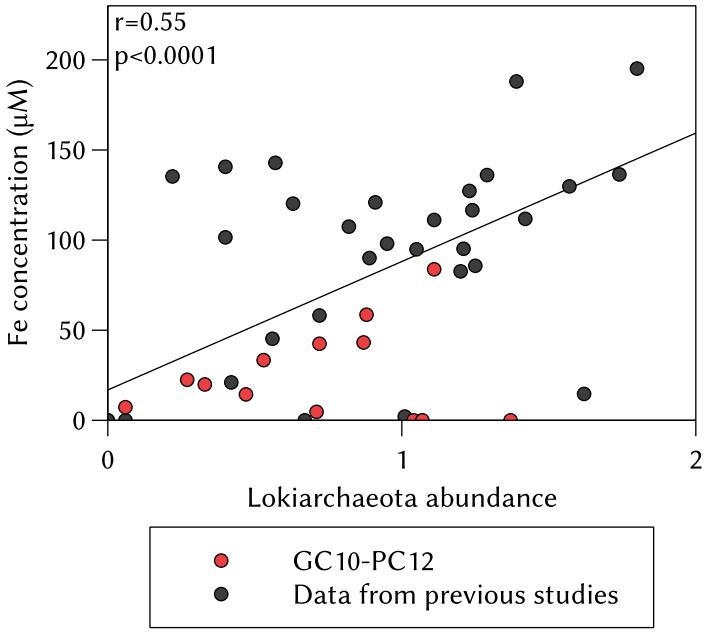


Figure 4.1: Pearson correlation between the relative abundance of Lokiarchaeota (based on qPCR of 16S rRNA genes) and the concentration of dissolved iron in the porewater of several sediment cores. Lokiarchaeota abundance is given as the logarithm of the relative abundance in %. The grey points are from GC12, GC14 and PC15 described in Jørgensen et al. 2013.

water. That means iron reduction could occur without Fe(II) accumulating in the sediment (Lovley & Phillips 1988a). This could also help explain the apparent negative correlation between dissolved iron and manganese in the sediment core (Figure 3.5).

If the iron reduction zone overlap with the sulfate reduction zone, a different chemical complication could arise. The sulfide produced by reducing sulfate reacts with iron oxides, producing Fe(II) (Canfield 1989). Thus, the Fe(II) concentration in the porewater could be elevated even though no microbial reduction of iron takes place. However, based on the porewater sulfate profile there was no evidence of sulfate reduction in the core (Supplementary table S.1), so this is not likely to have happened in this case.

No causal relationship can – of course – be inferred from correlation analysis alone. The increased levels of Fe(II) in the porewater could be the result of other, less direct metabolic mechanisms – for example fermentation using Fe(III) as an electron sink. Many fermentative microbes can use Fe(III) and Mn(IV) reduction as a minor pathway in their metabolism. However, normally only a small component of the reducing equivalents are transferred to iron and manganese (Lovley 1987, Lovley & Phillips 1988a). If Lokiarchaeota are fermentative iron and manganese reducers, the enrichment strategy employed in this study would not be feasible as they would not depend on the availability of Fe(III) and Mn(IV) for energy conservation. However, this metabolism has not been shown to cause increase in cell yield (Lovley 2013), and it is therefore unlikely to have caused a correlation between iron and abundance of Lokiarchaeota.

The abundance of 16S rRNA genes measured by qPCR in the samples from PC12 was typically on the order of 10^4–10^5 copies/cm^3, which is several orders of magnitude lower than what is normally found in marine sediments (Parkes et al. 1994). Specifically, the abundance of Lokiarchaeota genes was in the range of 0–10^4 copies/cm^3. When compared to previous estimates from the area (Jørgensen et al. 2013, Jørgensen et al. 2012 and unpublished data) this might suggest that the carbon content is lower on the western flank of the ridge valley, as all other cores has been taken either in or on the eastern flank. This might be due to an influx of organic material from the Bear Island fan system.

Low abundance of template DNA is potentially a problem for PCR reliability, as it has been found to reduce the reproducibility of amplifications. This is believed to be due to stochastic variations in the formation of the primer-template-polymerase complex (molecular sampling error) (Chandler et al. 1997, Smith et al. 2006). Consequently, the qPCR results in this case, particularly those related to Lokiarchaeota, are less reliable than they would have been had the gene counts been higher, and any conclusions drawn from them must be regarded with a certain degree of caution.

Other factors could also affect the reliability of qPCR amplification. First of all, the entire analysis depends critically on the proper extraction of DNA from the sediment samples. In this case, a few measures were taken to optimise the efficiency of the extraction. Firstly, polyadenylic acid (poly-A) was added to the samples prior to cell lysis. The poly-A binds to particles in the sediment as well as to the beads in the bead solution. This minimises adsorbtion of DNA to said particles – as the DNA has to compete with the poly-A for adsorbtion sites – and thus helps to optimise the yield of the extraction (Hugenholtz et al. 1998). Secondly, samples from the enrichments were washed with an oxalate buffer before DNA extraction. This has the intended effect of removing iron oxides from the samples (Lovley & Phillips 1988b). The presence of iron can negatively impact the efficiency of DNA extractions, as DNA adsorbs easily to iron oxide particles (He et al. 2005). A comparison was made between DNA extractions with and without oxalate washing where the extractions that had been washed with oxalate yielded significantly more DNA (data not shown).

Coextraction of humic substances can potentially interfere with downstream application of DNA, for example by inhibiting DNA polymerase in PCR (Albers et al. 2013, Tebbe & Vahjen 1993, Wintzingerode et al. 1997). A common way to deal with this is to dilute the PCR template prior to amplification to reduce the concentration of humic substances in the reaction. A test run with 1:10 and 1:50 dilutions of a few of the horizons were performed, but no difference in amplification efficiency was observed (data not shown). Thus, all the amplifications were done using undiluted template, since low template concentration can have its own adverse effects on PCR (as described above).

A range of issues regarding primers can influence the results of PCR-based community analyses. Problems such as primer mismatches and degenerate positions may promote preferential binding of the primers to genes of certain groups of organisms, thus skewing the results in favour of those organisms. Excessive replication cycle numbers have also been shown to bias the compo-

sition of the PCR product. This can be a problem with low biomass samples – as in this case – where high cycle numbers may be necessary (Sipos et al. 2007, Wintzingerode et al. 1997).

Even if the amplification of 16S rRNA genes by PCR was not biased, it might not accurately reflect the active community of microrganisms in the sample. There is no way of knowing whether the genes amplified stem from viable or dead cells, or from extracellular DNA. Additionally, the number of 16S rRNA gene copies in the genome varies between organisms. For Bacteria, the average number is 4.0 copies/cell, and for Archaea the average is 1.6 copies/cell (from the Ribosomal RNA Database at https://rrndb.umms.med.umich.edu, Stoddard et al. 2015). Thus, there is not necessarily a linear relationship between gene copies and cell number in a sample, which could bias the community composition.

In sum, caution must be taken when interpreting qPCR results in general – and in this case in particular, since the template concentrations were so low. This inference is reinforced by the fact that in four horizons (176, 236, 1226 and 1376 cm), the gene count for Lokiarchaeota actually exceeded that for Archaea (Figure 3.6, Supplementary table S.2), a result that clearly does not reflect reality. Interestingly, three of these four horizons are the same that were excluded from the correlation analysis (Figure 3.7). If this is taken to suggest that Lokiarchaeota was overamplified in those horizons, it further supports their exclusion from the analysis. However, it also lends less credence to the results as a whole. Furthermore, there is a discrepancy between the qPCR and sequencing results from GC14 with regards to the ratio between Bacteria and Archaea. The sequencing showed an approximately equal amount of bacterial and archaeal genes (54.8 and 43.7 %) whereas the qPCR indicated 37 times more bacterial than archaeal genes. This indicates a problem with either the qPCR amplification or the preparation and sequencing of the amplicon library.

5 Conclusion

The results of this study point toward Lokiarchaeota being dissimilatory iron reducers. However, the evidence is not very strong. The enrichment attempts largely failed to yield any Lokiarchaeota; only one – containing pyruvate as electron donor and poorly crystalline Fe(III) oxide as electron acceptor – showed possible growth. The analysis of the depth profile of the sediment core revealed a possible correlation between iron concentration and Lokiarchaeota abundance, but the results are uncertain due to low template concentrations in the qPCR and a limited dataset.

Further studies could attempt creating enrichments using different combinations of electron donors – especially fermentation acids such as acetate – and possibly other forms of Fe(III) and Mn(IV) as electron acceptors, as well as try to reproduce the enrichment using poorly crystalline Fe(III) oxide. Additional correlation analyses between abundance data and geochemical parameters in sediment depth profiles using higher spatial resolution are also warranted.

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Supplementary data

Table S.1: Iron, manganese and sulfate concentrations in sediment core GS10-PC12 measured by inductively coupled plasma mass spectometry (ICP-MS) and ion chromatography.

Depth (cm)	Iron (μM)	Manganese (μM)	Sulfate (mM)	Depth (cm)	Iron (μM)	Manganese (μM)	Sulfate (mM)
180	0	0	29.2	990	52	111	29.6
210	0	0	29.1	1021	59	107	29.3
240	0	12	28.4	1050	70	104	27.7
270	0	29	29.3	1081	83	106	28.6
304	0	49	29.2	1110	77	103	27.7
329	0	61	28.5	1141	68	102	28.9
360	0	72	28.2	1171	76	104	28.6
390	0	79	28.9	1201	70	114	27.5
420	5	87	28.9	1230	84	116	28.8
453	0	96	29.2	1260	83	123	27.5
480	3	101	29.0	1290	59	125	28.7
511	0	111	28.8	1321	43	127	28.3
540	0	117	28.9	1351	21	135	28.1
570	2	120	29.0	1381	0	137	28.1
600	0	120	28.3	1410	7	142	28.0
629	7	124	28.3	1441	14	136	28.0
660	8	127	28.3	1471	31	131	28.6
690	5	130	28.6	1500	20	123	28.0
721	3	134	28.2	1560	33	113	28.1
750	0	138	28.5	1591	42	107	28.1
781	0	137	28.4	1620	59	99	28.1
811	12	131	29.5	1650	59	89	26.8
841	20	125	28.9	1682	362	84	27.8
871	23	123	29.5	1711	94	81	27.6
900	25	117	29.5	1741	105	74	27.8
930	29	116	28.7	1770	123	73	27.6
960	38	113	29.4				

Table S.2: Abundance of 16S rRNA genes in sediment core GS10-PC12 based on qPCR. The abundance is given as gene copies per ml.

Depth (cm)	Archaea	Bacteria	Lokiarchaeota
176	456	11400	1174
206	6903	23126	—
236	3837	19364	5209
266			—
446	1897	19451	—
596	3556	18890	—
626	0	19339	—
656			8899
686	2005	19277	883
776	3925	58559	—
866	146775	505751	5664
926	0	8523	—
986	431	9220	—
1076		23014	—
1136	2504	11703	—
1166	1015	12262	—
1196	373	7728	—
1226	0	14177	1676
1256			—
1286	94708	250720	22585
1316	86990	202614	18749
1346			316
1376	2851	37398	4299
1406	5485	814319	1284
1436	8446	64189	1439
1466			3666
1496	14854	359654	4203
1526			—
1556	2861	92847	2305
1586	17511	51702	2925
1616			—
1646			249
1676			271
1706			257
1736			296
1766		10947	—

Table S.3: Relative abundance of 16S rRNA genes in sediment core GS14-GC12 based on Ion Torrent sequencing.

Phylum	Slurry	Fe-oxide, no H_2	Fe-oxide and H_2
Ancient Archaeal Group	0.00 %	0.14 %	0.00 %
Crenarchaeota	0.00 %	0.55 %	0.00 %
Euryarchaeota	0.34 %	3.37 %	4.93 %
Lokiarchaeota	0.01 %	6.10 %	0.22 %
Thaumarchaeota	0.01 %	0.69 %	0.64 %
Acidobactera	0.89 %	0.71 %	1.51 %
Actinobacteria	0.11 %	26.71 %	2.47 %
Armatimonadetes	0.00 %	0.12 %	0.00 %
Bacteriodetes	1.44 %	0.98 %	1.44 %
BD1-5	0.00 %	0.03 %	0.00 %
BHI80-139	0.00 %	1.23 %	0.00 %
Candidate division AC1	0.00 %	0.03 %	0.00 %
Candidate division BRC1	0.00 %	0.03 %	0.03 %
Candidate division Caldithrix	0.00 %	0.07 %	0.00 %
Candidate division NC10	0.03 %	0.02 %	0.22 %
Candidate division OD1	0.00 %	0.08 %	0.00 %
Candidate division OP3	0.03 %	0.46 %	0.54 %
Candidate division OP8	0.00 %	0.40 %	0.00 %
Candidate division OP9	0.01 %	3.03 %	0.02 %
Candidate division TM6	0.00 %	1.34 %	0.11 %
Candidate division WS3	0.00 %	0.07 %	0.00 %
Chlorobi	0.00 %	0.06 %	0.00 %
Chloroflexi	0.64 %	6.61 %	6.77 %
Cyanobacteria	0.00 %	0.05 %	0.13 %
Deinococcus-Thermus	0.00 %	0.05 %	0.00 %
Elusimicrobia	0.00 %	0.05 %	0.29 %
Fibrobacteres	0.00 %	0.08 %	0.00 %
Firmicutes	0.23 %	7.34 %	1.36 %
Fusobacteria	0.02 %	0.08 %	0.06 %
Gemmatimonadetes	0.09 %	0.34 %	0.88 %
Lentisphaerae	0.00 %	0.42 %	0.00 %
Nitrospirae	0.01 %	0.02 %	0.04 %
PAU34f	0.00 %	0.04 %	0.00 %
Planctomycetes	0.86 %	3.13 %	6.89 %
Proteobacteria	95.16 %	29.22 %	70.45 %
RF3	0.01 %	0.00 %	0.00 %
SAR406	0.06 %	0.23 %	0.65 %
Spirochaetes	0.04 %	0.58 %	0.08 %
Thermotogae	0.00 %	0.02 %	0.00 %
Verrumicrobia	0.01 %	0.18 %	0.00 %
WCHB1-60	0.00 %	0.07 %	0.00 %
Eukaryota	0.01 %	1.57 %	0.10 %
No hits	0.00 %	3.68 %	0.18 %

Table S.4: Relative abundance of 16S rRNA genes in sediment core GS10-GC14 based on Ion Torrent sequencing.

Phylum	Slurry	Fe-citrate	Fe-oxide	Fe- and Mn-oxide	No e^--acceptor
Crenarchaeota	0.00 %	0.00 %	0.15 %	0.00 %	0.00 %
Euryarchaeota	2.67 %	0.01 %	0.00 %	0.02 %	0.01 %
Lokiarchaeota	24.63 %	1.10 %	0.01 %	0.00 %	0.00 %
Thaumarchaeota	16.37 %	1.14 %	0.00 %	0.00 %	0.01 %
Acidobactera	0.03 %	0.32 %	0.00 %	0.00 %	0.16 %
Actinobacteria	1.90 %	1.03 %	3.12 %	3.46 %	1.55 %
Armatimonadetes	0.03 %	0.00 %	0.00 %	0.00 %	0.00 %
Bacteriodetes	2.84 %	26.04 %	1.18 %	2.81 %	16.66 %
BHI80-139	0.82 %	0.00 %	0.09 %	0.00 %	0.00 %
Candidate division AC1	0.00 %	0.00 %	0.00 %	0.17 %	0.00 %
Candidate division BRC1	0.06 %	0.00 %	0.01 %	0.00 %	0.00 %
Candidate division OP3	0.49 %	0.00 %	0.00 %	0.00 %	0.00 %
Candidate division OP8	0.13 %	0.00 %	0.03 %	0.00 %	0.00 %
Candidate division OP9	0.01 %	0.03 %	0.07 %	0.02 %	0.01 %
Candidate division TM6	0.61 %	0.00 %	0.00 %	0.00 %	0.00 %
Candidate division WS3	0.06 %	0.00 %	0.00 %	0.00 %	0.00 %
Chlorobi	0.02 %	0.00 %	0.00 %	0.00 %	0.00 %
Chloroflexi	1.45 %	0.30 %	0.38 %	0.00 %	1.22 %
Cyanobacteria	0.00 %	0.55 %	0.47 %	0.00 %	0.09 %
Firmicutes	0.16 %	1.07 %	8.70 %	1.60 %	1.74 %
Fusobacteria	0.00 %	0.07 %	0.01 %	1.07 %	0.01 %
Gemmatimonadetes	0.17 %	0.00 %	0.00 %	0.00 %	0.03 %
LD1-PA38	0.06 %	0.00 %	0.00 %	0.00 %	0.00 %
Nitrospirae	2.16 %	0.01 %	0.03 %	0.01 %	0.00 %
Planctomycetes	16.52 %	0.33 %	0.00 %	0.02 %	0.00 %
Proteobacteria	26.12 %	67.98 %	84.82 %	90.60 %	78.52 %
RF3	0.00 %	0.00 %	0.44 %	0.00 %	0.00 %
SAR406	0.06 %	0.01 %	0.00 %	0.01 %	0.00 %
Spirochaetes	1.06 %	0.03 %	0.01 %	0.00 %	0.00 %
Thermodesulfobacteria	0.02 %	0.00 %	0.00 %	0.00 %	0.00 %
Verrumicrobia	0.00 %	0.00 %	0.24 %	0.23 %	0.00 %
Eukaryota	0.00 %	0.00 %	0.23 %	0.00 %	0.00 %
No hits	1.58 %	0.00 %	0.00 %	0.01 %	0.00 %