Max Planck Institute for Marine Microbiology

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Universität Bremen*

*EXZELLENT.

Effect of humic substances on microbial community composition and iron reduction in marine sediments

Dissertation

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

- Dr. rer. nat. -

dem Fachbereich Biologie/Chemie der

Universität Bremen

vorgelegt von

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Bremen, Dezember 2015

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Tag des Promotionskolloquiums: 3 February, 2016

To my parents

To my family

To my wife

"The role of the infinitely small in nature is infinitely great"

Louis Pasteur

Summary

Humic substances (HS) are a complexed mixture of organic compounds formed from decomposition of organic matter. They are known to play a role in anaerobic respiration as extracellular electron shuttling molecules. This is due to their unique molecular structure that contains quinone moieties as the redox active sites. HS are known to be biotically reduced, and in turn donate abiotically their electrons to iron oxides. Most iron reducing bacteria were found to be able to reduce HS, but also other microorganisms, such as methanogens and fermenters, are able to do so. Therefore, there is a high potential for their impact on the iron cycle. Although the impact of humics on populations and kinetics of iron reduction were shown before in soils and fresh water sediments, little is known about their impact in marine sediments.

In this thesis, I investigated the effect of the humic analog 9,10-anthraquinone-2,6-disulfonate (AQDS) on microbial populations and iron reduction in marine surface sediments. Three marine sites were studied: (1) The Wadden Sea tidal flats (Dorum-Neufeld), (2) the Helgoland mud area (North Sea), and (3) the shallow hydrothermal vent systems at the island of Dominica (Lesser Antilles). Anoxic sediment incubations were performed and iron reduction rates were determined. For investigating the microbial community, which is involved in humic and iron respiration, I used different methods. Microbial ribosomal RNA (rRNA) gene community fingerprints were analyzed by terminal restriction fragment length polymorphism (TRFLP). For quantification I used most probable number (MPN) incubations. For identification of the active population, which can assimilate acetate (^{13}C-labeled) and couple it to iron and humic reduction, stable isotope probing of RNA (RNA-SIP) was used.

Iron reduction was significantly stimulated by addition of AQDS. The stimulation resulted in up to ~ 4.5 times more Fe^{2+} formed than in control incubations. Furthermore, low AQDS concentrations such as 0.5 and 5 µM resulted in higher stimulation of iron reduction than using 50 and 1000 µM. These results suggest that iron reduction was limited by the availability of quinone moieties in slurry incubations. In incubations with sediment from Wadden Sea and North Sea, iron reduction was stimulated as a result of acetate addition, suggesting that availability of electron donors for iron reduction was also limiting. When using sediment from Dominica hydrothermal vents, no stimulation was observed.

Quantification of AQDS-reducing microorganisms by most probable number cultivation resulted in ~ 50 times higher numbers than with iron oxide as sole electron acceptor. Additionally, differences in microbial community fingerprinting structure were observed. When using sediment from Dorum or Helgoland, community structure was

affected mainly by electron donor amendment. In contrast, in incubations from Dominica, microbial community structure was affected by AQDS amendment, suggesting that quinone respiration is a more common property in Wadden Sea and North Sea sediments.

Using RNA-SIP approach, I showed that *Desulfuromonadales* spp. are the main microorganisms who could couple acetate assimilation to AQDS and iron reduction in sediments from Dorum and Helgoland, implying that humic respiration coupled to acetate oxidation is carried out by iron reducing bacteria. In incubations with sediment from Dominica when AQDS was amended the *Halobacteriales* group DHVEG-6 was found as main acetate assimilating microorganism. This result gives direct evidence for the ability of an uncultivated archaeal group to utilize acetate with AQDS.

Overall, the results presented in this thesis provide insight to the barely studied field of the *in-situ* utilization of HS in marine sediments. They suggest that there is a high potential to use HS for respiration in marine sediments. Therefore, input of organic carbon in the form of HS will likely result in a stimulation of carbon mineralization and enhance iron reduction through electron shuttling in marine sediments.

Zusammenfassung

Huminstoffe (HS) sind eine komplexe Mischung aus organischen Verbindungen, die durch Abbau organischer Substanzen entstehen. Es ist bekannt, dass HS als extrazelluläre Elektonentransportmoleküle eine Rolle in der anaeroben Atmung spielen. Dies kann auf ihre einzigartige Molekularstruktur zurückgeführt werden, welche Chinonreste als redoxaktive Gruppen aufweist. Es ist, bekannt dass HS biotisch reduziert werden können und dass sie wiederum abiotisch Elektronen an Eisenoxide abgeben können. Es ist außerdem bekannt, dass sowohl die meisten eisenreduzierenden Bakterien, als auch andere Mikroorganismen, wie z.B. Methanogene und Fermentierer, HS reduzieren können. Dies bedeutet, dass diese Organismen sehr wahrscheinlich einen direkten Einfluss auf den Eisenkreislauf haben. Obwohl die Auswirkungen von Huminstoffen auf die Populationen und die Kinetik von eisenreduziererenden Mikroorganismen in verschiedenen Studien gezeigt wurden, ist wenig über deren Bedeutung in marinen Sedimenten bekannt.

In dieser Arbeit habe ich die Auswirkungen der huminanalogen Substanz 9,10-Anthrachinon-2,6- Disulfonat (AQDS) auf mikrobielle Populationen und Eisenreduzierer in marinen Oberflächensedimenten erforscht. Drei marine Sedimente wurden untersucht: (1) Das Wattenmeersediment (Dorum-Neufeld), (2) das Helgoländer Schlickgebiet bei Helgoland (Nordsee), und (3) die Flachwasser Hydrothermalquellensysteme um Dominica (Kleine Antillen). Inkubationen mit anoxischem Sediment wurden angesetzt und die Eisenreduktionsraten bestimmt. Zur Untersuchung der mikrobiellen Gemeinschaft, die in Huminstoff- und Eisenrespiration involviert ist, habe ich verschiedene Methoden verwendet. Mikrobielle Gemeinschaftsprofile wurden durch Analyse der terminalen Restriktionsfragmentlängenpolymorphismen (T-RLFP) des Gens für ribosomalen RNA untersucht.. Zur Abschätzung der Anzahl von Mikroorganismen habe ich MPN (most probable number) Inkubationen verwendet. Um aktive Populationen zu identifizieren, welche Acetat- (^{13}C-markiert) assimilieren und diese an die Eisen- und Huminstoffreduktion koppelt, wurde stablile RNA Isotopenbeprobung (RNA-SIP) verwendet.

Eisenreduktion wurde signifikant durch die Zugabe von AQDS angeregt. Durch die Stimulation wurde 4,5-fach mehr Fe^{2+} geblidet als in Kontrollinkubationen. Außerdem führten geringe Konzentrationen an AQDS (0,5 und 5 µM) zu einer höheren Anregung der Eisenreduktion als höhere Konzentrationen (50 und 1000 µM). Dies deutet darauf hin, dass Eisenreduktion in Schlamminkubationen durch die Verfügbarkeit von Chinonresten limitiert war. In Inkubationen mit Sediment des Wattenmeeres und der

Nordsee erfolgte die Anregung der Eisenreduktion nach Zugabe von Acetat, was darauf hindeutet, dass auch die Verfügbarkeit von Elektronendonatoren für die Eisenreduktion limitierend war. In Dominica-Sedimenten konnte keine Anregung der Eisenreduktion nachgewiesen werden.

Die Quantifizierung der AQDS-reduzierenden Mikroorganismen durch MPN (most probable number)-Kultivierung resultierte in einer ~ 50-fach höheren Zellzahl als unter Verwendung von Eisenoxid als einzigem Elektronenakzeptor. Zusätzlich wurden Unterschiede im mikrobiellen Populationsfingerabdruck festgestellt: Die Populationsstruktur von Dorum- und Helgolandsediment wurde hauptsächlich durch Zusatz von Elektronendonatoren beeinflusst. Im Gegensatz dazu wurde die Populationsstruktur in Dominica-Sedimenten durch die Zugabe von AQDS beeinflusst, was darauf hindeutet, dass Chinonrespiration in Wattenmeer- und Nordseesedimenten stärker verbreitet ist.

Durch Verwendung der RNA-SIP-Methode konnte ich zeigen, dass hauptsächlich *Desulfuromonadales* spp. Acetat assimilieren während AQDS- und Eisen(III) in Dorum- und Helgolandsedimenten reduziert wurde. Dies legt den Schluss nahe, dass eisenreduzierende Bakterien Huminstoffatmung mit Acetatoxidation koppeln. In Inkubationen mit Dominica-Sedimenten, in welche AQDS zugegeben wurde, wurde die *Halobacteriales* Grouppe DHVEG-6 als hauptsächlich Acetat assimilierende Mikroorganismen identifiziert. Diese Ergebnisse liefern einen eindeutigen Hinweis darauf, dass diese bisher nicht kultivierten Archaeen die Fähigkeit haben, Acetat mit AQDS zu verstoffwechseln.

Die Ergebnisse dieser Arbeit liefern Einblicke in das kaum erforschte Gebiet der *in situ* Verwertung von HS in marinen Sedimenten. Sie deuten an, dass es ein hohes Potenzial zur respirativen Nutzung von HS in marinen gibt Sedimente ein hohes Potenzial aufweisen, HS zu veratmen. Organischer Kohlenstoffeintrag in Form von HS wird deshalb sehr wahrscheinlich zu einer erhöhten Kohlenstoffmineralisierung und Eisenreduktion durch Elektronentransportprozesse in marinen Sedimenten führen.

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List of abbreviations

AHQDS	anthrahydroquinone 2,6, disulfonate
ANOSIM	Analysis of similarity
AQDS	Anthraquinone disulfonate
ASW	Artificial sea water
bp	Base pairs
BSA	Bovine serum albumin
CFB	*Cytophaga-Flavobacteriia-Bacteroides*
CI	Chloroform isoamylalcohol
C_{org}	Organic carbon
CsTFA	Cesium trifluoroacetate
DEPC	Diethylpyrocarbonate
DHVEG-6	Deep sea hydrothermal vent group 6
DNA	Deoxyribonucleic acid
DOC	Dissolved organic carbon
DOM	Dissolved organic matter
EES	Extracellular electron shuttle
EET	Extracellular electron transfer
FA	Fulvic acid
HA	Humic acid
HFO	Hydrous ferric oxides
HS	Humic substances
IR	Infrared

MFC	Microbial fuel cells
MPN	Most probable number
NMDS	Non metric multidimensional scaling
NMR	Nuclear magnetic resonance
OM	Organic matter
PCI	Phenol chloroform isoamylalcohol
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
qPCR	Quantitative PCR
rfu	relative fluorescence units
RNA	Ribonucleic acid
RNA-SIP	Stable isotope probing of RNA
rRNA	ribosomal RNA
TEA	Terminal electron acceptor
TRFLP	Terminal restriction length polymorphism
UV	Ultraviolet

Chapter 1 Introduction

1.1 Respiratory processes in marine sediments

The marine environment covers 70 % of the Earth's surface. Marine sediments are a big reservoir of organic carbon (C_{org}) with burial rates of ~ 0.12 Pg C yr^{-1} (Sarmiento and Sundquist, 1992; Tranvik et al., 2009). Organic matter (OM) in the marine environment can be of terrestrial or marine origins (differences are discussed in section 1.3.2). Most of riverine OM is mineralized in seawater or in surficial marine sediments. Where ~ 10 % of marine primary productivity remains at depths of up to several hundred meters, only ~ 1 % gets to the deep ocean (> 4000 m depth), and less than 0.5 % of global OM production buried in ocean sediments (Hedges and Keil, 1995).

OM degradation in marine sediments is connected to most biogeochemical processes during early diagenesis and mainly driven by microbiological mineralization (Rullkötter, 2006; Schulz, 2006). Degradation occurs in such a way that easily degradable OM (labile) is utilized first, whereas less reactive OM accumulates and is buried in deeper sediment (Rullkötter, 2006). OM degradation includes a variety of aerobic and anaerobic respiration processes as well as fermentation, which take place based on the available free energy of reactions (Thamdrup and Canfield, 2000; Jørgensen, 2006) (Table 1). As a result, a "vertical" redox zonation is created in the sediment. The general cascade along depth of terminal electron acceptors (TEA) used to oxidize OM is O_2 → NO_3^- → Mn(IV) → Fe(III) → SO_4^{2-} → CO_2 (Froelich et al., 1979; Jørgensen, 2006). During aerobic respiration, microorganisms can oxidize more complex organic compounds. Anaerobic respiratory microorganisms are more dependent on fermentation products, and electron acceptors, which provide higher

energy yield, will be used first (Lovley and Phillips, 1987; Chapelle and Lovley, 1992; Jørgensen, 2006) (Fig. 1). However, availability and reactivity (e.g. concentration, soluble *vs.* non-soluble) of TEA for respiration plays an important role. Therefore, there is a difficulty to define a clear cut for zonation and spatial overlaps occur (Canfield et al., 1993b; Thamdrup, 2000; Canfield and Thamdrup, 2009).

Table 1. Redox reactions of TEA for carbon oxidation and their standard energy (Gibbs free energies- ΔG°) per mole of organic carbon; after Jørgensen (2006)

Pathway	**Reaction**	**ΔG° (kJ mol^{-1})**
Aerobic respiration	$CH_2O + O_2 \rightarrow CO_2 + H_2O$	-479
Denitrification	$5CH_2O + 4NO_3^- \rightarrow 2N_2 + 4HCO_3^- + CO_2 + 3H_2O$	-453
Mn(IV) reduction	$CH_2O + 2CO_2 + H_2O + 2MnO_2 \rightarrow 2Mn^{2+} + 4HCO_3^-$	-349
Fe(III) reduction	$CH_2O + 7CO_2 + 4Fe(OH)_3 \rightarrow 4Fe^{2+} + 8HCO_3^- + 3H_2O$	-114
Sulfate reduction	$2CH_2O + SO_4^{2-} \rightarrow H_2S + 2HCO_3^-$	-77
Methanogenesis	$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$	-28

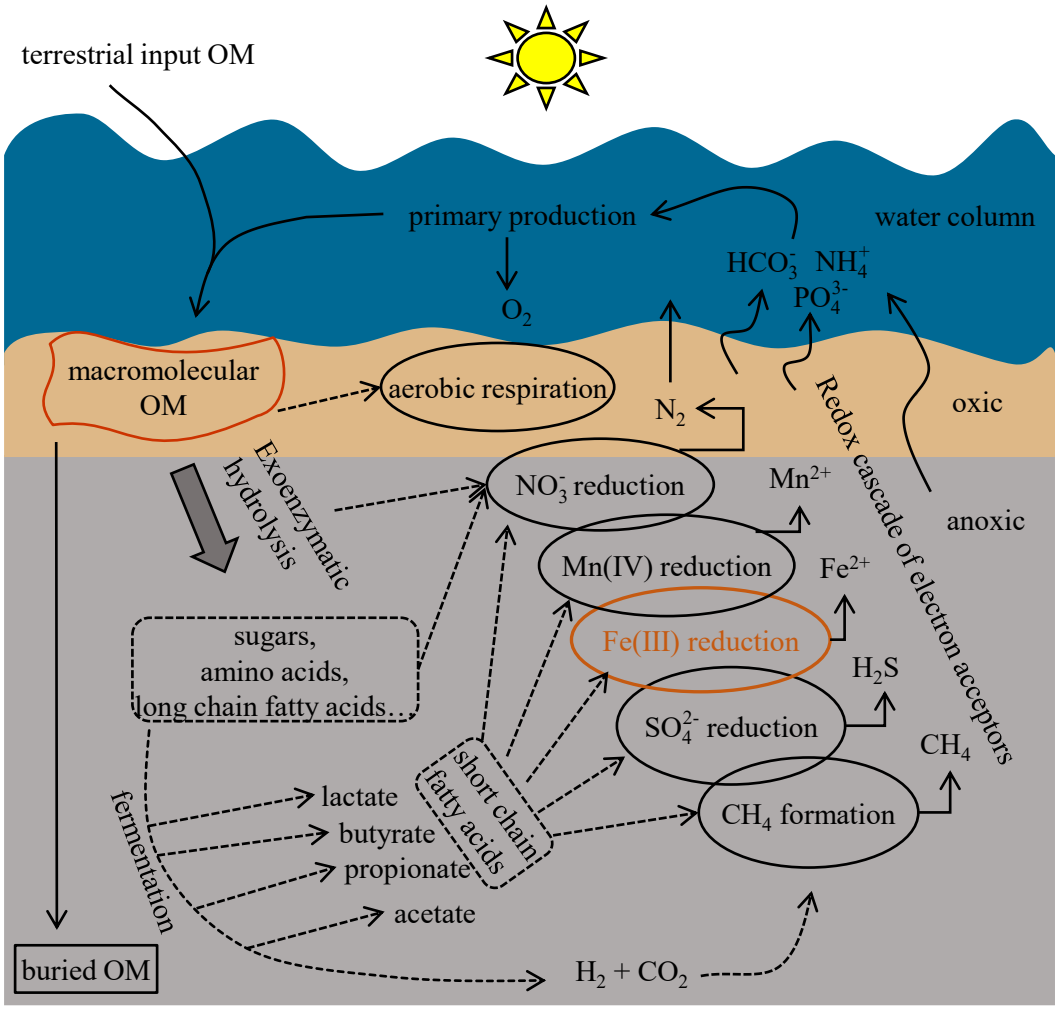

Figure 1. Scheme of redox cascade of TEA and geochemical zonations for OM degradation in marine sediments. Modified from Jørgensen (2006); and Ahke (2007).

1.2 Iron reduction in marine sediments

Iron is the fourth most abundant element in the continental crust. In marine sediments, it can make up to 6 % of total content. Under circumneutral pH iron oxides are extremely insoluble (reviewed in: Kraemer, 2004; Weber et al., 2006). Amorphous iron (hydr)oxide (poorly crystalline Fe, e.g. ferrihydrite; will be regarded as iron oxide) is the most important phase for microbial reduction (although not the only) and can account for up to 20 % of the total iron content (Thamdrup, 2000; Haese, 2006, and citations within). Nonetheless, iron reduction couples carbon and sulfur cycles and

occurs in almost all environmental niches in nature (Melton et al., 2014). Microbial iron reduction is controlled by availability of organic matter, concentration and reactivity of iron oxides, which is strongly connected to sediment reoxidation by bioturbation, waves, and currents (Jensen et al., 2003).

1.2.1 Importance of iron reduction in marine sediments

Iron reduction is an important pathway in C_{org} mineralization and usually takes place in the upper surface sediment (3 – 10 cm) (Thamdrup, 2000). Microbial iron reduction seems to be the most important source of dissolved iron in near shore marine sediments (Canfield, 1989). Contribution of iron reduction to total carbon mineralization can vary between different locations with an average of ~ 17 % of total mineralization (Thamdrup, 2000). Although sulfate reduction is considered to be the prominent pathway for carbon mineralization, iron reduction can be as significant as sulfate reduction (Sørensen, 1982; Canfield et al., 1993a), and it was found to account for more than 60 % at some sites (Jensen et al., 2003).

1.2.2 Mechanisms of biotic iron reduction

Unlike soluble electron acceptors, iron oxide is more difficult to access for respiration and cannot be easily taken up by cells. Therefore, microorganisms have to transfer electrons outside of the cell to the oxides using outer membrane bound cytochromes, requiring direct contact to the mineral surface (as reviewed in: Lovley, 2000; Kappler and Straub, 2005; Bücking et al., 2012; Levar et al., 2012) (Fig. 2 I). As surface area of minerals and motility of microorganisms can be limiting factors, different strategies are used by microorganisms to overcome these limitations and transfer electrons to iron oxides. The use of bacterial pili as nano-wires by *Geobacter*

sulfurreducens was proposed for transferring electrons to iron oxides (Reguera et al., 2005) (Fig. 2 II). Several microorganisms like *Shewanella oneidensis* can secrete redox active molecules (e.g., flavins) that can be used to shuttle electrons to the insoluble iron oxides (Newman and Kolter, 2000; Marsili et al., 2008; Wang et al., 2010). Others use chelators to solubilize the iron oxides, which can subsequently diffuse back to cells (as reviewed in: Nevin and Lovley, 2002; Melton et al., 2014) (Fig. 2 III). Furthermore, microorganisms can use natural organic molecules as humic substances (HS) as electron shuttle molecule (Lovley et al., 1996) (Fig. 2 IV). The latter will be discussed extensively in section 1.3.

1.3 Humic substances involved in extracellular electron transport

1.3.1 Composition and properties- general characterization

Humic substances (HS) are a mixture of heterogeneous organic compounds formed after decomposition of organic matter from plants, animals, and microbial cells (Livens, 1991; Stevenson, 1994; MacCarthy, 2001; Van Trump et al., 2006; Piepenbrock et al., 2011). Although these molecules are considered to be relatively recalcitrant, they can still be further degraded (Esham et al., 2000; MacCarthy, 2001; Van Trump et al., 2006; Kisand et al., 2008; Rocker et al., 2012a). HS are ubiquitous in the environment and can be found in soils, waters, and sediments (MacCarthy, 2001; Van Trump et al., 2006). They do not have an identified skeletal backbone, and there is no possibility to assign a molecular structure to fully describe the characteristic of these materials (MacCarthy, 2001). They possess alkyl aromatic units, carboxylic acid, phenolic, alcoholic, and

quinonic groups (Livens, 1991; Schulten et al., 1991; Stevenson, 1994; MacCarthy, 2001; Sutton and Sposito, 2005; Van Trump et al., 2006) (Fig. 3).

HS are classified into three groups: humins, humic acids (HA), and fulvic acids (FA), which are defined by their solubility. The humin fraction is insoluble at all pH values, HA are completely insoluble at pH < 2.0, and FA are soluble at all pH values. Those characteristics are the basis behind the first step of HS's extraction procedure from the environment. In a second step, the HS are separated over different XAD columns. The extraction procedure with strong acid and alkaline solutions can cause chemical changes to the HS molecule (Livens, 1991; Stevenson, 1994; Straub et al., 2005; Van Trump et al., 2006; Piepenbrock and Kappler, 2012).

HS can account for ~ 10 % of total content (by weight) of soils and sediments (reviewed in:Van Trump et al., 2006). Likewise, they are an important part of the total dissolved organic matter (DOM) pool and can account for 50 – 80 % in fresh water, riverine, marine sediments and coastal waters (Ertel and Hedges, 1984; Dittmar and Kattner, 2003; Rocker et al., 2012b). Dissolved organic carbon (DOC) flux to the ocean from riverine is estimated at ~ 0.36 Pg C yr^{-1} (Carlson and Hansell, 2015). As HS are also transported via rivers, estuaries, and near shore areas are expected to have higher input of terrestrial HS.

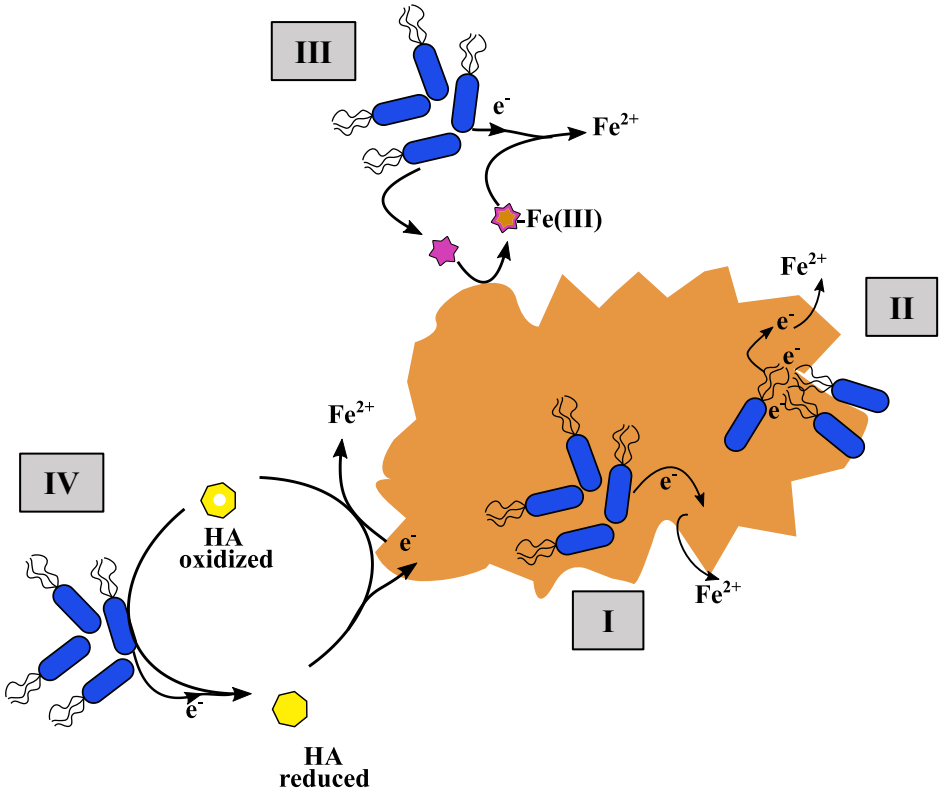

Figure 2. Mechanisms of microbial iron reduction. I, Electron transfer by direct contact. II, Use of conductive pili. III, Secretion of chelating agents, which solubilize iron and allow diffusion back to the cells. IV, Use of redox active molecule (humic acid- HA, as a HS representative) as electron shuttles for iron reduction.

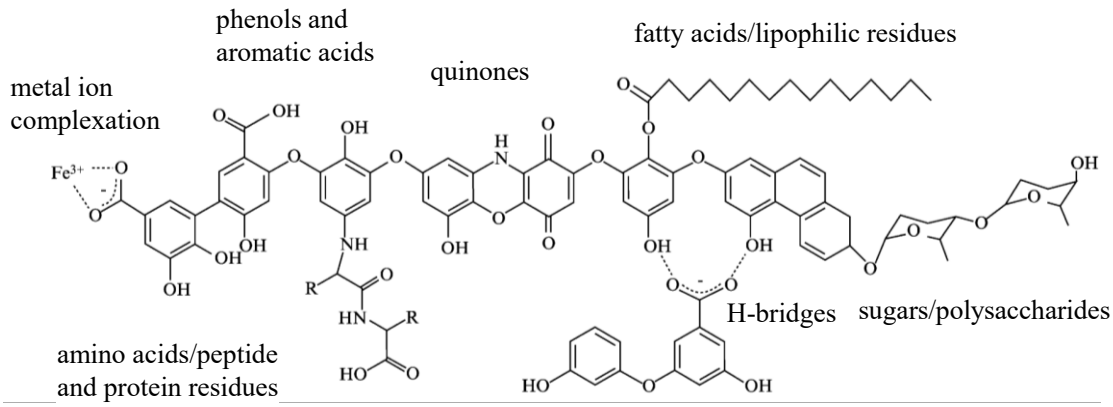

Figure 3. Representative molecular structure of HS molecule. Figure was taken from Straub et al. (2005) with publisher's permission.

HS are known to be redox active and have a wide range of redox potentials ranging from -300 to +400 mV which correlates to the redox active moieties (Struyk and Sposito, 2001; Straub et al., 2005; Aeschbacher et al., 2010; Aeschbacher et al., 2011; Piepenbrock and Kappler, 2012). As a result, HS can serve as electron sinks for fermentative bacteria, electron acceptors for microbial respiration, or for abiotic oxidation of inorganic compounds. Furthermore, they can donate electron for microbial respiration, or chemically reduce metals. Thus, they can act as electron shuttle molecules to mediate between bacterial oxidation of organic matter and reduction of metal oxides (Curtis and Reinhard, 1994; Lovley et al., 1996; Benz et al., 1998; Lovley et al., 1999; Nevin and Lovley, 2000; Cervantes et al., 2002; Kappler et al., 2004; Heitmann and Blodau, 2006; Van Trump et al., 2006; Lipson et al., 2010; Roden et al., 2010; Van Trump et al., 2011). Alternatively, HS have the ability to complex metals and to sorb on mineral and metals surfaces (Rashid, 1985; Livens, 1991; Van Trump et al., 2006; Riedel et al., 2013; Shimizu et al., 2013). Thus, HS have high potential to play a role as redox mediators in the environment.

1.3.2 Humics in the marine environment

HS share similarity in the basic composition despite differences in geographic location, climate condition and origin from which they are formed (MacCarthy, 2001). Nonetheless, it is possible to identify HS origin based on functional groups, which vary and depend on origin and age of the material (Van Trump et al., 2006). Characterization of HS is done using elemental composition ratios (H:C, O:C, N:C, etc.), IR and ^{13}C NMR spectrometry, and UV-visible spectrometry using ratios between absorbance at wave lengths of 465 and 665 nm (E4/E6) as well as 270 and 407 nm (A2/A4) (Schnitzer and Khan, 1972; Fooken and Liebezeit, 2000). Combination of E4/E6 and A2/A4 ratios,

followed by total carbon and hydrogen content were found to be the most representative factors for comparing the origins and characterization of different HS (Fooken and Liebezeit, 2000; Moreda-Piñeiro et al., 2006). Nevertheless, differences in HS composition can be due to differences in the extraction procedure (section 1.3.1).

HS found in marine sediments can originate from two different OM sources: aquatic-marine organisms, or terrestrial OM which is transferred into sediments from fields, soils, and streams. Terrestrial HS are derived from higher plants, and lignin and phenolic moieties have been identified as major components (Rasyid et al., 1992; Moreda-Piñeiro et al., 2006). They were found to have a high A2/A4 ratio (~ > 3.4), and H:C atomic ratios of ~ 1.068 – 1.324 have been reported (Fooken and Liebezeit, 2000; Moreda-Piñeiro et al., 2006). Soil and sediment derived HS tend to have higher electron uptake capacities than aquatic HS, due to higher content of aromatic moieties (Aeschbacher et al., 2010; reviewed in: Piepenbrock and Kappler, 2012). Marine derived HS are mainly of planktonic origins, are less aromatic, and have higher carbohydrate and protein contents. Marine derived HS were reported to have lower A2/A4 ratios with values down to ~ 1.9 (where coastal sediments have intermediate values), and H:C atomic ratios of ~ 1.29 – 1.52 (Rasyid et al., 1992; Fooken and Liebezeit, 2000; Moreda-Piñeiro et al., 2006, and citations within). Generally, as the humic molecule is younger and less aromatic, a higher ratio of E4/E6 is expected (Schnitzer and Khan, 1972).

Table 2 summarizes examples of HS characteristics from different locations- river sites sediments, estuarine, bays, and open water. Similar values concerning carbon content and H:C ratio can be seen. It is important to remember that, as stated earlier, differences in extraction procedure will affect the HS characteristics. Generally, it was

found that sediments closer to coastal areas possess HS which are more similar to terrestrial origin (Fooken and Liebezeit, 2000; Fooken and Liebezeit, 2003). In addition, currents and water circulations play an important role in distributing the OM of different origins in marine sediments (De la Rosa et al., 2011).

1.3.3 Extracellular electron transfer in sediments

Electron transfer and especially extra cellular electron transfer (EET) in sediments have become of interest recently in geomicrobiology (e.g. Nielsen et al., 2010; Roden et al., 2010; Malvankar et al., 2015; Nielsen and Risgaard-Petersen, 2015). Filamentous bacteria were shown to be conductive (cable bacteria) and proposed to facilitate electron transfer in sediments (Pfeffer et al., 2012). Another mechanism for EET involves conductive minerals such as magnetite and pyrite as was shown in pure cultures and sediment incubations (Kato et al., 2012; Malvankar et al., 2015). Dissolved HS were first shown to act as extra cellular electron shuttle (EES) molecule for microbial iron reduction by Lovley et al. (1996). In addition, Roden et al. (2010) showed that solid-phase HS can act as electron shuttle molecules as well. HS were shown to increase electric current in microbial fuel cells (MFC) incubated with marine sediments (Holmes et al., 2004). However, there is lack of data on the utilization of HS as electron shuttle molecules *in-situ* in marine sediments.

Table 2. Examples of HS extracted from fresh water and marine sediments of various locations. Content of carbon hydrogen and nitrogen, and atomic ratios. Ranges are from several locations at the sites.

Sediment origin	Depth	~C (%)	~H (%)	~N (%)	~H:C	~N:C	Reference
River sediment	20 cm	~9-50	na	5-4.4	na	0.07-0.09	a
River sediment	14-15 m	43	4.9	3.3	1.4	0.06	b
River sediment	38-39 m	47	6.3	3.5	1.6	0.06	b
Estuary	20 cm	46-50	na	4.3-4.7	na	0.07-0.09	a
Manila Bay	surface	48-53	5-5.8	5.8-7.3	1.1-1.4	0.09-0.13	c
Bolinao Bay	surface	49-52	5.6-6	7.7-9.2	1.4	0.13-0.16	c
Gulf of Mexico	na	55	4.5	5.6	1.2	0.07	c
Pacific Pelagic	na	54	5.6	5.9	1.3	0.09	c

a (De la Rosa et al., 2011).
b (Polvillo et al., 2009).
c (Llaguno, 1997).
na- not available.

1.3.4 Humic substances as electron shuttles for iron reduction

Quinone moieties are believed to be the main redox active sites in HS (Dunnivant et al., 1992; Scott et al., 1998; Lovley and Blunt-Harris, 1999). Furthermore, it was suggested that the electron uptake capacity of OM is correlated to the content of total aromatic moieties in the samples (Chen et al., 2003). Thus, quinone-like moieties are an important factor in the electron transfer properties of HS.

Lovley et al. (1996) were the first to show the ability of microorganisms (*Geobacter metallireducens* and *Shewanella alga*) to reduce HS by oxidizing an organic carbon source and to conserve energy (support growth) from the process. The process further stimulated reduction of iron oxides and depended on the presence of bacterial cells. Hence, HS were used as electron shuttle molecules in microbial metabolism.

The HS mediated electron shuttling process occurs in two steps (Fig. 4) (Lovley et al., 1996): the first step is the microbial reduction of the HS coupled to the oxidation of organic matter. The second step is the abiotic reduction of the iron oxide by the reduced HS and is controlled by the concentration and diffusion of the HS (Lovley et al., 1996; Kappler et al., 2004; Weber et al., 2006; Jiang and Kappler, 2008; Piepenbrock and Kappler, 2012; Melton et al., 2014). Hence, the HS molecule can be recycled (Klüpfel et al., 2014) and increase iron reduction rates. In addition, HS complexation can reduce Fe^{2+} concentrations, thereby increasing the thermodynamic driving force for further iron reduction (Royer et al., 2002a; Royer et al., 2002b).

The exact mechanisms of HS reduction by microorganisms are not completely known. But there are indications that the electrons are being transferred from the cells via similar, although with a broader range, outer membrane protein complexes which are used for direct iron reduction (Lies et al., 2005; Gescher et al., 2008; Voordeckers et al., 2010). In the abiotic step, reduced HS can transfer electron to a variety of iron oxides, including more crystalline forms which are harder to reduce directly by microorganisms (Lovley et al., 1998), hence a greater impact on the iron pool.

Due to complexity and low solubility of commercial HS, they are not generally used in incubation experiments. Therefore, other model compounds such as the quinone analog 9,10-anthraquinone-2,6- disulfonate (AQDS; Fig. 5) are used (Curtis and

Reinhard, 1994; Lovley et al., 1996; Coates et al., 1998; Straub et al., 2005; Van Trump et al., 2006). AQDS was found to be one of the most efficient quinone analogs of HS. Additionally, it does not have chelating properties, has a mid-redox potential of -184 mV (pH 7), and AQDS sorption to iron minerals is more than one order of magnitude lower than that of HS (Wolf et al., 2009).

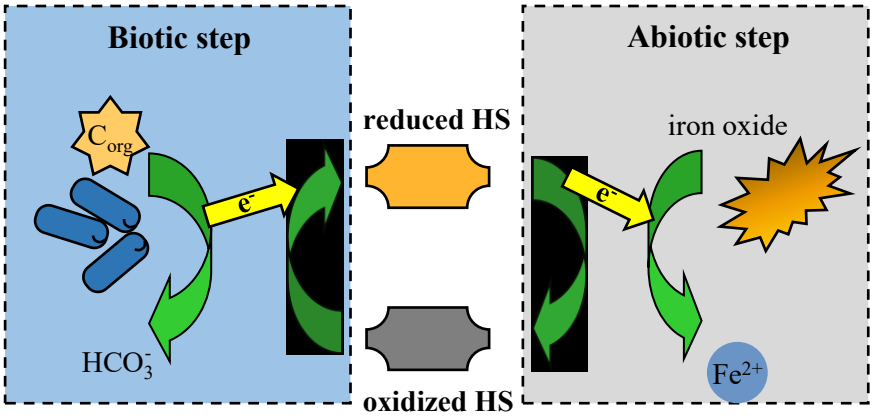

Figure 4. Two step reaction model of iron reduction via HS as electron shuttles. In the biotic step, organic carbon is oxidized coupled to reduction of HS. In the abiotic step, the reduced HS molecule is oxidized by iron oxide and is ready for another cycle, resulting in release of Fe^{2+}.

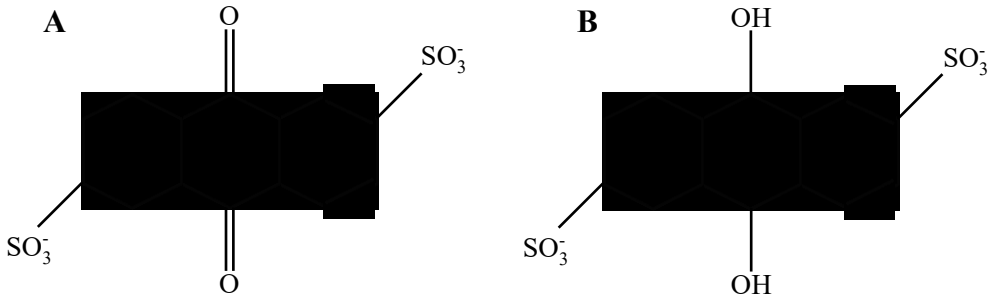

Figure 5. Chemical structure of the HS quinone analog AQDS in an oxidized form (A) and in the most reduced form (B).

1.3.5 Effect of humic substances on geochemical cycles in marine sediments

The effect of HS on marine sediments is not restricted to direct OM respiration and iron reduction. HS can also effect the sulfur cycle by complexation or oxidation of sulfide species (Heitmann and Blodau, 2006; Aranda-Tamaura et al., 2007; Yu et al., 2015; Yu et al., 2016). By complexation of sulfide with HS, sulfide concentrations will decrease, keeping the energy yield (ΔG') favorable for continuous sulfate reduction, potentially coupled to OM oxidation. Hence, it has an indirect effect of HS on carbon mineralization in sediments. Alternatively, after chemical reduction of HS by sulfide, the reduced HS can donate the electrons to iron oxides. This process results in an indirect iron reduction with electrons which originated from sulfate reduction. Reduced HS can also affect the nitrogen cycle by biotic reduction of nitrate and nitrite coupled to quinone oxidation. It also lowers emissions of the greenhouse gas N_2O (Lovley et al., 1999; Aranda-Tamaura et al., 2007; Van Trump et al., 2011). Additionally, respiration of HS was found to decrease emissions of CH_4, another greenhouse gas (Blodau and Deppe, 2012; Martinez et al., 2013). Finally, HS can also take active part in interspecies electron transfer (Lovley et al., 1999). Therefore, an input of organic carbon in the form of HS will likely result in a stimulation of carbon mineralization, enhanced iron reduction through electron shuttling in marine sediments, as well as other effects in several geochemical cycles.

1.4 Identification and quantification of humic and iron reducing microorganisms

Iron and humic reducing microorganisms can be found in different phylogenetic phyla (reviewed in: Weber et al., 2006; Lovley, 2013; Martinez et al., 2013) (Fig. 6), regardless whether they gain energy for growth from the process or not. Most iron reducing microorganisms are able to reduce humics. Additionally, respiration of quinones is assumed to be widespread among anaerobes (Stams et al., 2006). However, not only iron reducers can respire HS, also halorespiring, sulfate reducing and methanogenic microorganisms are able to perform this process. Additionally, fermenting bacteria can use HS as electron sink (Benz et al., 1998; Cervantes et al., 2002). Thus, iron reduction via electron shuttling of HS can be mediated by a large variety of microorganisms. As a result, there is no phylogenetic marker (such as the 16S rRNA gene), nor any specific key gene, that can be used for molecular identification, and quantification *in-situ*.

For quantifying iron and humic reducing microorganisms, the most probable number (MPN) technique can be applied (Kappler et al., 2004; Straub et al., 2005; Lin et al., 2007; Vandieken et al., 2012; Vandieken and Thamdrup, 2013; Piepenbrock et al., 2014) (Comparison table: chapter 3, Table 4). Identification of iron and humic reducing microorganisms can be achieved by sequencing of: enrichment cultures, microorganisms in highest dilutions of MPN tubes, or isolates. Alternatively, it is possible to identify key players *in-situ* by stable isotope probing of RNA (RNA-SIP). RNA-SIP is a powerful method to link identity of uncultivated microorganisms to physiology. This is done by incorporation of substrate which is enriched in stable isotope composition (e.g., ^{13}C) into RNA molecules (Manefield et al., 2002; Dumont

and Murrell, 2005). The ^{13}C-labeled RNA can be separated from the total RNA via density gradient centrifugation (Manefield et al., 2002). ^{13}C-acetate is usually used for identifying iron reducing microorganisms (Hori et al., 2010). It is possible as acetate oxidation to CO_2 can occur only in the presence of suitable electron acceptors (during respiration) or in syntrophic acetate oxidizing consortia (Schink, 1997; Nüsslein et al., 2001) due to low energy yield ($acetate^- + H^+ + 2H_2O \rightarrow 2CO_2 + 4H_2$; $\Delta G°' = +95$ kJ mol^{-1}) (Thauer et al., 1977). Furthermore, by using Illumina sequencing technique it is possible to identify the active population even when their (labeled) RNA proportion of the total RNA pool is as low as 0.001 % (Aoyagi et al., 2015).

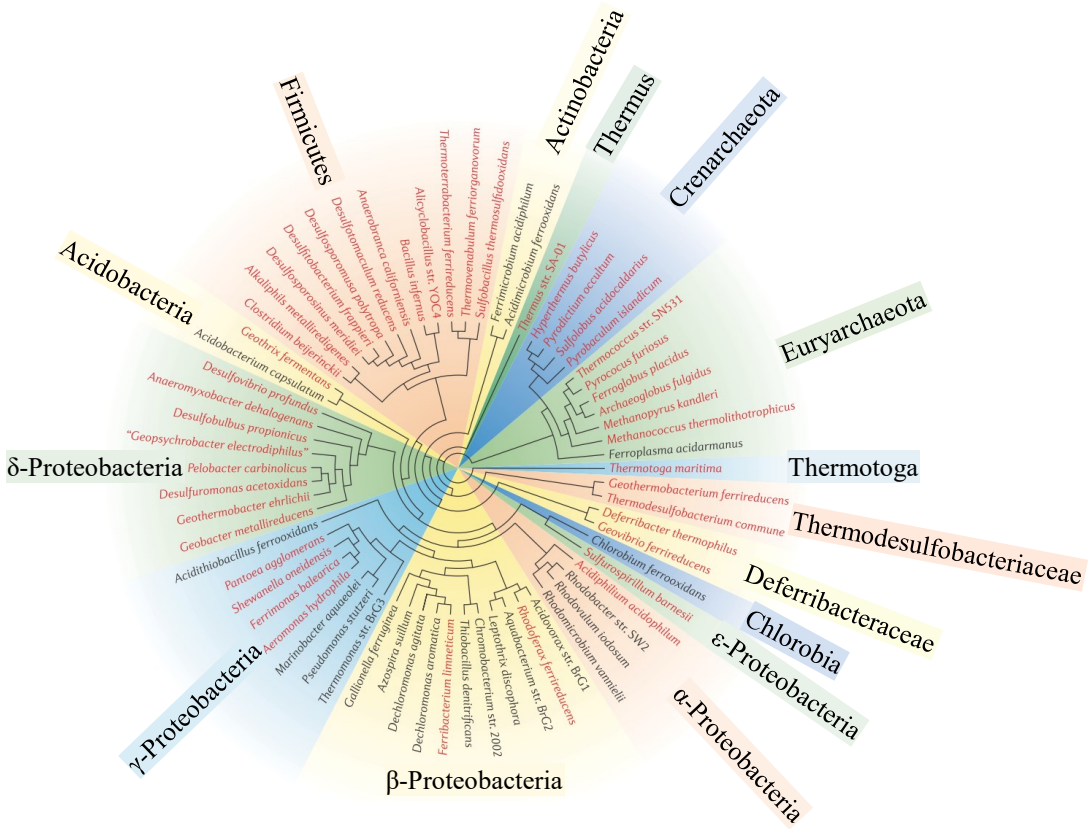

Figure 6. Phylogenetic affiliation of microorganisms involved in iron cycling. Iron reducers are marked in red, iron oxidizers in black. Figure was taken from Weber et al. (2006) with publisher's permission.

1.5 Objectives and research questions

The potential of using HS as electron shuttling molecules for respiration processes, especially as mediators for iron reduction was shown previously mainly by using pure cultures and synthetic iron oxides, but was shown to occur also in soils, marine sediment MFCs, and fresh water sediments (Lovley et al., 1996; Coates et al., 1998; Nevin and Lovley, 2000; Holmes et al., 2004; Kappler et al., 2004; Wolf et al., 2009; Piepenbrock and Kappler, 2012). Marine sediments, especially near shore environments, can have DOC content as high as 24 mg L^{-1} (Billerbeck et al., 2006) and therefore are prone to support electron shuttling via HS as suggested by Jiang and Kappler (2008). Furthermore, the reduction of solid/phase humics for respiration (Roden et al., 2010) suggests that HS reduction is likely to be underestimated when only the dissolved HS are taken into account. Only few attempts were done to identify HS reducing microorganisms in marine sediments if by isolation (Coates et al., 1998) or in sediment dilutions enriched together with iron oxides or in MFC (Holmes et al., 2004; Lin et al., 2007). However, no extensive work is available on identification and quantification of humic reducing microorganisms in marine sediments. As a result, there is a gap in our knowledge where HS can be used *in-situ* in marine sediments as EET molecules for supporting iron reduction.

In this work, I will show the microbial potential to use HS in marine systems by using slurry incubations, MPN counts, and identification by RNA-SIP approach from different marine sites. The experiments conducted will answer the following research questions and will check the validity of the hypotheses.

1.5.1 Is there a potential for utilizing HS as electron shuttle molecules for iron reduction in marine sediments?

Hypothesis: Fe^{2+} formation rates are higher in HS amended slurry incubations.

In order to answer this question, slurry incubations from three different sites were conducted. The incubations were amended with different types and concentrations of HS (natural or the analog molecule AQDS). The experiments and results are presented in chapters 3 and 5 as separate units to be submitted for publications.

1.5.2 Who are the active microorganisms that use HS as electron shuttle/sink in marine sediments?

Hypothesis: Oxidation of acetate, as an important carbon source, is mediated by different populations in the absence or presence of HS.

For answering this, we used incubations parallel to those mentioned above, incubating with ^{13}C-labeled acetate and applying the RNA-SIP approach coupled with high throughput sequencing. The experiments and results are presented in chapters 4 and 5 as separate units to be submitted for publications.

1.5.3 What are the abundances of the active humic and iron reducing populations in marine sediments?

Hypothesis: HS reducing bacteria are more abundant in marine sediments than iron reducing bacteria.

This question was addressed by applying MPN counts to estimate the numbers of microorganisms which can use acetate as electron donor with; iron oxides as TEA, HS

as TEA, or iron as TEA and HS as electron shuttle molecule. The results are presented in chapter 3.

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Chapter 2 Methods

2.1 Sediment sampling

Surface sediment samples were collected from: 1. The Helgoland mud area (Fig. 1 A) in July 2013 during RV HEINCKE cruise HE406 using a multicorer (water depth 30 m, plexiglass tubes 9.5 cm in diameter down to 50 cmbsf) from stations HE406/04-1 (54.1N, 7.9845E) and HE406/08-01 (54.0835N, 7.96683E); samples were stored until further processing at 4 °C (ca. 3 weeks). 2. Dorum Neufeld (Wadden Sea, 53.738281N, 8.504825E) in January 2013, April 2013, and March 2014 using plexiglass tubes (5.2 cm in diameter, 30 cm long) (Fig. 1 B); samples were brought back to the lab and immediately processed. 3. An active hydrothermal vent site at the coast of Soufriere (Dominica, Lesser Antilles) in April 2013 (south west of the island 15.232N, 61.3616W) from a water depth of 5 m and sediment temperature of 55 °C (Fig. 1 C). Sediment cores (plexiglass tubes 5.2 cm in diameter, 30 cm long) were retrieved using scuba diving after identifying hot spots with temperature probes as described earlier (Price et al., 2013a; Price et al., 2013b; Gomez-Saez et al., 2015; Kleint et al., 2015). Samples were stored until processing in the lab at 4 °C (9 days).

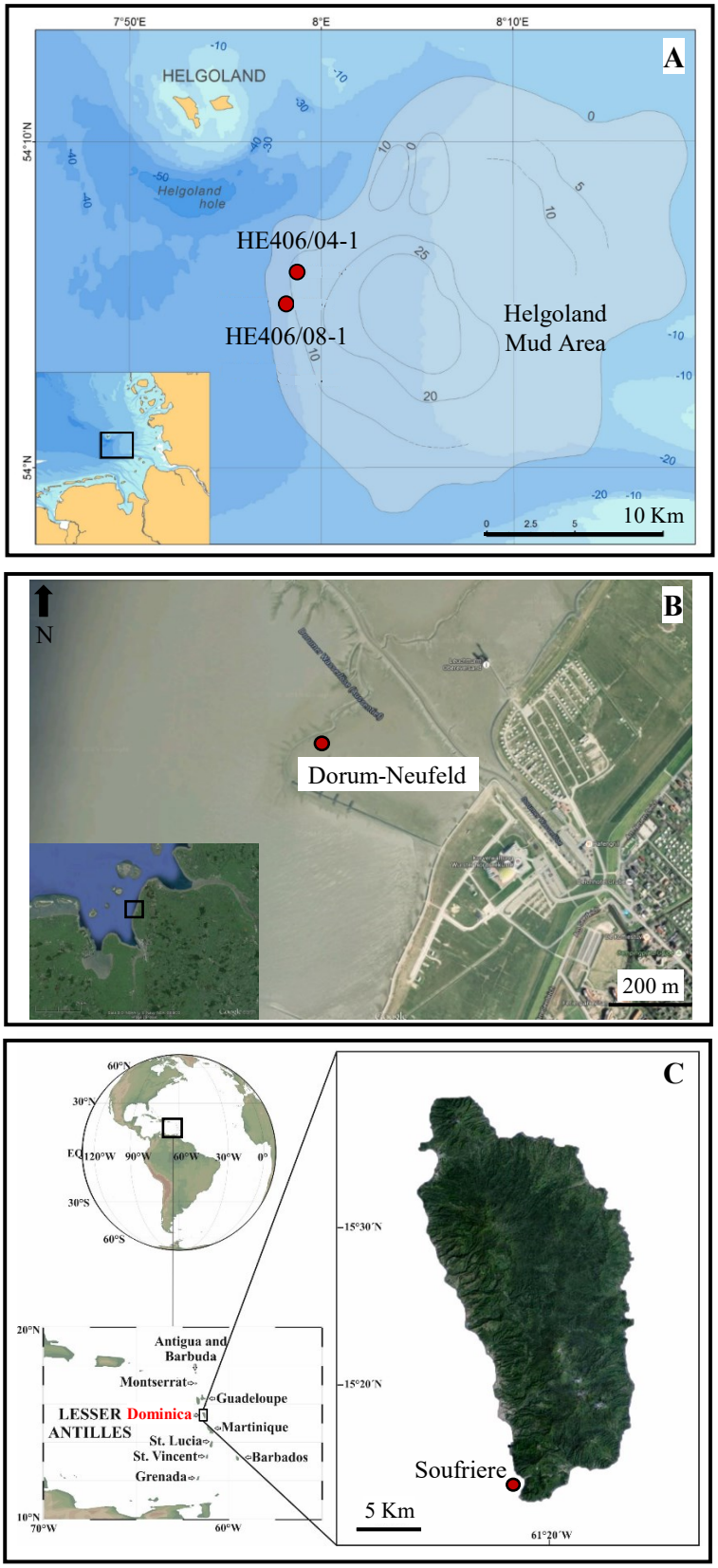

Figure 1. Sampling sites locations. **A.** Helgoland mud area (courtesy R. Martinez). **B.** Dorum-Neufeld. **C.** Soufriere bay Dominica Lesser Antilles. Maps were created using Google Earth (http://earth.google.com) (B, C) and after Gomez-Saez et al. (2015) using Ocean Data View (R. Schlitzer, http://odv.awi.de) (C).

2.2 Sediment slurry preparations

For sediment incubations, slurries were prepared under continuous flow of N_2 gas using the upper 5 to 10 cm of sediment cores by mixing the sediment with autoclaved anoxic sulfate-free artificial seawater (ASW) which contained (per liter of deionized water) 26.4 g NaCl, 11.2 g $MgCl_2 \cdot 6H_2O$, 1.5 g $CaCl_2 \cdot 2H_2O$, 0.7 g KCl. Sediment slurry was transferred into vials, supplements were added under a stream of N_2, and vials were sealed with butyl rubber stoppers. Sodium acetate or sodium lactate was used as electron donors. For labeling experiments, 1,2- $^{13}C_2$ 99 % sodium acetate was used (Cambridge Isotope Laboratories, USA). Lepidocrocite powder (Υ – FeOOH; Bayferrox 943, Lanxess, Leverkusen, Germany) was supplemented as an electron acceptor (chapters 3-4). AQDS (AB136728, ABCR, Karlsruhe, Germany) was used as electron shuttle or terminal electron acceptor. Aldrich humic acid (Sigma-Aldrich Chemie GmbH, Steinheim Germany) was prepared in 30 mM phosphate buffer pH 7, and used as an electron shuttle at a final concentrations of 167 µg mL^{-1} or 16.7 µg mL^{1-} (chapter 3).

2.3 Most probable number incubations (MPN)

For MPN incubations (chapter 3), an anoxic, bicarbonate buffered (30 mM) marine Widdel medium (modified after Straub et al. (2005)) was used; per liter of deionized water, the medium contained: 26.4 g NaCl, 11.2 g $MgCl_2 \cdot 6H_2O$, 1.5 g $CaCl_2 \cdot 2H_2O$, 0.7 g KCl, 0.023 g $MgSO_4 \cdot 7H_2O$. The medium was prepared anoxically in a 5 L Widdel flask, autoclaved, and cooled under N_2:CO_2 (80 % : 20 % v:v) atmosphere (Widdel and Bak, 1992). The medium was supplemented with 1 mL L^{-1} of: ammonium

chloride (stock solution 0.62 M; 0.62 mM final concentration), non-chelated trace element mixture (SL 10), and selenite-tungstate solution (Widdel and Bak, 1992). Vitamin solutions were modified after (Winkelmann and Harder, 2009) and added to a final concentration of: 0.04 mg L^{-1} 4-aminobenzoic acid, 0.02 mg L^{-1} biotin, 0.1 mg L^{-1} nicotinic acid, 0.05 mg L^{-1} pantothenic acid, 0.15 mg L^{-1} pyridoxine HCl, 0.04 mg L^{-1} folic acid, 0.02 mg L^{-1} lipoic acid, and 0.05 mg L^{-1} B-12. Riboflavin and thiamin HCl were added to 0.05 mg L^{-1} each. 10 g of sediment was added to 90 mL of ASW in a glass bottle. The bottle was sealed with a rubber stopper and the headspace was made anoxic by alternating vacuum and flushing with N_2 (99.999%) for 3 times 3 min each. Dilution series (1:10, in triplicates) were made from the slurry in Hungate tubes filled with 9 mL of medium. Sodium acetate was used as electron donor at a final concentration of ~ 2 mM. Ferrihydrite was used as terminal electron acceptor and was prepared after Schwertmann and Cornell (1991) and Straub et al. (2005); briefly, ~ 80.5 g of $FeCl_3$ was dissolved in 1 L of water, pH was adjusted with 1 M NaOH to pH 7. The precipitated ferric iron was washed 7 times by centrifugation, decantation of the supernatant, and re-elution of the precipitate with deionized water. The ferrihydrite stock solution was made anoxic as described above and autoclaved (121 °C, 20 min) (Piepenbrock et al., 2011). Ferrihydrite was added to a final concentration of ~ 8 µmol cm^{-3} incubation. AQDS was added as electron shuttle or as terminal electron acceptor (5 µM and ~ 440 µM, respectively). Most probable number dilutions were incubated for 14 weeks in the dark at room temperature. Microbial reduction of AQDS was indicated by a color change from transparent to an increase in yellow color. Iron oxide reduction was accompanied by a change in color from reddish (ferrihydrite) to brown/black and to colorless compared to control tubes without sediment inoculum, and by the iron concentration compared to the control using the ferrozine assay (see below).

Most probable numbers were estimated using KLEE software (Klee, 1993), and Cornish and Fisher lower and upper 95 % confidence limits (Cornish and Fisher, 1938) were calculated by the software. DNA was extracted from the highest positive dilution tubes.

2.4 Chemical analysis

At each time point, liquid samples were taken anoxically using an N_2 flushed syringe, filtered through 0.2-µm regenerated cellulose filter (Sartorius, Göttingen Germany) and transferred into 0.5 M HCl (Fig. 2) (trace metal grade, HN53.1, Carl-Roth, Karlsruhe, Germany). Fe^{2+} was measured spectrophotometrically following the ferrozine assay (Stookey, 1970; Hegler et al., 2008) with modifications (50 % w:v ammonium acetate, 0.1 % w:v ferrozine). $FeCl_2$ (in 0.5 M HCl) anoxic solution (under N_2 atmosphere) was used as standard for calibration with 6 points (0 – 25 µM final concentration in ferrozine). Samples (in HCl) and ferrozine solution were mixed in 1:1 ratio, allowed to react for 5 min, and absorbance was measured at wavelength of 562 nm. Samples, which had higher absorbance than the calibration curve (~ 0.7), were diluted once more (in HCl) to achieve an absorbance value between the calibration points. Minimum volume of samples transferred for dilution was usually > 50 µL and never below 30 µL. Sulfate concentration was measured at the laboratories of the AWI (Bremerhaven, Germany) using a Metrohm Compact IC 761 ion chromatograph. Dissolved manganese was measured after filtration and acidification with HNO_3 (conc.). Measurements were done at AWI (Bremerhaven, Germany) via inductively coupled plasma optical emission spectrometry (Thermo Scientific IRIS Intrepid instrument, Bremen, Germany) (Riedinger et al., 2014).

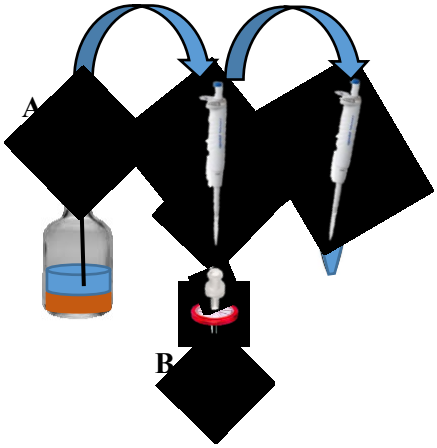

Figure 2. Anoxic sampling for iron measurements. A. Sample was taken using N_2 pre-flushed syringe. B. Sample was filtered (0.2 µm) to a second syringe. C. The first aliquot was discarded, then using a pipette the desired amount was taken at the lowest point possible to minimize effect of oxygen diffusion. D. Sample was added to 0.5 M HCl.

2.5 DNA and RNA extractions from sediment slurries

DNA and RNA extractions were done as described by Henckel et al. (1999) and Lueders et al. (2004). Approximately 0.7 g of sediment was added to a 2 mL screw-cap vial, prefilled with ~ 0.7 g of baked (3 h, 180 °C) 0.1 mm (diameter) zirconia/silica beads (11079101z, BioSpec, USA). For DNA extraction from highest positive MPN tubes, cells were concentrated from 1.8 mL of culture medium by centrifugation for 70 min at 20,800 rcf, 4°C, the supernatant was discarded, and zirconia/silica beads were added. For quantification of 16S rRNA gene copy numbers at the beginning of MPN incubations, 1 mL of the inoculation slurry (~ 0.1 mL wet sediment) was used. The vials were filled with 750 µL of 120 mM $NaPO_4$ buffer (Table 1) and 250 µL TNS solution (Table 1) and placed in a bead beater for 45 s at 6.5 m s^{-1}. Immediately after that the vials were centrifuged for 10 min at 20,800 rcf and 4 °C. Supernatants were transferred to new 2 mL vials. One volume of phenol/chloroform/isoamylalcohol (P/C/I; 25:24:1

v:v:v; pH 8; A156.1, Carl-Roth GmbH, Karlsruhe Germany) was added. Vials were vigorously shaken for 20 s and then centrifuged for 5 min at 20,800 rcf and 4 °C. Supernatants were transferred to new 2 mL vials, and one volume of chloroform/isoamylalcohol (C/I; 24:1 v:v; Carl-Roth GmbH, Karlsruhe Germany) was added. Vials were vigorously shaken for 20 s and then centrifuged for 5 min at 20,800 rcf and 4 °C. Supernatants were transferred to new 2 mL vials, ~ 2.5 volumes of polyethylene glycol (PEG; Table 1) were added for precipitation and the vials were centrifuged for 90 min at 20,800 rcf and 4 °C. Supernatants were discarded, the pellets were washed with 70 % ethanol (4 °C) and centrifuged for additionally 30 min. Supernatants were discarded, pellets were left for air drying (~5 min) and resuspended with 50 µL nuclease free diethylpyrocarbonate (DEPC) treated water (T143.3, Carl-Roth GmbH, Karlsruhe Germany).

In order to get pure RNA, DNA was digested using RQ1 DNase (Promega) according to the manufacturer's protocol, which was scaled up to 200 µL of volume. Further P/C/I (25:24:1 v:v:v) and C/I (24:1 v:v) extractions were performed (Lueders et al., 2004) for removing DNase, followed by overnight precipitation in one volume of isopropanol, one fifth volume of 3 M sodium acetate at -20 °C and 60 min centrifugation at the morning after. The extracts were then washed in 70 % ethanol (4 °C), centrifuged for 30 min, air dried, eluted in nuclease free DEPC treated water and quantified using RiboGreen assay (Invitrogen Quant-iT Ribogreen RNA assay kit, Life technologies).

Table 1. Solutions used for DNA/RNA extractions and ultracentrifugation. All solutions were prepared with DEPC treated water, in baked glassware (3 h, 180 °C)

Solutions	Ingredients
120 mM $NaPO_4$ buffer	112.88 mM Na_2HPO_4, 7.12 mM NaH_2PO_4
TNS solution	500 mM Tris-HCl pH 8, 100 mM NaCl, 10 % SDS (w:v)
PEG	30 % (w:v) polyethylene glycol 6000 in 1.6 M NaCl
Gradient buffer	0.1 M Tris-HCl pH 8, 0.1 M KCl, 1 mM EDTA

2.6 Reverse transcription and amplification of 16S rRNA genes

cDNA was prepared using Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Life Technologies, Darmstadt, Germany) following the manufacturer's protocol. 16S rRNA (genes) were amplified from DNA extracts or cDNA using primer sets listed in Table 2 (see sections 2.7, 2.8, and 2.10). The reaction mixture contained in a total volume of 25 µL (all in final concentrations): 1 x PCR buffer II (Applied Biosystems), 200 µM of each dNTP, 3 mM $MgCl_2$, 200 µg mL^{-1} bovine serum albumin (BSA), 0.3 mM of each primer and 0.025 U μL^{-1} of Ampli *Taq* DNA polymerase (Applied Biosystems). Amplification was performed using a Gene Amp 9700 PCR System (Applied Biosystems), and PCR conditions were: initial denaturation at 94 °C for 2 min followed by 30 cycles of: denaturation at 94 °C for 30 s, annealing at 52 °C for 1 min, initial elongation at 72 °C for 1 min, adding a final extension step at 72 °C for 7 min. PCR products were purified using the MinElute PCR purification kit (Qiagen, Hilden, Germany).

2.7 Terminal restriction length polymorphism (TRFLP) analysis

120 ng of PCR products (primers set 8f-FAM labeled/907r; Table 2; PCR cycle as mentioned above) were digested using *MspI* (Promega) as described by Egert and Friedrich (2003). Digested products were purified using SigmaSpin Post reaction clean up columns (Sigma-Aldrich, Germany), and 3 µL were mixed with (per reaction) 14.7 µL of Hi-Di formamide (Applied Biosystems) and 0.3 µL of ROX-labeled MapMarker 1000 (BioVentures, Murfreesboro, TN), denatured (5 min at 95 °C) and kept at 4 °C until processing. Size separation on an ABI Prism 3130 XL Genetic Analyzer (Applied Biosystems) was performed as described earlier (Schauer et al., 2010). For analysis, only peaks between 50 and 800 base pairs (bp) in length which had more than 50 relative fluorescence units (RFU) were used. Peak analysis was performed with TREX-software (Culman et al., 2009) with a noise reduction based on 1.5 standard deviations of peak height. Automated alignment using 1 bp shift was used for binning. Peaks were relativized within each sample, and RFU were recalculated based on peak area.

Table 2. Primer sets used for amplification of 16S rRNA or its gene. See chapters 3-5 for details, of which primer set was used each time.

Purpose	Primer set	Sequence 5'→3'	Reference
TRFLP/amplicons for sequencing	8f/FAM labeled	AGAGTTTGATCCTGGCTCAG	(Turner et al., 1999)
	907r	CCGTCAATTCCTTTRAGTTT	(Muyzer et al., 1995)
qPCR	8f	AGAGTTTGATCCTGGCTCAG	(Turner et al., 1999)
	1492r	GGTTACCTTGTTACGACTT	(Turner et al., 1999)
qPCR	338f	CCTACGGGAGGCAGCAG	(Muyzer et al., 1993)
	518r	ATTACCGCGGCTGCTGG-	(Muyzer et al., 1993)
sequencing	27f	AGRGTTTGATCMTGGCTCAG	(Kuske et al., 2006)
	519r	GWATTACCGCGGCKGCTG	(Turner et al., 1999)
sequencing / amplicons for sequencing	341f	CCTACGGGNGGCWGCAG	(Herlemann et al., 2011)
	805r	GACTACNVGGGTATCTAATCC	(Takahashi et al., 2014)

2.8 Quantitative PCR (qPCR)

Bacterial 16S rRNA genes were quantified using an absolute qPCR method. Standard template was prepared by extracting DNA from *Escherichia coli* strain SB1. The 16S rRNA gene was amplified using the bacterial primers 8f and 1492r (Table 2) and purified as described above. Concentrations of purified standard template and DNA extracts from MPN dilutions were quantified using Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies, Darmstadt, Germany). Quantitative PCR (cycler: StepOnePlus, Real-Time PCR System, Applied Biosystems) was carried out using bacterial primers 338f and 518r (Table 2). Each qPCR reaction contained in total volume of 20 µL (all in final concentrations): 1 x of MESA Blue qPCR MasterMix Plus (Eurogentec, Cologne, Germany), 0.2 mg mL^{-1} BSA, and 0.1 µM of each primer. Standard curve analysis was

performed using 10-fold serial dilutions of the standard template ranging from 1 pg μL^{-1} to 0.1 fg μL^{-1} with 94 % efficiency (R^2=0.992). Quantity of DNA extracts was obtained based on the standard curve analysis. Gene copy numbers were quantified using the following formulas:

$$Gene\ copies\ = \frac{M\ \times T}{QU\ \times 914878\ (Da)}$$

$$M\ = \frac{QO}{1.66054\ \times 10^{-24}\ (g)}$$

Where:

M = Mass of DNA amplified (Da)

T = Total DNA extracted (ng)

QU = Quantity of DNA used for qPCR (ng)

QO = Quantity of DNA obtained from q-PCR (g)

914878 (Da) = Mass of one gene copy of standard template

2.9 Isopycnic separation and fractionation

Density gradient centrifugation was modified following Lueders et al. (2004). Briefly, RNA (exact quantities are described in chapters 4 and 5) was mixed with gradient buffer (Table 1) to a volume of 1.3 mL, 240 µL of deionized formamide and 6 mL of cesium trifluoroacetate (CsTFA; illustra CsTFA- 17084702, GE Healthcare, UK). The starting density of the gradient medium was adjusted to ~ 1.796 g mL^{-1} using an AR200 digital refractometer (Reichert Analytical, NY USA). Afterwards, ~ 6.5 mL

of the mixture was transferred to Beckman polyallomer Quick Seal 16 x 45 mm tubes (Catalog number 345830, Beckman Coulter, USA) sealed and spun in a VTI 65.1 vertical rotor in an Optima XE-90 ultracentrifuge (both Beckman Coulter) at 124,000 rcf and 20 °C for 65 h. Gradients were fractionated (into 1.5 mL vials) from bottom to top by displacement with water at a flow rate of 1 mL min^{-1} for 25 seconds (Aladdin syringe pump, AL-1000, WPI, Berlin Germany). From each sample, 14 equal fractions (~ 416 µL) were collected. Density was measured (75 µL) using a refractometer. RNA is expected to be found in gradient fractions with a density of ~ 1.78 g mL^{-1} (1.776 – 1.79 g mL^{-1}) (Lueders et al., 2004), and fully ^{13}C-labeled RNA is expected to be found at the fraction with a density of ~ 1.82 g mL^{-1} (> 1.795 – 1.83 g mL^{-1}) (Lueders et al., 2004). RNA was precipitated from gradient fractions using isopropanol and sodium acetate followed by washing with ethanol as described above. The RNA pellet was then eluted in 20 µL nuclease free water and quantified using the RiboGreen assay. A schematic overview of the RNA-SIP approach is shown in Fig. 3.

Escherichia coli cells were grown on fully ^{13}C-labeled or unlabeled medium (*E. coli* OD2 ^{13}C labeled- 110201102; *E. coli* OD2- 100002, Silantes, Munich Germany), and RNA was extracted in order to provide known ^{13}C-labeled and unlabeled RNA as gradient markers of the isotopically "heavy" (~ 1.82 g mL^{-1}) and "light" (~ 1.78 g mL^{-1}) gradient fraction densities after centrifugation. RNA from the corresponding densities was used for cDNA libraries and further sequencing.

2.10 Sequence analysis

16S rRNA sequences were amplified from cDNA or DNA extracts using bacterial primer set 8f and 907r (Table 2; chapters 3-4) or prokaryote primer set 341f and 805r (Table 2; chapter 5). PCR products were purified as described above and sequenced using Illumina's paired end method at MR. DNA Molecular Research LP (Texas, USA). For sequencing, the primer set 27f and 519r (Table 2; chapters 3-2) or 341f and 805r (Table 2; chapter 5) with barcodes on the forward primers were used in a 5 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94 °C for 3 minutes, followed by 28 cycles of 94 °C for 30 seconds, 53 °C for 40 seconds and 72 °C for 1 minute, after which a final elongation step at 72 °C for 5 minutes was performed. Following PCR, multiple samples were pooled together in equal concentrations and purified using calibrated Ampure XP beads (Agencourt Bioscience Corporation, MA, USA). DNA library was prepared following Illumina TruSeq DNA library preparation protocol, and sequencing was performed on a MiSeq following the manufacturer's guidelines. Joined sequences were depleted of primers and barcodes using split_libraries.py as implemented in QIIME (version 1.8.0; Caporaso et al. (2010)).

The sequence reads were processed by the NGS analysis pipeline of the SILVA rRNA gene database project (SILVAngs 1.2) (Quast et al., 2013) using the default settings and as described by Ionescu et al. (2012). Briefly, reads were aligned (and quality-controlled) using the Silva Incremental Aligner (SINA v1.2.10 for ARB SVN (revision 21008)) against the SILVA SSU rRNA gene database project (Schauer et al., 2010). Unique reads (OTUs) were clustered and classified on a per sample basis. OTUs were clustered using cd-hit-est (version 3.1.2; http://www.bioinformatics.org/cd-hit).

Classification was performed by a local nucleotide BLAST search against the non-redundant version of the SILVA SSU Ref dataset (http://www.arb-silva.de) using blastn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) with standard settings. Low identity and artificial BLAST hits, which did not exceed the value of 93 (chapters 3-4) or 50 (chapter 5) from the function "(% sequence identity + % alignment coverage)/2", remained unclassified. Raw sequences can be found on supplemented DVD.

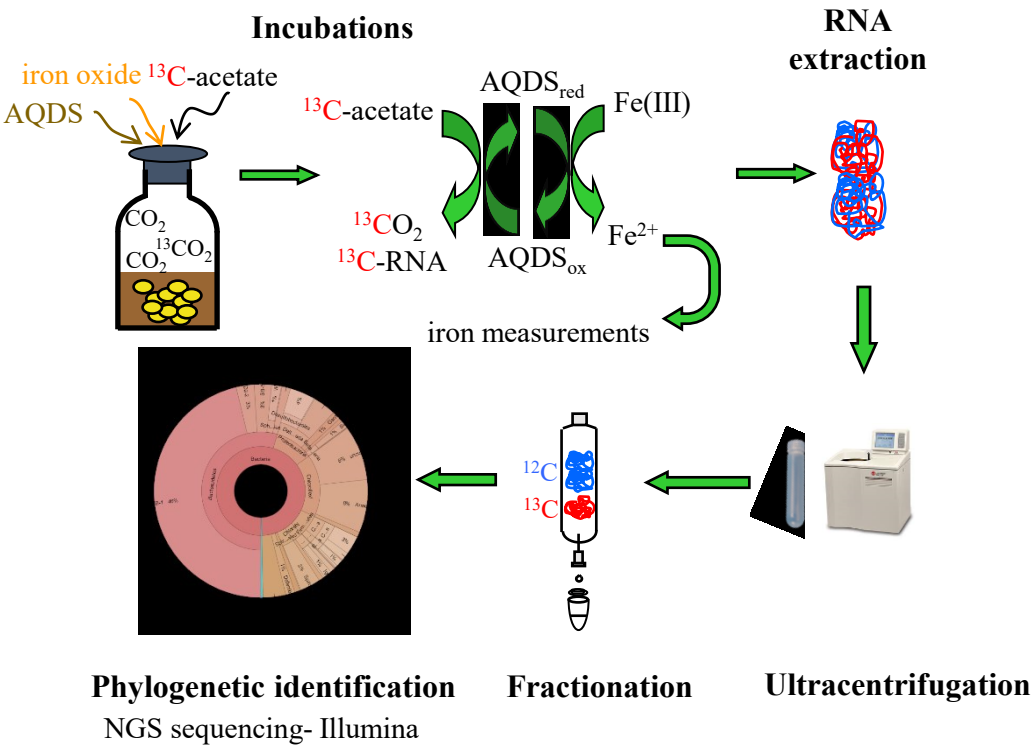

Figure 3. Schematic overview of the RNA-SIP approach. Briefly, slurry incubations were amended with ^{13}C-labeled acetate as carbon source (and electron donor). Iron reduction was monitored by measuring Fe^{2+} formation and expected to be simulated by AQDS addition. RNA was extracted and separated over a density gradient by ultracentrifugation. Density gradients were fractionated and RNA was retrieved from the fractions. Non-labeled RNA light (^{12}C) was found at a density of ~ 1.78 g mL^{-1} and labeled RNA (^{13}C) was found at a density of ~ 1.82 g mL^{-1}. Finally, RNA from the desired densities was sequenced for phylogenetic affiliation.

2.11 Statistic and community analyses

Statistical analyses were performed with the open-source software R (The R project for Statistical Computing v.3.0.2; http://www.r-project.org/) using the "vegan" package (version 2.0-40.; Oksanen et al. (2013)). In chapter 3, for community analyses, non-metric multidimensional scaling (NMDS) ordination plots were created using Bray-Curtis coefficient of dissimilarities. Separations of groups which were identified on the plots were tested using the non-parametric Analysis of Similarity (ANOSIM) (Clarke, 1993). In chapter 5, Cluster analysis (paired group algorithm) plots were created with Bray-Curtis similarity coefficient using PAlaeontological STatistics (PAST version 2.17c; http://folk.uio.no/ohammer/past) (Hammer et al., 2001).

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List of manuscripts and contribution of authors

Humic substances stimulate iron reduction in marine surface sediments. (Chapter III)

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Manuscript in preparation

Author's contributions

S.Y.S. and M.W.F. developed the concept. S.Y.S. sampled the cores from Dorum, performed the experiments analyzed the data and wrote the manuscript. C.R. helped with data analysis, editing and writing the manuscript. A.K. performed the qPCR analysis, helped with sampling and incubation preparations and gave input to the manuscript. S.K. organized the research cruise to Helgoland mud area, provided the samples and performed sulfate measurements. M.W.F. helped with sampling, editing and writing of the manuscript.

Phylogenetic identification of humic acid and iron reducing acetate oxidizing bacteria in marine sediments using RNA Stable Isotope Probing (RNA-SIP). (Chapter IV)

Saar Y. Sztejrenszus, Carolina Reyes, Sabine Kasten, and Michael W. Friedrich.

Manuscript in preparation

Author's contributions

S.Y.S. and M.W.F. developed the concept. S.Y.S. sampled the cores from Dorum, performed the experiments, analyzed the data and wrote the manuscript. C.R. helped with data analysis, editing, and writing the manuscript. S.K. organized the research cruise to Helgoland mud area, provided the samples and performed manganese measurements. M.W.F. helped with sampling, editing, and writing of the manuscript.

DHVEG-6 archaea couple acetate oxidation to humic compound mediated iron reduction in shallow hydrothermal vent sediment, Dominica Island, Lesser Antilles. (Chapter V)

Saar Y. Sztejrenszus, Solveig I. Bühring, and Michael W. Friedrich.

Manuscript in preparation

Author's contributions

S.Y.S. and M.W.F. developed the concept. S.Y.S. performed the experiments, analyzed the data, and wrote the manuscript. S.I.B. organized the research expedition, and helped with editing and writing the manuscript. M.W.F. helped with editing and writing of the manuscript.

Chapter 3

Humic substances stimulate iron reduction in marine surface sediments

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Abstract

Humic substances (HS) are known as electron acceptor and extracellular shuttling molecules, but their importance in electron transfer reactions in marine sediments is not known. Here, we have studied the effect of humic acids as electron shuttles on iron reduction and microbial populations in marine surface sediments from the Wadden Sea tidal flats (Dorum-Neufeld) and the Helgoland mud area (North Sea) by incubation experiments, most-probable-number (MPN) estimates, terminal restriction fragment length polymorphism (TRFLP) community profiling, and sequencing of 16S rRNA genes. In slurry incubations amended with the HS analog anthraquinone disulfonic acid (AQDS), iron reduction was significantly stimulated already at low concentrations (5 μM) compared to control treatments. Based on cluster analysis of TRFLP patterns, the community structure was affected mainly by electron donor amendment in slurry incubations. With AQDS as sole electron acceptor, MPN counts were $10^{4.6}$ cells (mL wet tidal flat sediment)$^{-1}$ with (1 – 14) x 10^4; 95 % confidence limits, which was 50 times higher than with iron oxide as electron acceptor. Based on MPN analyses, most abundant were the members of the *Alteromonadaceae* (AQDS as terminal electron acceptor) and *Desulfuromonadales* with ferrihydrite only as well as with ferrihydrite plus AQDS. Our data show that (i) HS utilization is more widespread in marine sediments among microbial populations than iron reduction and (ii) that iron reduction in sediments appears to be limited by the availability of electron shuttling compounds such as HS. Climate-based increases of terrestrial runoff rich in organic matter and humics may therefore be affecting the liberation of larger amounts of iron through HS shuttling into the water column in coastal areas.

3.1 Introduction

Iron(III) oxides serve as important electron acceptors in anaerobic respiration processes in sediments (Lovley, 1991; Thamdrup, 2000; Lovley et al., 2004; Jørgensen, 2006). They can be found in soils, fresh water, and marine sediments (Lovley et al., 2004). However, iron oxides are characterized by a very low bioavailability due to their low solubility at neutral pH, e.g. $<10^{-9}$ M for the rather amorphous ferrihydrite (Kraemer, 2004; Weber et al., 2006). In order to be able to transfer electrons to iron oxides, microorganisms use several different mechanisms: I) direct electron transfer through direct contact with iron oxides, II) production of siderophores to act as chelators, III) the use of a molecule as an electron shuttle (Lovley et al., 1996; Lovley, 1997; Weber et al., 2006). It is known that dissolved humic substances (HS) can act as an extracellular electron shuttle (EES) and mediate the biogenic oxidation of organic compounds and the inorganic reduction of metals like Fe(III) (Lovley et al., 1996; Kappler et al., 2004; MacDonald et al., 2011). HS are chemically heterogeneous polymeric organic compounds that are produced by the decay of organic matter (Stevenson, 1994; Piepenbrock et al., 2011). They can be found in soils, waters and sediments and can serve as electron acceptor for the biogenic oxidation of organic compounds (e.g. acetate), electron shuttles, or electron donors (Lovley et al., 1996; Nevin and Lovley, 2000; Kappler et al., 2004; Lipson et al., 2010; Roden et al., 2010) due to redox active quinone moieties (Scott et al., 1998; Lovley and Blunt-Harris, 1999) that are a part of their polycyclic structure (Straub et al., 2005). The electron shuttling process occurs in two steps: the first step is the microbial reduction of the humic substances coupled to the oxidation of organic matter. The second step is the abiotic reduction of the metal oxide by the reduced humic substances and it is controlled by the

concentration and diffusion of the HS (Lovley et al., 1996; Kappler et al., 2004; Jiang and Kappler, 2008).

Quinone respiration seems to be widespread among anaerobes (Stams et al., 2006). In addition, a wide range of microorganisms are capable of reducing HS or the model analog anthraquinone-2-4-disulfonic acid (AQDS) (Lovley et al., 1998; Lovley et al., 2004; Field and Cervantes, 2005; Van Trump et al., 2006; Martinez et al., 2013). The role of HS as electron acceptor and shuttle for the reduction of metal oxides in fresh water sediments and pure cultures has been shown by previous studies (Nevin and Lovley, 2000; Nevin and Lovley, 2002; Kappler et al., 2004; Wolf et al., 2009; Piepenbrock et al., 2011). However, the role of HS as electron shuttle for the terminal reduction of metal oxides in the marine environment and their impact on the iron and manganese cycles in marine sediments is still unclear.

In this study, we addressed the role of HS as electron acceptor and shuttle for the terminal reduction of iron oxides by marine microorganisms present in sediments collected from two different sites. The first site, a coastal tidal flat (Dorum Neufeld, North Sea) is influenced by terrestrial input. The second site, the Helgoland mud area (North Sea), represents an open water site. Our aim was to find out whether HS addition alone or in combination with additional electron acceptor (iron oxides) and different electron donors could stimulate the transfer of electrons to iron oxides. Sediment slurries amended with HA or AQDS were analyzed for community composition via TRFLP, Illumina sequencing and MPN cell counts.

3.2 Methods

3.2.1 Sediment sampling

Surface sediment samples were collected from the Helgoland mud area in July 2013 on the RV HEINCKE cruise HE406 using a multicorer sediment core (water depth 30 m, plexiglass tubes 9.5 cm in diameter down to 50 cmbsf) from stations HE406/04-1 (54.1N, 7.9845E) and HE406/08-01 (54.0835N, 7.96683E). Samples were stored at 4 °C until further processing (ca. 3 weeks). Samples from Dorum Neufeld (Wadden Sea, 53.738281N, 8.504825E) were taken using plexiglass tubes (5.2 cm in diameter, 30 cm long) in January 2013 (Dorum1), April 2013 (Dorum2), and March 2014. Samples were brought back to the lab and immediately processed.

3.2.2 Sediment incubations

For sediment incubations, slurries were prepared under continuous flow of N_2 gas. We prepared sediment slurries from the upper 5-10 cm of sediment cores by mixing sediment with autoclaved anoxic sulfate-free artificial seawater (ASW) which contained (per liter of deionized water) 26.4 g NaCl, 11.2 g $MgCl_2 \cdot 6H_2O$, 1.5 g $CaCl_2 \cdot 2H_2O$, 0.7 g KCl at a 1:1 ratio. Sediment slurries were transferred into vials, supplements were added under a stream of N_2, and vials were sealed with butyl rubber stoppers as described in Table 1. Lepidocrocite powder (Υ – FeOOH; Bayferrox 943, Lanxess, Leverkusen, Germany) was supplemented to a final concentration of ~ 10 µmol cm^{-3} as an electron acceptor, AQDS (AB136728, ABCR, Karlsruhe, Germany) was used as electron shuttle (5 or 50 µM) or terminal acceptor (1 mM). Sodium acetate was used as electron donor at a final concentration of 0.5 mM.

In Dorum2 incubations without lepidocrocite, sodium acetate and sodium lactate were used as electron donors at a final concentration of 0.5 mM, and samples were supplemented with additional electron donor (0.5 mM) on the ninth day. Aldrich humic (HA) (Sigma-Aldrich Chemie GmbH, Steinheim Germany) was prepared in 30 mM phosphate buffer pH 7 and used as an electron shuttle at final concentrations of 167 µg mL^{-1} or 16.7 µg mL^{1-} (Table 1).

3.2.3 Most probable number (MPN) analysis

For MPN analysis, an anoxic, bicarbonate buffered (30 mM) marine Widdel medium modified after Straub et al. (2005) was used; per liter of deionized water, the medium contained: 26.4 g NaCl, 11.2 g $MgCl_2 \bullet 6H_2O$, 1.5 g $CaCl_2 \bullet 2H_2O$, 0.7 g KCl, 0.023 g $MgSO_4 \bullet 7H_2O$. The medium was prepared anoxically in a Widdel flask, autoclaved, and cooled under N_2:CO_2 (80 % : 20 % v:v) atmosphere (Widdel and Bak, 1992). The medium was supplemented with 1 mL L^{-1} of: ammonium chloride (0.62 mM final concentration), non-chelated trace element mixture (SL 10), and selenite-tungstate solution (Widdel and Bak, 1992). Vitamin solutions were modified after (Winkelmann and Harder, 2009) and added to a final concentration of: 0.04 mg L^{-1} 4-aminobenzoic acid, 0.02 mg L^{-1} biotin, 0.1 mg L^{-1} nicotinic acid, 0.05 mg L^{-1} pantothenic acid, 0.15 mg L^{-1} pyridoxine HCl, 0.04 mg L^{-1} folic acid, 0.02 mg L^{-1} lipoic acid, and 0.05 mg L^{-1} B-12. Riboflavin and thiamin HCl were added to 0.05 mg L^{-1} each. The upper 5 cm from sediment cores collected in March 2014 were homogenized, and 10 g of sediment were added to 90 mL of ASW in a glass bottle. The bottle was sealed with a rubber stopper, and the headspace was made anoxic by alternating vacuum and flushing with N_2 (99.999%) for 3 times and 3 min each. Dilution series (1:10, in triplicates) were made from the slurry in Hungate tubes filled with 9 mL of medium. Sodium acetate was

used as electron donor at a final concentration of ~ 2 mM. Ferrihydrite was used as terminal electron acceptor and was prepared after Schwertmann and Cornell (1991) and Straub et al. (2005); briefly, ~ 80.5 g of $FeCl_3$ was dissolved in 1 L of water, pH was adjusted with 1 M NaOH to pH 7. The precipitated ferric iron was washed 7 times by centrifugation, decantation of the supernatant, and re-elution of the precipitate with deionized water. The ferrihydrite stock solution was made anoxic as described above and autoclaved (121 °C, 20 min) (Piepenbrock et al., 2011). Ferrihydrite was added to a final concentration of ~ 8 µmol cm^{-3} incubation. AQDS was added as electron shuttle or as terminal electron acceptor (5 µM and ~ 440 µM, respectively). Most probable number dilutions were incubated for 14 weeks in the dark at room temperature. Microbial reduction of AQDS was indicated by a color change from transparent to yellow-orange. Iron oxide reduction was accompanied by a change in color from reddish (ferrihydrite) to brown/black and to colorless compared to control tubes without sediment inoculum, and by the iron concentration compared to the control using the ferrozine assay (Stookey, 1970). Most probable numbers were estimated using KLEE software (Klee, 1993), and Cornish and Fisher lower and upper 95 % confidence limits (Cornish and Fisher, 1938) were calculated by the software. DNA was extracted from the highest positive dilution tubes.

3.2.4 Chemical analyses

At each time point, liquid samples were taken anoxically using an N_2 flushed syringe, filtered through 0.2-µm regenerated cellulose filter (Sartorius, Göttingen Germany) and transferred into 0.5 M HCl (trace metal grade, HN53.1, Carl-Roth, Karlsruhe, Germany). Fe^{2+} was measured following the ferrozine assay (Stookey, 1970; Hegler et al., 2008) with modifications (50 % w:v ammonium acetate, 0.1 % w:v

ferrozine). Sulfate concentration was measured at the laboratories of the AWI (Bremerhaven, Germany) using a Metrohm Compact IC 761 ion chromatograph. Initial sulfate concentrations in incubations were 4.47 mM (Helgoland sediment), 2.96 mM (Dorum1), and 3.33 mM (Dorum2). From each time point, sediment samples were stored at -80 °C for further molecular biology work, and duplicate incubations from Helgoland were pooled.

3.2.5 DNA extraction

From each incubation DNA was extracted (Table 1) as described by Henckel et al. (1999), and Lueders et al. (2004) with the following modifications: DNA was precipitated using 2-3 volumes of polyethylene glycol (PEG) and centrifugation for 90 min at 20,800 rcf and 4 °C. For DNA extraction from highest positive MPN tubes, cells were concentrated from 1.8 mL of culture medium by centrifugation for 70 min at 20,800 rcf, and 4°C, the supernatant was discarded, and extraction was done as described above. For quantification of 16S rRNA gene copy numbers at the beginning of the MPN incubations, 1 mL of the inoculation slurry (~ 0.1 mL wet sediment) was used and the DNA extracted as described above.

3.2.6 Terminal restriction fragment length polymorphism (TRFLP) analysis

For TRFLP analysis, 16S rRNA genes were amplified from DNA extracts using bacterial primers 8f-FAM labeled (Turner et al., 1999) and 907r (Muyzer et al., 1995). The reaction mixture contained in a total volume of 25 µL (all in final concentrations): 1 x PCR buffer II (Applied Biosystems, Life Technologies), 200 µM of each dNTP, 3 mM $MgCl_2$, 200 µg mL^{-1} bovine serum albumin (BSA), 0.3 mM of each primer and 0.025 U μL^{-1} of Ampli *Taq* DNA polymerase. Amplification was performed using a

Gene Amp 9700 PCR System (Applied Biosystems, Life Technologies, Darmstadt, Germany), and PCR conditions were: initial denaturation at 94 °C for 2 min followed by 30 cycles of: denaturation at 94 °C for 30 s, annealing at 52 °C for 1 min, initial elongation at 72 °C for 1 min, adding a final extension step at 72 °C for 7 min. PCR products were purified using the MinElute PCR purification kit (Qiagen, Hilden, Germany). PCR products (120 ng) were digested using *MspI* (Promega) as described by Egert and Friedrich (2003). Digested products were purified using SigmaSpin Post reaction clean up columns (Sigma-Aldrich, Germany), and 3 µL were mixed with 14.7 µL of Hi-Di formamide (Applied Biosystems) and 0.3 µL of ROX-labeled MapMarker 1000 (BioVentures, Murfreesboro, TN), denatured (5 min at 95 °C) and kept at 4 °C until processing. Size separation was performed as described earlier (Schauer et al., 2010). For analysis, only peaks between 50 and 800 base pairs (bp) in length which had more than 50 relative fluorescence units (RFU) were used. Peak analysis was performed with TREX-software (Culman et al., 2009) with a noise reduction based on 1.5 standard deviations of peak height. Automated alignment using 1 bb shift was used for binning. Peaks were normalized within each sample and RFU were recalculated based on peak area. For Dorum incubations, TRFLP profiles were averaged between technical replicates (Table 1).

3.2.7 Quantitative-PCR (qPCR)

In order to estimate cell abundances in Dorum sediment samples, bacterial 16S rRNA genes were quantified using an absolute qPCR method. Standard template was prepared by extracting DNA from *Escherichia coli* strain SB1. The 16S rRNA gene was amplified using the bacterial primers 8f and 1492r (Turner et al., 1999) and purified as described above. Concentrations of purified standard template and DNA extracts from

MPN dilutions were quantified using Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies, Darmstadt, Germany). Quantitative PCR was carried out using bacterial primers 338f and 518r (Muyzer et al., 1993). Each q-PCR reaction contained in total volume of 20 µL (all in final concentrations): 1 x of MESA Blue qPCR MasterMix Plus (Eurogentec, Cologne, Germany), 0.2 mg mL^{-1} BSA, and 0.1 µM of each primer. Standard curve analysis was performed using 10-fold serial dilutions of the standard template ranging from 1 pg μL^{-1} to 0.1 fg μL^{-1} with 94 % efficiency (R^2=0.992). Quantity of DNA amplified from the MPN DNA extracts was obtained based on the standard curve analysis. Gene copy numbers were quantified using the following formulas:

$$Gene\ copies\ = \frac{M\ \times T}{QU\ \times 914878\ (Da)}$$

$$M\ = \frac{QO}{1.66054\ \times 10^{-24}\ (g)}$$

Where:

M = Mass of DNA amplified (Da)

T = Total DNA extracted (ng)

QU = Quantity of DNA used for qPCR (ng)

QO = Quantity of DNA obtained from q-PCR (g)

914878 (Da) = Mass of one gene copy of standard template

Estimation of bacterial cell number based on 16S rRNA gene copy number was made using the ribosomal RNA operon copy number database (rrnDB version 4.3.3; https://rrndb.umms.med.umich.edu/) (Stoddard et al., 2015) with an average of 4 gene copies per cell.

3.2.8 Sequence analysis

16S rRNA genes from DNA extracts of MPN incubations were amplified using bacterial primers 8f (Turner et al., 1999) and 907r (Muyzer et al., 1995) and purified as described above. PCR products were sequenced using Illumina's paired end method at MR. DNA Molecular Research LP (Texas, USA). Briefly, the primer set 27f (Kuske et al., 2006) and 519r (variable regions V1-V3) (Turner et al., 1999) with barcodes on the forward primer was used in a 5 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94 °C for 3 minutes, followed by 28 cycles of 94 °C for 30 seconds, 53 °C for 40 seconds and 72 °C for 1 minute, after which a final elongation step at 72 °C for 5 minutes was performed. Following PCR, multiple samples were pooled together in equal concentrations and purified using calibrated Ampure XP beads (Agencourt Bioscience Corporation, MA, USA). DNA library was prepared following Illumina TruSeq DNA library preparation protocol, and sequencing was performed on a MiSeq following the manufacturer's guidelines. Joined sequences were depleted of primers and barcodes using split_libraries.py as implemented in QIIME (version 1.8.0; Caporaso et al. (2010)) with default settings, and only sequences with size of 300-570 base pairs were used. The sequence reads were processed by the NGS analysis pipeline of the SILVA rRNA gene database project (SILVAngs 1.2) (Quast et al., 2013) using the default settings and as described by Ionescu et al. (2012). Briefly, reads were aligned (and quality-controlled) using the Silva Incremental Aligner (SINA v1.2.10 for ARB SVN (revision 21008)) against the SILVA SSU rRNA gene database project (Schauer et al., 2010). Unique reads (OTUs) were clustered and classified on a per sample basis. OTUs were clustered using cd-hit-est (version 3.1.2; http://www.bioinformatics.org/cd-hit). Classification was performed by a local nucleotide BLAST search against the non-redundant version of the SILVA

SSU Ref dataset (release 119; http://www.arb-silva.de) using blastn (version 2.2.28+; http://blast.ncbi.nlm.nih.gov/Blast.cgi) with standard settings. Low identity and artificial BLAST hits, which did not exceed the value of 93 from the function "(% sequence identity + % alignment coverage)/2", remained unclassified. Raw sequences can be found on supplemented DVD.

3.2.9 Statistical analyses

All statistical analyses were performed with the open-source software R (The R project for Statistical Computing v.3.0.2; http://www.r-project.org/) using the "vegan" package (version 2.0-40.; Oksanen et al. (2013)). For community analyses, non-metric multidimensional scaling (NMDS), ordination plots were created using Bray-Curtis coefficient of dissimilarities. Separations of groups, which were identified on the plots, were tested using the non-parametric Analysis of Similarity (ANOSIM) (Clarke, 1993).

3.3 Results

In order to determine the potential of the endogenous microbial community to utilize humic compounds for extracellular electron transfer (EET) in marine surface sediment, slurries were incubated for 8 and 9 days with sediment from Dorum1 and Helgoland sampling sites, respectively. The slurries were amended with AQDS as EES, acetate as electron donor and the iron oxide lepidocrocite as terminal electron acceptor.

3.3.1 Dorum and Helgoland acetate and lepidocrocite amended slurry incubations

Acetate amendment as electron donor stimulated iron reduction in both incubations. When added to Dorum sediment incubations, the net Fe^{2+} production was up to ~ 5

times higher than in the control (Fig. 1 A). When added to Helgoland sediment incubations, 2 times more Fe^{2+} was formed than in the un-amended control (Fig. 1 B). Incubations amended only with acetate and lepidocrocite did not result in increased iron reduction compared to the acetate only incubations, (Fig. 1A, B). With lepidocrocite as sole electron acceptor (no acetate addition), stark differences in iron reduction were observed between the two sediments tested. In incubations with Dorum sediment, 2.1 times more Fe^{2+} was produced than in the control after 5 days (Fig. 1 C), whereas in incubations with Helgoland sediment, no difference was observed between lepidocrocite amended and un-amended control (Fig. 1 D).

3.3.2 Dorum and Helgoland AQDS amended slurry incubations

When AQDS was amended as electron shuttle, iron reduction was significantly stimulated (ANOVA, $p < 0.01$) in incubations from both sites (Fig. 1). After one week of incubation, Fe^{2+} formation was ~ 1.6 - 2.1 and ~ 3.2 to 4.7 times higher in incubations amended with AQDS compared to the un-amended incubations with Dorum and Helgoland sediment, respectively (Fig. 1).

In order to check the effects of electron shuttle concentration on microbial iron reduction potentials, incubations with Dorum sediment were amended with AQDS at three different concentrations (0.5, 5, and 50 µM) (Fig. 2). The addition of AQDS at a concentration of 5 µM stimulated Fe^{2+} formation the most (2.5 times after 6 days of incubation, compared to no addition of AQDS as electron shuttle), followed by that at 0.5 µM (2 times) and 50 µM (1.6 times). The additional amendment of acetate as electron donor had mostly no effect on the formation of Fe^{2+} (Fig. 2 A).

3.3.3 Dorum slurry incubations amended with natural HA

In order to determine the effect of a natural humic substance on iron reduction kinetics, we used Aldrich HA as electron shuttle and acceptor in Dorum2 sediment incubations in combination with lactate and acetate as electron donors. Aldrich HA is similar in characteristics to the standard HA of the International Humic Substances Society (Ratasuk and Nanny, 2007; Aeschbacher et al., 2010; Piepenbrock et al., 2014). With 39 % carbon content C:N ratio of 37.9 and C:H ratio of 0.7 (Kappler et al., 2000) Aldrich HA is similar to HA extracted from various marine sediments with respect to C:H ratio, but have lower carbon content and higher C:N ratio (Llaguno, 1997; De la Rosa et al., 2011; Martin et al., 2014). The addition of Aldrich HA (167 µg mL^{-1}) stimulated net Fe^{2+} formation of up to ~ 1.3 fold after the first week of incubation (Fig. 3). At lower concentration of HA (16.7 µg mL^{-1}), iron reduction was stimulated up to ~ 1.2 folds after the first week of incubation (Fig. 3).

When lactate was used solely as electron donor, Fe^{2+} was formed faster during the first two days of incubation compared to incubations with acetate (1.4 – 1.5 times more Fe^{2+} was produced) (Fig. 3 B, C). Nevertheless, at the end of the incubations the addition of lactate resulted in only slightly higher Fe^{2+} concentrations (1.2 times) than with acetate as electron donor.

3.3.4 TRFLP analysis of bacterial 16S rRNA genes

In order to assess the impact of the different incubation settings, the microbial community composition was analyzed by TRFLP analysis of the 16S rRNA genes in samples from sediment incubations. The microbial community was mainly influenced by the electron donor present (Fig. 4). In incubations with Dorum1 sediment, samples

without electron donor (lactate or acetate) were significantly different from all samples with acetate as electron donor (Fig. 4 A; R= 0.8125., p= 0.001; one way ANOSIM); samples amended with acetate and AQDS as terminal electron acceptor formed a separate group (Fig. 4 A). In incubations with Helgoland sediment, all samples without electron donor grouped together and separated from samples with acetate addition (Fig. 4 B). Samples amended with acetate and AQDS grouped together with samples amended with acetate, lepidocrocite and AQDS, whereas samples amended only with acetate grouped together with samples amended with acetate and lepidocrocite (Fig. 4 B). In the second incubation with Dorum2 sediment, the same separation was observed, and all samples without electron donor were significantly separated from samples with electron donor regardless of any other addition (Fig. 4 C; R= 0.7259, p= 0.001; one way ANOSIM).

3.3.5 Most probable number estimations, qPCR and phylogenetic identification

In order to determine the proportion of the microbial community that is capable of using AQDS as electron shuttle and acceptor, MPN analyses in combination with qPCR analyses were carried out with sediment samples from Dorum. These samples contained 1.57×10^7 ($\pm 1.87 \times 10^6$) 16S rRNA gene copies (mL wet sediment)$^{-1}$, which corresponds to 3.92×10^6 ($\pm 4.67 \times 10^5$) cells (mL wet sediment)$^{-1}$, assuming an average of four 16S rRNA gene copies per cell. Using MPN analysis, the highest cell numbers were found in incubations with acetate as electron donor and AQDS as sole electron acceptor $\sim 4.3 \times 10^4$ cells (mL wet sediment)$^{-1}$ with $\sim (1 - 14) \times 10^4$; 95 % confidence limits (Table 2), which corresponds to 1 % of all cells present in sediment samples. Incubations with acetate as electron donor and only ferrihydrite as electron acceptor were similar to incubations with ferrihydrite and AQDS as electron shuttle molecule

with ~ 9.3 x 10^2 cells (mL wet sediment)$^{-1}$ with ~ (2 – 27) x 10^2; 95 % confidence limits and ~ 7.4 x 10^2 cells (mL wet sediment)$^{-1}$ with ~ (2 – 21) x 10^2; 95 % confidence limits (Table 2).

In order to identify the most abundant populations in MPN incubations, DNA was extracted from the highest positive MPN dilutions, and 16S rRNA gene sequences were obtained by Illumina paired-end sequencing. The Number of sequences that were analyzed by the SILVA pipeline, ranged from 85,900 to 125,260 per sample (average of 109,130). The average length was ~ 500 base pairs per sequence (Table S1). *Proteobacteria* related sequences mostly dominated all MPN incubations (total of ~ 77 % of all sequences). In incubations with ferrihydrite as sole electron acceptor, *Bacteroidetes* related sequences were the second most abundant (~ 44 %) and (~ 10.5 %) when AQDS was the sole electron acceptor (Table 3). When only AQDS was added as terminal electron acceptor, *Alteromonadaceae* related sequences were the most abundant group (~ 36 %), followed by *Bradyrhizobiaceae* (~ 27 %), and *Desulfuromonadales* related sequences (19 %). When only ferrihydrite was added as electron acceptor, *Desulfuromonadales* related sequences were ~ 35.6 % of the total community, and *Desulfobulbaceae* related sequences were ~ 12.7 % of the total community. When all supplements were added, the most dominant group was *Desulfuromonadales* with > 86 % of all sequences.

3.4 Discussion

Our experimental approach shows that there is a large potential for utilizing HS and quinones as EES molecules in the marine sediments tested. This potential is shown by high stimulation of Fe^{2+} formation in incubations amended with AQDS (up to ~ 4.5 times more than incubations without AQDS). Despite the high potential, this approach cannot answer, if HS are being used *in-situ* as electron acceptors or electron shuttle molecules as was shown by Kappler et al. (2004). Additionally, due to the ability of HS to complex metals they tend to coagulate at high salinity (Rashid, 1985). Therefore, addition of dissolved quinone molecules (AQDS) is expected to have a strong impact. We did not find stimulation of Fe^{2+} formation in incubations with Dorum sediment when AQDS was amended to a concentration of 1 mM. Yet, we can exclude lack of activity as the color of the incubation changed to orange, which is characteristic to accumulation of reduced AQDS (Lovley et al., 1996). Sulfate reduction cannot be completely excluded in the incubations, due to the initial presence of sulfate (3 – 4.5 mM). None the less, the continued accumulation of Fe^{2+} in all treatments suggests that free sulfide is limited and iron reduction was a result of direct microbial respiration (Canfield, 1989) or through respiration of HS.

At increased AQDS concentrations (5 µM vs. 0.5 µM, Fig. 2), Fe^{2+} formations were higher, which is corroborated by observations in pure cultures (O'Loughlin, 2008; Wolf et al., 2009). However, at the highest AQDS concentration (50 µM), less Fe^{2+} was formed, although still higher than the controls. Differences can be due to experimental conditions (pure cultures vs. sediment slurries), and it is possible that another geochemical background affected our system. The added electron donors stimulated Fe^{2+} formation (Fig. 1 A, B; Fig. 3 B, C), indicating that supply of electron donors

limited iron reduction. Our observations are different from those in the study of Finke and colleagues (2007), where increase in volatile fatty acid concentrations did not stimulate iron reduction, suggesting that the electron donors were not limited. Concluding, that in presence of enough electron donors autochthonous to the sediment, iron reduction cannot be further stimulated.

In contrast to the high stimulation of Fe^{2+} formation observed by AQDS and acetate additions, lepidocrocite addition did not stimulate Fe^{2+} formation in most of the incubations (Fig. 1). When incubations with Helgoland sediment were amended with lepidocrocite and AQDS, rates were similar to incubations amended with only AQDS (Fig. 1 B, D). Therefore, Fe^{2+} formations can be attributed to AQDS addition. These results suggest that iron oxide was not limited for respiration in the sediment.

In order to determine the effect of a natural HS, we used the Aldrich HA with sediment from Dorum tidal flats. Stimulation of iron reduction was observed (up to ~ 1.3 folds after one week) although to lower extend than AQDS. HA concentration of 16.7 $\mu g\ mL^{-1}$ corresponds to 6.5 mg C L^{-1} (Kappler et al., 2000), which is at the lower end of concentrations suggesting to support electron shuttling via HA (5 – 10 mg C L^{-1}) (Jiang and Kappler, 2008). Nonetheless, Wolf et al. (2009) used HA concentration of 0.59 mg C L^{-1} and observed stimulation of iron reduction. Piepenbrock et al. (2011) showed in their setups that HS concentrations of 210 $\mu g\ mL^{-1}$ did not stimulate iron reduction and even decreased the rate of reduction due to sorption of the HS to iron oxide. AQDS, however, shows little sorption to iron minerals (Wolf et al., 2009) which can account for some differences between the stimulation of iron reduction observed.

Not only did electron donor availability and type affect Fe^{2+} formation rates in incubations but also the underlying microbial community structure (Fig. 4). Lentini et

al. (2012) observed the same trend, where the community structure was influenced by the electron donor given: acetate, lactate and glucose. On the other hand, AQDS amendment had a minor effect only. Stams et al. (2006) suggested that quinone respiration is a widespread property among anaerobes, and these observations support that.

The difference between MPN estimates of AQDS and iron reducing microorganisms (~ 50 times more AQDS reducers), is in range of previous reports (summarized in Table 4). Our estimations of iron reducing microorganisms were ~ 3 orders of magnitude lower than previous reports from coastal sediments incubated for 10 months (Vandieken and Thamdrup, 2013). Nonetheless, differences can be due to sediment location and time of incubations. When AQDS was added as an electron shuttle molecule together with ferrihydrite, the number of cells estimated was slightly lower compared to ferrihydrite without AQDS. In contrast, Kappler et al. (2004) found higher cells counts with HA and iron oxide compared to iron oxide alone. Most members of the microbial population found in incubation with ferrihydrite and AQDS, were related mainly to *Desulfuromonadaceae* spp. (Table 4). This group harbors known iron reducing and humic reducing microorganisms (Lovley, 1993; Lovley, 2006; Vandieken and Thamdrup, 2013), suggesting that our count estimates were reflecting iron reducing microorganisms, which may use AQDS as electron shuttles when available. All highest positive dilutions of MPN incubations had populations belonging to *Desulfuromonadales* spp., which are known to reduce iron and AQDS in pure cultures and were found in MPN incubations for iron reducers (Lovley, 1993; Lovley, 2006; Vandieken and Thamdrup, 2013). Additionally, Coates et al. (1998) isolated humic reducing bacteria closely related to *Desulfuromonas* spp. from marine sediments with acetate as electron donor and AQDS as electron acceptor. Thus, we conclude that

members of the *Desulfuromonadales* play a key role in HA and iron reduction coupled to acetate in marine sediments.

Bacteroidetes related sequences were found in marine enrichments with iron oxides and associated with anodes of microbial fuel cells with acetate (Lin et al., 2007; Zhang et al., 2011). Therefore, it is possible that they contribute to iron reduction. Populations related to *Desulfobacterales* were as well found previously in MPN estimates for iron reducers, associated with the anode in marine microbial fuel cells, and are able to reduce iron oxide and AQDS (Holmes et al., 2004a; Holmes et al., 2004b; Vandieken and Thamdrup, 2013). Although we cannot completely exclude sulfate reduction in the tubes, we assume that iron reduction was the main respiration process.

Rhizobiales sp. was found to oxidize reduced AQDS coupled to nitrate reduction in river sediments (Coates et al., 2002). Other members of the family *Bradyrhizobiaceae* can grow anaerobically in saline environments (Marcondes de Souza et al., 2014). We assume that nitrate reduction did not take place in incubations, and it might be that *Bradyrhizobiaceae* are also able to reduce AQDS. Members of the *Alteromonadaceae* were shown to be able to reduce Fe(III) complexed by nitrilotriacetic acid (Handley et al., 2009; Handley et al., 2010). Other families within the *Alteromonadales* (*Shewanellaceae*) are capable of reducing iron and HA (Lovley, 2006). We suspect that the *Alteromonadaceae* found, harbor similar and can reduce AQDS as well.

3.5 Conclusions

Extracellular electron transfer in sediments has been of interest in the last years (e.g. Nielsen et al., 2010; Malvankar et al., 2015; Nielsen and Risgaard-Petersen, 2015). We found that HS addition significantly stimulate Fe^{2+} formation in our incubations, with minor effect on bacterial community fingerprint structure. Additionally, cell number estimations were higher for AQDS reducing than iron reducing microorganisms. We suggest that iron and carbon cycles in sediments are limited by the amount of HS, and even small additions will gradually stimulate those cycles. Hence, continuous input of terrestrial quinone rich HS to coastal areas (e.g. from agriculture runoff), might have strong effect on carbon mineralization and iron reduction in those sediments.

Acknowledgements

This study was supported and funded by the University of Bremen. We would like to acknowledge to the captains and the crew of the R/V HEINCKE and the team of HE406 during cruise HE406 in July. We would like to thank Edinson Puentes Cala for helping in lab work and measurements. In addition, we thank Dr. Christian Quast for helping with the SILVA pipeline and Dr. Danny Ionescu with technical assistance in analyzing the data.

Figures:

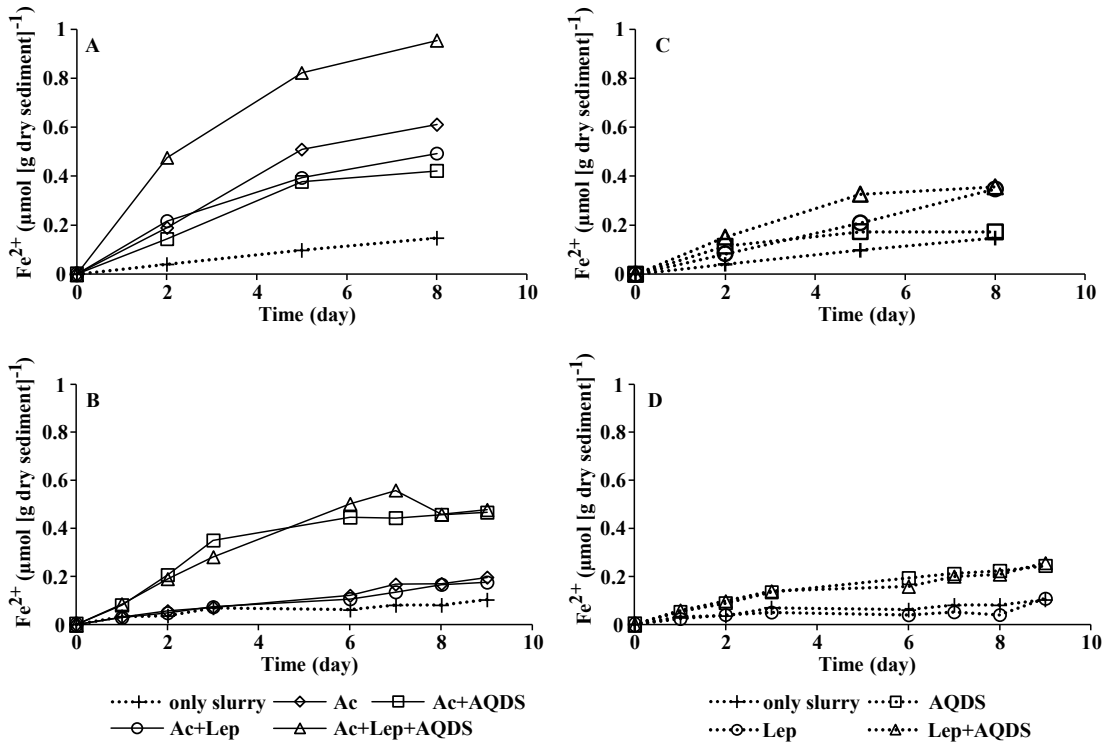

Figure 1. The effect of electron shuttle (AQDS) and electron acceptor (lepidocrocite) on Fe^{2+} formation in sediment incubations. All graphs show net formation of Fe^{2+} i.e., the production of Fe^{2+} from which the initial Fe^{2+} concentration was subtracted. Incubations amended with acetate with sediment from the Dorum tidal flats, January 2013, (**A**) and from Helgoland mud area (**B**). Incubations without acetate addition with sediment from Dorum tidal flats, January 2013 (**C**) and from Helgoland mud area (**D**). In incubations with sediment from Dorum tidal flats (**A** and **C**), AQDS was amended to a final concentration of 1 mM without lepidocrocite, and 50 µM with lepidocrocite. In incubations with sediment from Helgoland mud area (**B** and **D**), AQDS was amended to a final concentration of 5 µM. Ac- Acetate (0.5 mM), Lep- lepidocrocite (10 µmol cm^{-3}). Results represent average. For incubations using sediment from Dorum tidal flats n = 3, for incubations using sediment from Helgoland mud area n = 2 (Table 1). Error bars are not presented.

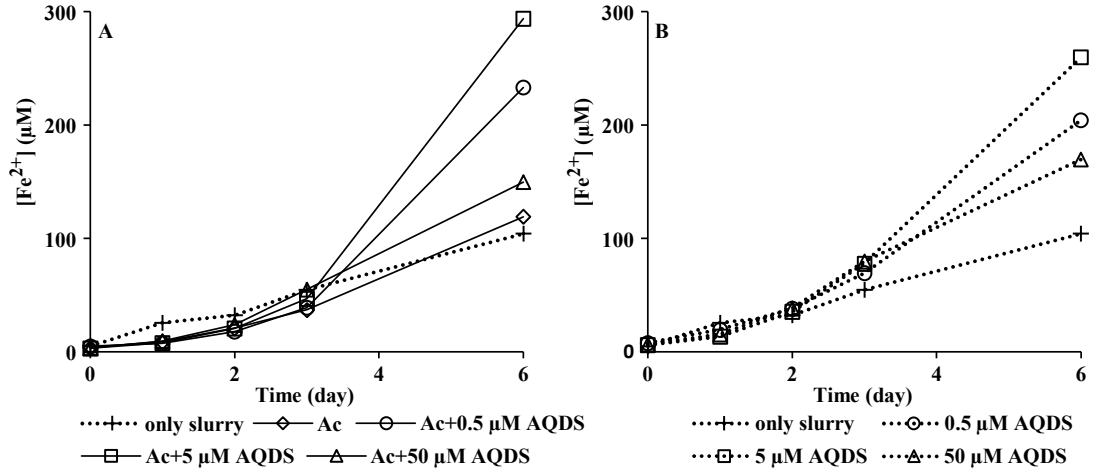

Figure 2. Effect of different AQDS concentrations (0.5, 5, 50 µM) on the formation of Fe^{2+} over time in incubations with Dorum sediment. **A**. Incubations amended with 0.5 mM acetate. **B**. Incubations without acetate. Highest stimulation was observed in incubations with 5 µM. Results represent average n = 2. Error bars are not presented. For each time point- duplicates were sacrificed for measurements.

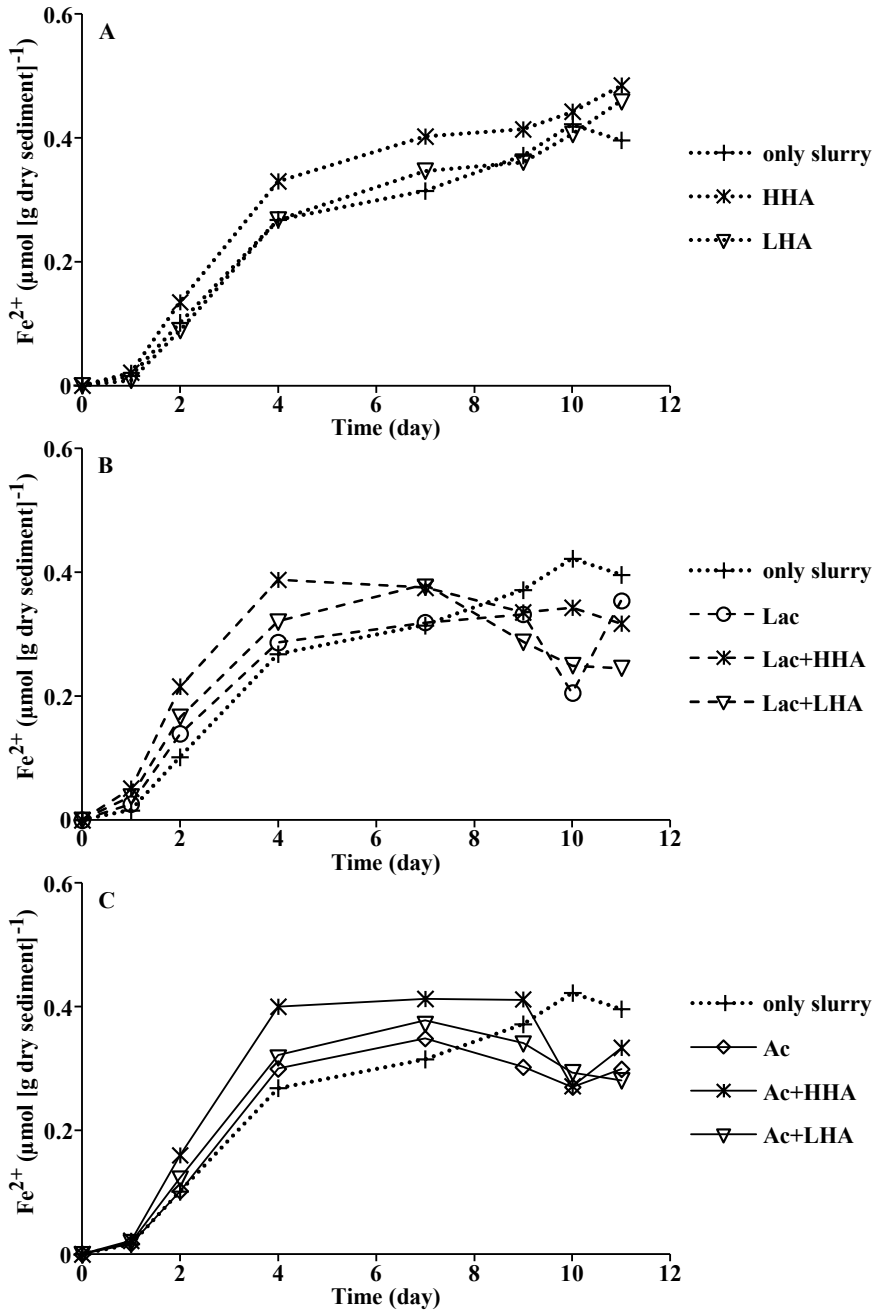

Figure 3. Effect of HA (Aldrich) and electron donor on Fe^{2+} formation in sediment incubations from Dorum tidal flats (April 2013). All graphs show net formation of Fe^{2+}. On day 9, a second dose of 0.5 mM acetate or lactate was added to the incubations. **A.** Incubations without addition of electron donor. **B**. Incubations with lactate as electron donor. **C**. Incubations with acetate as electron donor. HHA- Aldrich HA 167 µg mL^{-1}, LHA- Aldrich HA 16.7 µg mL^{-1}, Lac- lactate (0.5 mM), Ac- acetate (0.5 mM). Results represent average n = 2. Error bars are not presented. For each time point- duplicates were sacrificed for measurements.

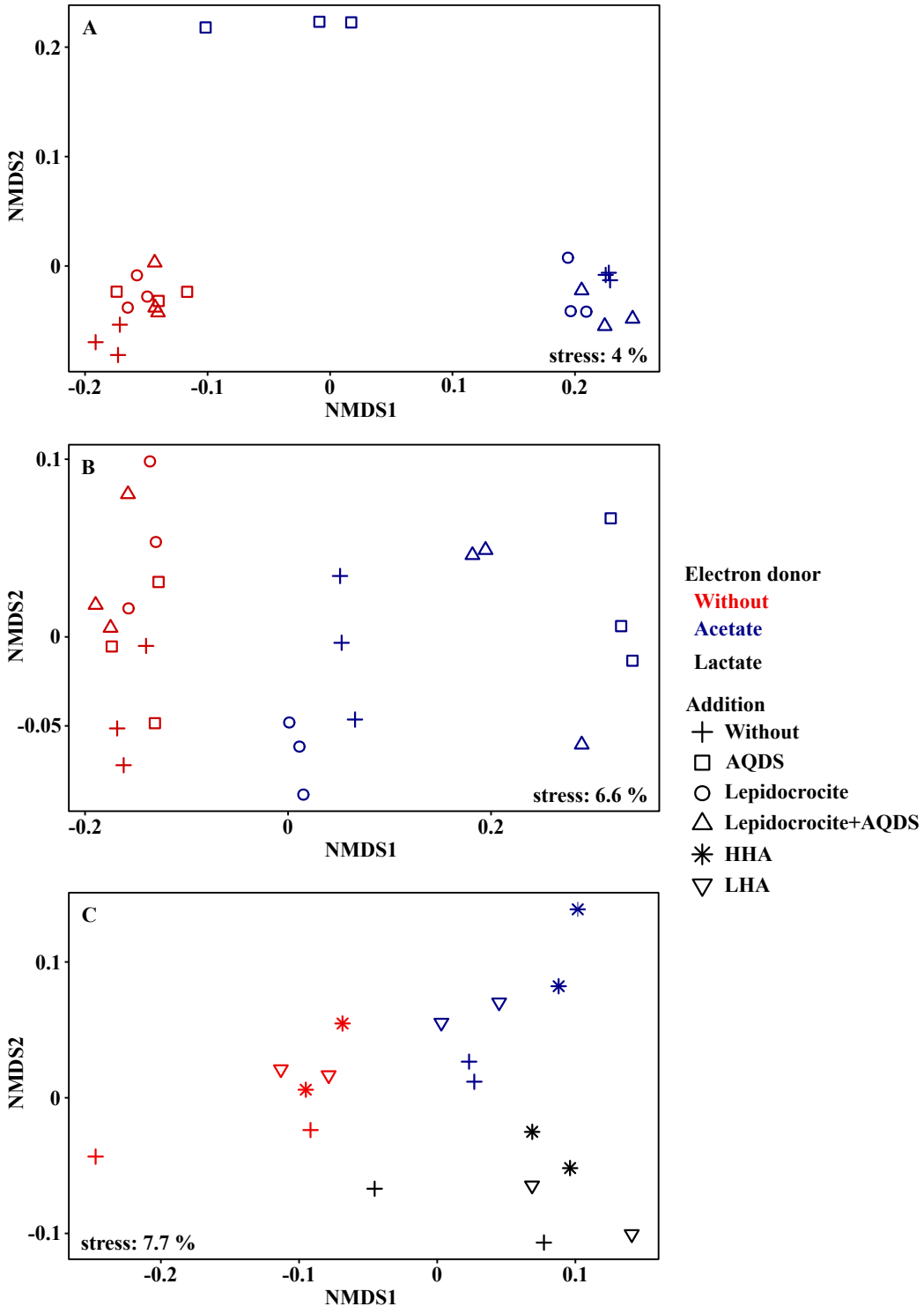

Figure 4. Non-metric multidimensional scaling (NMDS) ordination plots (based on Bray-Curtis distance matrix) of TRFLP profiles from the different incubations. Grouping pattern was mainly based on the presence of electron donor (acetate or lactate) observed in each of the incubations and with a minor extent by the addition of HS or AQDS. Acetate and lactate were added to a final concentration of 0.5 mM, lepidocrocite to a final concentration of 10 µmol cm^{-3}. **A**. Incubations with Dorum sediment, made in January (2013). AQDS was amended to a final concentration of 1 mM without lepidocrocite and 50 µM with lepidocrocite. **B**. Incubations with

Helgoland mud area sediment. Technical replicates (3) are shown. AQDS was amended to a final concentration of 5 µM. **C**. Incubations with Dorum sediment (April 2013). HHA and LHA- Aldrich HA in concentrations of 167 and 16.7 µg mL^{-1} for HHA and LHA, respectively.

Tables:

Table 1. Incubation conditions for estimating metabolic potentials and population analysis. All incubations were in the dark.

Sampling site	**Dorum Neufeld**	**Helgoland mud area**	**Dorum Neufeld**
Sampling and incubation date	January 2013	July 2013	April 2013
Incubation time	8 days	9 days	11 days
Sediment depth used	0-10 cm	0-10 cm	0-5 cm
Incubation vial volume	120 mL	30 mL	30 mL
Incubation volume	40 mL	12 mL	15 mL
Incubation temperature	10 °C	10 °C	20 °C
Replication	triplicate	duplicate	duplicate
Measurements	Continuous for the first 3 time points in which triplicates sacrificed for measurements.	For each time point- duplicates sacrificed for measurements.	For each time point- duplicates sacrificed for measurements.
Electron donor	Acetate 0.5 mM	Acetate 0.5 mM	Acetate or lactate 0.5 mM
Electron acceptor	Lepidocrocite	Lepidocrocite	Not added
Electron shuttle molecule	AQDS 50 µM or 1 mM	AQDS 5 µM	Aldrich HA 167 or 16.7 µg mL^{-1}
DNA extraction and TRFLP analysis	From each incubation replica, DNA extracted in duplicates. TRFLP profiles were averaged between technical replicates.	Duplicate incubations were pooled prior to extractions. DNA extracted in triplicates.	From each incubation replica, DNA extracted in triplicates. TRFLP profiles were averaged between technical replicates.

Table 2. Most probable number (MPN) estimations and lower and upper 95 % confidence limits of iron(III)- and AQDS-reducing microorganisms present in sediment samples from Dorum. Our qPCR estimates were 3.92×10^6 ($\pm 4.67 \times 10^5$) cells (mL wet sediment)$^{-1}$.

Treatment	**Cells (mL wet sediment)$^{-1}$**	**Confidence limits**
Acetate+AQDS	4.27×10^4	$(1.02\text{-}13.8) \times 10^4$
Acetate+ferrihydrite	9.33×10^2	$(2.09\text{-}26.9) \times 10^2$
Acetate+ferrihydrite+AQDS	7.41×10^2	$(1.73\text{-}21.4) \times 10^2$

Table 3. Relative abundance of 16S rRNA genes from the highest positive dilutions of MPN estimates with acetate as electron donor and AQDS as sole electron acceptor, ferrihydrite as sole electron acceptor; and ferrihydrite as electron acceptor and AQDS as electron shuttle molecule. Only sequences with relative families of more than 1 % across all treatments are shown.

	Frequency of sequences (%) of		
Phylogenetic affiliation	**AQDS**	**Ferrihydrite**	**Ferrihydrite+AQDS**
Proteobacteria			
α-Proteobacteria			
Rhizobiales			
Bradyrhizobiaceae	**27.3**	-	-
β-Proteobacteria			
Burkholderiales			
Comamonadaceae	6.2	-	-
Nitrosomonadales			
Gallionellaceae	1.5	-	-
δ-Proteobacteria			
Desulfobacterales			
Desulfobulbaceae	-	12.7	-
Desulfuromonadales			
Desulfuromonadaceae	-	-	**85.8**
Clone Sva1033[a]	**19**	**35.6**	-
γ-Proteobacteria			
Alteromonadales			
Alteromonadaceae	**35.7**	1.2	-
Shewanellaceae	-	2.1	-
Pseudomonadales			
Pseudomonadaceae	6.9	-	1.1
Bacteroidetes			
Flavobacteriia			
Flavobacteriales			
Flavobacteriaceae	-	4.2	-
SB-1	-	**40**	10.5
Sum (%) of sequences	96.6	95.8	97.4

[a]Isolated from surface sediment off the coast of Svalbard and described as related to *Desulfuromonas* sp. (Ravenschlag et al., 1999).

Table 4. Comparison of MPN estimates (log_{10}MPN) using iron oxides and HS from various sediments

		Electron donor	Electron acceptor		
			Iron oxide	AQDS	HA
Fresh water sediment	Wetland[a]	acetate	nt	4-5	nt
	Lake[b]	acetate	4-5.67	nt	5.78-6.66
		lactate	<4-5.75	nt	6.25-6.92
	Lake[c]	acetate	6.38-10	nt	nt
		lactate	5.92-8.18	nt	nt
	Anoxic aquifer[d]	lactate/acetate/ yeast extract	1-5	1-5	1-5
Marine sediment	Bay sediment[e]	acetate	2.97	nt	nt
	Black Sea shelf[f]	acetate	1.36	nt	nt
	Tidal flats[g]	Mixture of monomeric compounds	7.3-8.4	nt	nt
	Loihi Seamount[h]	acetate	3.4-3.9	nt	nt
	Intertidal sediment-estuary[i]	lactate/acetate / yeast extract	5.66 fresh, 4.38 brackish		nt
	Coastal[g]	acetate	6.4	nt	nt
		lactate	5.98	nt	nt

[a] Cells (g sediment)$^{-1}$, range from 5 different sediment depths (Coates et al., 1998).
[b] Cells (mL sediment)$^{-1}$ after 10 weeks of incubation, range from different sediment depth. Iron oxide- poorly crystalline ferric iron hydroxide, HA were extracted from the sediment and were used together with poorly crystalline ferric iron hydroxide (Kappler et al., 2004).
[c] Cells (g sediment)$^{-1}$ after 8 weeks of incubation, range from different media types. Iron oxide- ferrihydrite (Melton et al., 2014).
[d] Cells (g wet sediment)$^{-1}$ after 6 – 8 weeks of incubation, range from different incubations. Iron oxide- ferrihydrite, HA were purchased from Aldrich (Piepenbrock et al., 2014).
[e] Cells (g sediment)$^{-1}$ after 90 days of incubation with contaminated harbor sediment. Iron oxide- amorphous iron oxide (Coates et al., 1996)
[f] Cells cm-3 after 1 year of incubation. Iron oxide- ferrihydrite (Thamdrup et al., 2000).
[g] Cells (g sediment)-1 after 12 weeks of incubation, range from 2 sediment depths. Iron oxide- amorphous ferrihydrite (Köpke et al., 2005).

[h] Cells (mL of mat)$^{-1}$ after 6 weeks of incubation, range from 3 mat locations. Iron oxide- biogenic oxides from natural microbial mat samples (Emerson, 2009).
[i] Cells (g dry sediment)$^{-1}$ after 2 months of incubation with polluted sediment. Incubations from 2 locations at the estuary with different salinities (fresh water and brackish water), with 4 different types of iron oxides- Fe(III) citrate, ferrihydrite, hematite and hematite with AQDS (Lin et al., 2007).
[j] Cells cm-3 after 10 months of incubation. Iron oxide- ferrihydrite (Vandieken and Thamdrup, 2013).
nt- not tested.

Supplementary table:

Table S1. Statistic summary sequences data from the SILVA pipeline.

Sample Name	Number of sequences	Min. length	Avg. length	Max. length	% Classified	% Rejected
AQDS	85912	272	492	536	99.53	0
Ferrihydrite	116218	273	510	539	99.52	0
Ferrihydrite+AQDS	125262	272	495	540	99.74	0

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Chapter 4

Phylogenetic identification of humic acid and iron reducing acetate oxidizing bacteria in marine sediments using RNA Stable Isotope Probing (RNA-SIP)

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Abstract

Humic substances (HS) are known to have a strong effect on iron reduction and play an important role in anaerobic respiration. But, the effect on iron reduction in marine sediments is poorly studied, and the microorganisms involved are elusive. Here, we have used stable isotope probing of RNA (RNA-SIP) with ^{13}C-labeled acetate to identify microorganisms involved in reducing iron oxides through HS mediated extracellular electron shuttling in incubations along 8 – 9 days, with marine surface sediments from the Wadden Sea tidal flats (Dorum-Neufeld) and the Helgoland mud area (North Sea). In tidal flat sediment incubations, *Desulfuromonadales* related sequences were most dominant in assimilating acetate regardless of any other addition. On the other hand, in Helgoland mud sediment incubations, the community structure shifted from *Campylobacterales* and *Desulfuromonadales* related sequences as most dominant when only acetate was amended, to only *Desulfuromonadales* related sequences as most dominant when iron oxide, AQDS or both were added. Hence, our data show that microorganisms, which are known to reduce metal and sulfur, have the capacity to use acetate as electron donor and HS as electron acceptor in the marine sediments tested.

4.1 Introduction

Marine surface sediments have an important role in recycling of organic matter. Oxygen is usually depleted in the upper few mm of the sediment followed by nitrate and manganese as electron acceptors for microbial respiration (Jørgensen and Revsbech, 1989; Thamdrup, 2000; Vandieken and Thamdrup, 2013). Fermentation products and especially acetate serve as important electron donors for iron and sulfate reduction (Sørensen et al., 1981; Lovley and Phillips, 1986), which compete with each other in marine sediments (Canfield et al., 1993a; Canfield et al., 1993b). Furthermore, under low sulfate concentrations (~ 3 mM) iron oxide addition inhibited sulfate reduction (Lovley and Phillips, 1987). Acetate oxidation to CO_2 in the absence of other terminal electron acceptors is energetically unfavorable ($\Delta G^{\circ\prime}$ = 95 kJ mol^{-1}) (Thauer et al., 1977), however, it can happen during respiration processes or through syntrophic acetate oxidation (Schink, 1997; Nüsslein et al., 2001). Therefore, it is commonly used as electron donor in iron reduction studies from different sediment sources (Lovley and Phillips, 1989; Hori et al., 2010).

Humic substances (HS) have been shown to have a strong effect on metal reduction, and to play a major role in anaerobic respiration (Lovley et al., 1996; Lovley et al., 1998; Kappler et al., 2004; Lipson et al., 2010; Lipson et al., 2013). HS addition was found to stimulate electrical current production in microbial fuel cells incubated with marine sediments (Holmes et al., 2004b). In addition, iron reduction was stimulated up to ~ 4 fold when HS was added to North Sea sediment incubations, indicating high potential for extra cellular electron transfer (EET) processes in those sediments (chapter 3). Nonetheless, there were only few attempts to identify humic reducing microorganisms in marine sediments (e.g. Coates et al., 1998; Lin et al., 2007),

and not much is known about the identity of microorganisms that can reduce HS coupled to acetate oxidation.

Stable isotope probing of RNA (RNA-SIP) is a powerful method to link identity and function of uncultivated microorganisms in their natural habitats (Manefield et al., 2002; Dumont and Murrell, 2005). SIP capitalizes on incorporating substrates enriched in stable isotope composition (e.g. ^{13}C-acetate) into cellular compounds. During RNA-SIP, the labeled carbon is incorporated into ribosomal RNA molecules, thus increasing their buoyant density. Using isopycnic centrifugation, ^{13}C-labeled RNA is separated from all un-labeled RNA. With subsequent PCR based tools, it is possible to identify the target populations in heavy gradient fractions (Manefield et al., 2002). Furthermore, by using Illumina sequencing technique, it is possible to identify the active population even when their (labeled) RNA proportion of the total community RNA is as low as 0.001 % (Aoyagi et al., 2015).

A wide range of microorganisms is known to be able to reduce or oxidize HS (Coates et al., 1998; Van Trump et al., 2011; Martinez et al., 2013). However, specific setups are needed to identify the population that can link HS reduction to oxidation of a substrate difficult to ferment, such as acetate (Gutierrez-Zamora and Manefield, 2010; Hori et al., 2010). Where identification of acetate oxidizing microorganisms using RNA-SIP in marine sediments has been done before (Miyatake et al., 2009; Vandieken and Thamdrup, 2013), there is no direct indication for linking acetate oxidation to humic reduction. Our goal in this study was to identify active acetate oxidizing, HS/iron reducing microorganisms in marine surface sediments using RNA-SIP combined with Illumina sequencing analysis. We hypothesized ^{13}C labeling will result in different labeling patterns based on the availability of the terminal electron acceptor. To answer

that, marine sediment from two sites (described in chapter 3) were incubated with ^{13}C-acetate, iron oxide and the humic acid (HA) analog anthraquinone disulfonate (AQDS).

4.2 Methods

4.2.1 Sediment sampling

Surface sediment samples were collected from Dorum Neufeld (Wadden Sea, 53.738281N, 8.504825E) in January 2013 using plexiglass tubes (5.2 cm in diameter, 30 cm long), brought back to the lab and immediately processed. Surface sediment samples from Helgoland mud area were collected in July 2013 on the RV HEINCKE cruise HE406 using a multicorer sediment cores (water depth 30 m, plexiglass tubes 9.5 cm in diameter down to 50 cmbsf) from stations HE406/04-1 (54.1N, 7.9845E) and HE406/08-01 (54.0835N, 7.96683E). Samples were stored until further processing at 4 °C (approx. 3 weeks).

4.2.2 Incubation set-ups

For incubations, slurries were prepared using the upper 10 cm of sediment cores by mixing the sediment with autoclaved sulfate-free artificial seawater (ASW) as described in chapters 2 and 3. Sodium acetate was used as electron donor at a final concentration of 0.5 mM (chapter 3). For labeling, 1,2- $^{13}C_2$ 99 % sodium acetate (0.5 mM) was used (CAS 56374-56-2, Cambridge Isotope Laboratories, USA). Lepidocrocite powder was used as terminal electron acceptor (final concentration of ~ 10 µmol cm^{-3}) and AQDS as electron shuttle (50 or 5 µM for Dorum or Helgoland incubations, respectively) or terminal electron acceptor (1 mM, Dorum incubations) (chapters 2 and 3). Sediment

slurries were incubated in the dark at 10 °C for 8 days (incubations from Dorum) and 9 days (incubations from Helgoland).

4.2.3 Chemical analyses

At each time point, samples were taken and filtered (chapters 2 and 3). Fe^{2+} was measured using the ferrozine assay (Stookey, 1970) as previously described (Hegler et al., 2008; chapters 2 and 3). Sediment samples were stored at -80 °C for nucleic acid extractions; duplicate incubations from Helgoland were pooled. Manganese (Mn^{2+}) was measured at the beginning of incubations and at the last time point after filtration and acidification with HNO_3 (conc.). Measurements were done at AWI (Bremerhaven, Germany) via inductively coupled plasma optical emission spectrometry (Thermo Scientific IRIS Intrepid instrument, Bremen, Germany) (Riedinger et al., 2014) (Table 1).

4.2.4 Nucleic acid extraction

RNA and DNA were extracted from the last day of all incubations (chapters 2 and 3). DNA was digested using RQ1 DNase (Promega), using the manufacturer protocol which was scaled up to 200 µL. Further phenol-chloroform-isoamyl alcohol (PCI, 25:24:1 vol:vol:vol) and chloroform-isoamyl alcohol (CI, 24:1 vol:vol) extractions were performed (Lueders et al., 2004), followed by overnight precipitation in one volume of isopropanol and one fifth volume of 3 M sodium acetate at -20 °C and 60 min centrifugation. The extracts were then washed in 70 % ethanol, centrifuged for 30 min, air-dried, eluted in nuclease free water and quantified using RiboGreen assay (Invitrogen Quant-iT RiboGreen RNA assay kit, Life technologies).

4.2.5 Pure cultures standards

Escherichia coli cells were grown on fully ^{13}C-labeled or unlabeled medium (*E. coli* OD2 ^{13}C labeled- 110201102; *E. coli* OD2- 100002, Silantes, Munich, Germany) and RNA was extracted in order to provide known ^{13}C-labeled and unlabeled RNA to use as gradient markers after the centrifugation.

4.2.6 Isopycnic separation and fractionation

Density gradient centrifugation was modified following Lueders et al. (2004), Briefly, ~750 ng of RNA was mixed with gradient buffer to a volume of 1.3 mL, 240 µL of deionized formamide and 6 mL of cesium trifluoroacetate (CsTFA; illustra CsTFA- 17084702, GE Healthcare, UK). Starting density was adjusted to ~ 1.796 g mL^{-1} using an AR200 digital refractometer (Reichert Analytical, NY USA). Afterwards, ~ 6.5 mL of the mixture was transferred to Beckman polyallomer Quick Seal 16 x 45 mm tubes (Catalog number 345830, Beckman Coulter, USA) sealed and spun in a VTI 65.1 vertical rotor in an Optima XE-90 ultracentrifuge (both Beckman Coulter) at 124,000 rcf and 20 °C for 65 h. Gradients were fractionated at a flow rate of 1 mL min^{-1} for 25 seconds (Aladdin syringe pump, AL-1000, WPI, Berlin, Germany), and density of fractions was measured using a refractometer. RNA was precipitated from the fractions using isopropanol and sodium acetate followed by ethanol as described above. The RNA pellet was then eluted in 20 µL nuclease free water and quantified using the RiboGreen assay.

4.2.7 Reverse transcription and terminal restriction fragment length polymorphism (TRFLP) analysis sequencing and sequence analysis

From each sample, we used isotopically "light" and "heavy" fractions (~ 1.78 and ~1.82 g mL^{-1}, respectively), which corresponded to densities where ^{13}C-labeled and un-labeled *E. coli* RNA were found. cDNA was prepared using Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Life technologies) following the manufacture protocol. For identifying shifts in community composition between the heavy and light fractions, TRFLP fingerprinting analysis was performed. The samples were amplified using bacterial primers 8f-FAM labeled (Turner et al., 1999) and 907r (Muyzer et al., 1995). PCR products were digested using *Msp*I (chapters 2 and 3). Size separation was performed (Schauer et al., 2010), and peaks were analyzed using TREX software (Culman et al., 2009; chapters 2 and 3).

4.2.8 Sequencing and sequence analysis

For sequencing, samples from heavy fractions were amplified using bacterial primers 8f (Turner et al., 1999) and 907r (Muyzer et al., 1995) and sequenced using Illumina's paired end method at MR. DNA Molecular Research LP (Texas, USA) as described in chapters 2 and 3. Joined sequences were depleted of primers and barcodes using split_libraries.py as implemented in QIIME (version 1.8.0; Caporaso et al. (2010)) with default settings and only sequences with size of 300-570 base pairs were used. The sequences reads were processed by the NGS analysis pipeline of the SILVA rRNA gene database project (SILVAngs 1.2) (Quast et al., 2013) as fully described in chapters 2 and 3. Raw sequences can be found on supplemented DVD.

4.3 Results

4.3.1 Iron reduction in ^{13}C amended incubations

In order to assess the effect of ^{13}C-acetate, AQDS and iron oxide addition, iron reduction was measured over time. Iron reduction in sediment incubations amended with un-labeled acetate or without any acetate addition was described and discussed extensively (chapter 3). Briefly, we found that iron reduction was strongly stimulated by the addition of AQDS (up to ~4.5 folds) and acetate (up to ~5 folds) (chapter 3). Using ^{13}C-labeled acetate in the current incubations similar results were observed. At the end of the incubations, the addition of ^{13}C acetate stimulated iron reduction up to 2 and 2.3 folds higher compared to control incubations (for Dorum and Helgoland sediment samples, respectively) (Fig. 1). In Dorum sediment incubations, when AQDS was added as terminal electron acceptor (1 mM), we observed a fast accumulation of Fe^{2+}. On the second day we found ~ 2.2 times more iron when AQDS and ^{13}C-acetate were amended than in the ^{13}C acetate only incubations (Fig. 1 A). Later, the differences between the two treatments became smaller, until the eighth day when the ^{13}C acetate amendment had more iron than the ^{13}C acetate and AQDS amended incubations (Fig. 1 A). Color change of AQDS in this treatment confirmed that AQDS was reduced (data not shown). On the other hand, in incubations with sediment from Helgoland mud area, AQDS was used in lower concentrations (5 μM), and at the end of the incubation, the stimulation was 1.9 fold higher than in incubations with only ^{13}C acetate added (Fig. 1 B). Incubations amended with ^{13}C acetate and lepidocrocite showed ~ 1.3 times more stimulation of iron reduction in Dorum (Fig. 1 A) and ~ 1.1 times more in Helgoland incubations (Fig. 1 B) than at the initial time point. The addition of ^{13}C acetate, lepidocrocite, and AQDS together yielded the highest stimulation of iron reduction

resulting in ~ 1.9 more Fe^{2+} produced than only ^{13}C-acetate with lepidocrocite (in both incubations) and ~ 2.5 for Dorum (Fig. 1 A) and 2.1 for Helgoland (Fig. 1 B) more Fe^{2+} produced compared to only ^{13}C acetate amendment treatments

4.3.2 Microbial community shifts in RNA-SIP fractions

After 8 or 9 days (for incubations from Dorum or Helgoland respectively) RNA was extracted. The RNA was separated on a density gradient, using isopycnic centrifugations, and fractionated in 13 – 14 fractions with a density range of 1.76 – 1.84 g mL^{-1}. RNA was detected in most fractions (supplementary Figs. S1, S2). For further analysis, we used heavy (~ 1.82 g mL^{-1}) and light (~ 1.78 g mL^{-1}) fractions from each sample, which corresponded to fractions where maximum *E. coli* RNA was found (supplementary Figs. S1, S2). Using TRFLP finger printing analysis of the fractions, a shift in community composition between the heavy and light fractions of samples amended with ^{13}C-acetate was observed. It indicated that RNA found in those fractions was labeled with ^{13}C originated from bacteria assimilated the ^{13}C-acetate. This shift was not observed in samples amended with non-labeled acetate (supplementary Figs. S3-S10).

4.3.3 Identification of ^{13}C acetate assimilating microorganisms

We used the heavy fractions from all samples for Illumina paired end sequencing. The number of sequences that were analyzed by the SILVA pipeline, ranged ~36,000 – 151,500 per sample (average of ~ 56,000). The average length was ~ 500 base pairs per sequence (Table S1). When comparing relative abundances of sequences between heavy fractions of ^{13}C-acetate amended incubations and ^{12}C-acetate amended incubations, it is possible to identify the microbial population that incorporated ^{13}C into the RNA. ^{13}C-

labeled RNA accumulated in the heavy fractions (enriched). Resulting in an increase or appearance in relative abundances of the corresponding sequences compared to ^{12}C amended incubations. Generally, *Proteobacteria* related sequences were the most abundant in all heavy fractions (Fig. 2).

In un-labeled incubations (acetate and control incubations) with Dorum sediment, the most abundant classes were *Alphaproteobacteria* (21-29 % of total sequences), *Planctomycetacia* (when incubated with lepidocrocite and AQDS; 24 %) and *Deltaproteobacteria* (when incubated with acetate and lepidocrocite; 19 %) (Fig. 2 A). In incubations with Helgoland sediments the most abundant classes were *Deltaproteobacteria* (30-39 % of total sequences), *Planctomycetacia* (when without any amendment; 23 %) and *Gammaproteobacteria* (when incubated with acetate and AQDS; 28%) (Fig. 2 B).

Incubations with ^{13}C acetate showed a different composition of microbial population than incubations with ^{12}C acetate, indicating that the heavy fractions from the ^{13}C incubations represent the population that actively assimilated the labeled acetate (Fig 2 A, B; Fig. 3 A, B). Thus, we only investigated those samples in order to assess the acetate oxidizing, iron/humic reducing microorganisms. In Dorum sediment incubations, the most dominant sequence type within all ^{13}C labeled acetate incubations fell into the order *Desulfuromonadales* (77 – 83 % of all sequences), closest related to clone sequence Sva1033 (Table 2). Sva1033 clone is closely related to *Desulfuromonas* sp. (Ravenschlag et al., 1999). In Helgoland sediment incubations the most dominant taxa varied between treatments (Fig. 2 B, Table 3). When only ^{13}C acetate was amended, two different *Proteobacteria* classes were the most abundant. *Epsilonproteobacteria* was represented by *Campylobacterales* (40 % of all sequences)

and *Deltaproteobacteria* was represented by *Desulfuromonadales* (36 % of all sequences). All other treatments with ^{13}C acetate from Helgoland mud area were more similar to Dorum incubations respecting relative abundance of sequences with *Desulfuromonadales* related sequences, which were found as the most dominant (66 – 70 % of all sequences). From the *Desulfuromonadales* that were found, the most dominant sequence was similar to the clone sequence Sva1033.

4.4 Discussion

Iron oxide is an important electron acceptor in marine sediments (Thamdrup, 2000). HS have been shown to act as electron shuttle molecules and by that enhance the rate of iron reduction (Lovley et al., 1996; Kappler et al., 2004; Wolf et al., 2009; Lipson et al., 2010). We found that AQDS stimulated iron reduction (~ 2 fold) in incubations with marine sediment from Dorum and Helgoland. This indicates high potential to use HS as electron shuttle molecules. In addition, the amendment of ^{13}C-acetate as electron donor stimulated iron reduction in all treatments compared to un-amended controls (average ~ 2 folds of Fe^{2+} formed by the end of incubations; data not shown). Acetate oxidation to CO_2 is energetically favorable only when it is coupled to respiration processes (e.g. iron or HS) or in a syntrophic acetate oxidizing consortia (Thauer et al., 1977; Schink, 1997; Nüsslein et al., 2001). Thus, it is a suitable substrate for identifying microbial communities which can reduce iron (Hori et al., 2010). Although we do not have a direct indication for acetate oxidation to CO_2, we can assume it was turned over during iron and AQDS reduction based on the stimulation observed. The microbial populations that incorporate ^{13}C-acetate into their RNA can be identified in heavy gradient fractions (based on sequence relative abundance) and can be linked to anaerobic respiration

(Kittelmann and Friedrich, 2008a; Kittelmann and Friedrich, 2008b; Hori et al., 2010). Here, we link ^{13}C-acetate assimilation to iron and humic respiration.

Populations related to *Alphaproteobacteria* were found active, regarding ribosome synthesis, in sediment incubations from Dorum, as can be seen by the relative abundance in the none labeled incubations. However, they did not assimilate acetate (Fig. 2 A). Members of *Rhizobiales* can oxidize reduced AQDS (Coates et al., 2002). Additionally, they were found in most probable number (MPN) incubations for AQDS reducers with acetate as sole electron donor (chapter 3). It might be that under *in-situ* conditions they take part in recycling of HS, but do not utilize acetate. In incubations with Helgoland sediment, *Desulfobacterales* spp. were found to be active. They are known to reduce sulfate, and some can couple this to acetate oxidation (Coleman et al., 1993; Lovley et al., 1993). As sulfate was available at the beginning of incubations (up to ~ 4.5 mM, chapter 3), they might have had reduced it. Additionally, members of *Desulfobacterales* were found to be able to reduce iron and AQDS (Coleman et al., 1993; Lovley et al., 1993; Holmes et al., 2004a). Nonetheless, they did not take part in acetate oxidation in these incubations.

When comparing the populations from the ^{12}C-acetate and the ^{13}C-labeled acetate, the results clearly indicate that only a small part of the population was assimilating ^{13}C-acetate in the sediments. In Dorum incubations, the main active acetate oxidizing populations within all incubations were members of the *Desulfuromonadales* (Table 2). Microorganisms within the *Desulfuromonadales* are capable of reducing iron as well as manganese and elemental sulfur coupled to the oxidation of acetate (Pfennig and Biebl, 1976; Lovley, 1993; Roden and Lovley, 1993; Lovley, 2013; Vandieken and Thamdrup, 2013). They were found in MPN incubations with acetate and iron or manganese

identified after assimilating ^{13}C-labeled acetate using SIP of RNA or DNA with different sediment sources and recovered from marine sediments as AQDS reducers (Coates et al., 1998; Hori et al., 2007; Hori et al., 2010; Webster et al., 2010; Vandieken and Thamdrup, 2013). Additionally, they were found in highest positive MPN incubations with acetate and AQDS in sediment from Dorum (chapter 3). Therefore, our results suggest that the indigenous acetate oxidizing community in Dorum sediments, is likely to play an important part in iron and humic reduction.

On the other hand, when using Helgoland sediment, we found a change in the community structure between treatments amended only with ^{13}C-labeled acetate and all other ^{13}C treatments (Fig. 1 B, Table 3). In incubations amended only with ^{13}C-acetate, we found ~ 40 % of the population to be *Campylobacteraceae* related spp. Members of this group (*Arcobacter* spp.) were previously found to assimilate ^{13}C-acetate using DNA and RNA SIP from marine environments with large distribution of the relative abundances (10 – 52 % of clones) (Webster et al., 2010; Vandieken et al., 2012; Berg et al., 2013; Vandieken and Thamdrup, 2013). They were suggested to be involved in manganese reduction coupled to acetate oxidation, as they were also found in MPN counts (Thamdrup et al., 2000; Vandieken et al., 2012). Additionally, an *Arcobacter* sp. was isolated from a marine microbial fuel cell fed with acetate (Fedorovich et al., 2009). Their proportion in the community decreased when AQDS/lepidocrocite were added, and the community shifted towards microorganisms capable of iron reduction (e.g. *Desulfuromonadales* related spp.). The increased proportion of potential iron reducing bacteria was corroborated by higher iron reduction occurring in incubations amended with ^{13}C-acetate and electron acceptors/shuttles (lepidocrocite, AQDS) than in incubations amended with acetate only. It appears that the indigenous bacterial community in Helgoland mud area sediment, which can oxidize acetate, has the

potential to reduce metals such as manganese and iron as well as HS. However, the occurrence of manganese reducers in incubations is not supported by elevated Mn^{2+} concentrations (Table 1). In contrast, more manganese was reduced when Helgoland sediment incubations were amended with AQDS (Table 1). Recently, AQDS was shown to increase manganese reduction in pure culture (Zhang et al., 2015). Therefore, our results hint towards the possibility of manganese reduction through electron shuttling molecule as proposed by Lovley (2013).

4.5 Conclusions

Humic substances were shown to be important key players in the reduction of iron oxides in marine sediments. The two sites tested slightly differ in the indigenous communities that can assimilate acetate. In incubations from Dorum tidal flats, the whole community was able to reduce iron and AQDS, whereas in incubations from Helgoland mud area a shift from a mix of manganese and iron reducers towards iron and AQDS reducing community was observed, when iron and AQDS reduction conditions were given. The differences can be due to the different locations. Dorum tidal flats sampling site is a coastal location and more influenced by terrestrial input, in addition, the sediment is exposed to cycles of oxidation events during the tides. Helgoland mud area, on the other hand, represents an open marine site, which is not subjected to oxidation cycles. The terrestrial input and tidal waves in Dorum sediments can contribute to the re-supply of fresh iron oxides to the system continuously, which can explain the community found. Nevertheless, both sites have a high potential to use electron shuttle molecules for respiration. Our observation of higher Mn^{2+} concentrations when AQDS was amended as electron shuttle, regardless of the lower

abundance of manganese reducers, suggests microorganisms have the ability to reduce other metals besides iron through electron shuttling.

Acknowledgements

This study was supported and funded by the University of Bremen. We would like to acknowledge to the captains and the crew of the R/V HEINCKE and the team of HE406 during cruise HE406 in July. We would like to thank Ajinkya Kulkarni for helping in lab work and measurements. In addition, we thank Dr. Christian Quast for helping with the SILVA pipeline and Dr. Danny Ionescu for technical assistance with sequence analysis.

Figures:

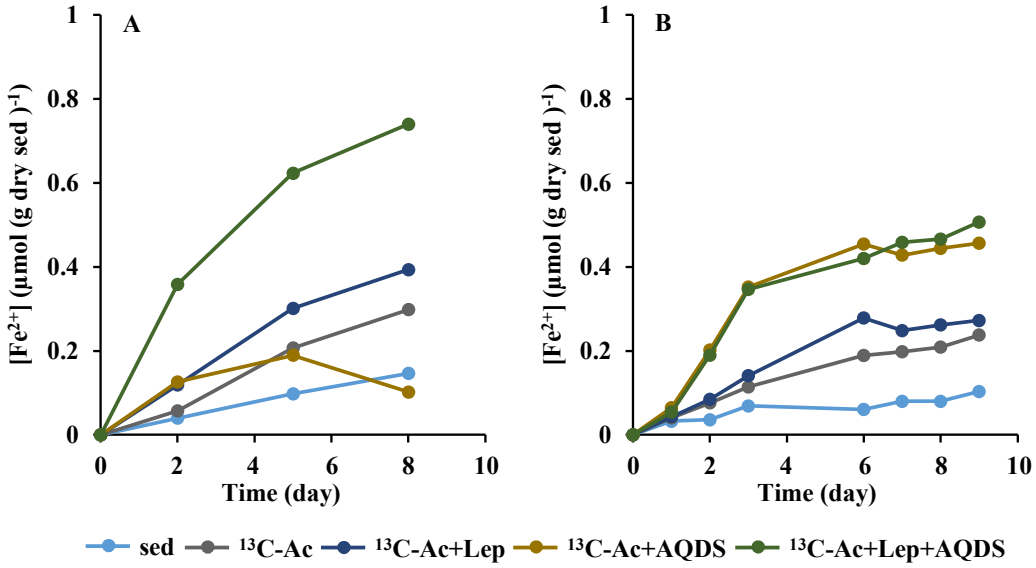

Figure 1. The effect of AQDS as electron shuttle and electron acceptor on the formation of Fe^{2+} in sediment incubations amended with ^{13}C-acetate. All graphs show net Fe^{2+} production. **A.** Incubations with Dorum tidal flat sediment. **B.** Incubations with Helgoland mud area sediment. Sed- incubations with only sediment slurry. ^{13}C-Ac- ^{13}C-labeled acetate (0.5 mM). Lep- lepidocrocite (10 µmol cm^{-3}). Dorum tidal flat sediment samples amended with AQDS at two concentrations: 1 mM as terminal electron acceptor (without lepidocrocite), and 50 µM as electron shuttle when lepidocrocite was added. In incubations from Helgoland mud area, AQDS was amended to a final concentration of 5 µM. Results represent average. For incubations using sediment from Dorum tidal flats n = 3, for incubations using sediment from Helgoland mud area n = 2. Error bars are not presented.

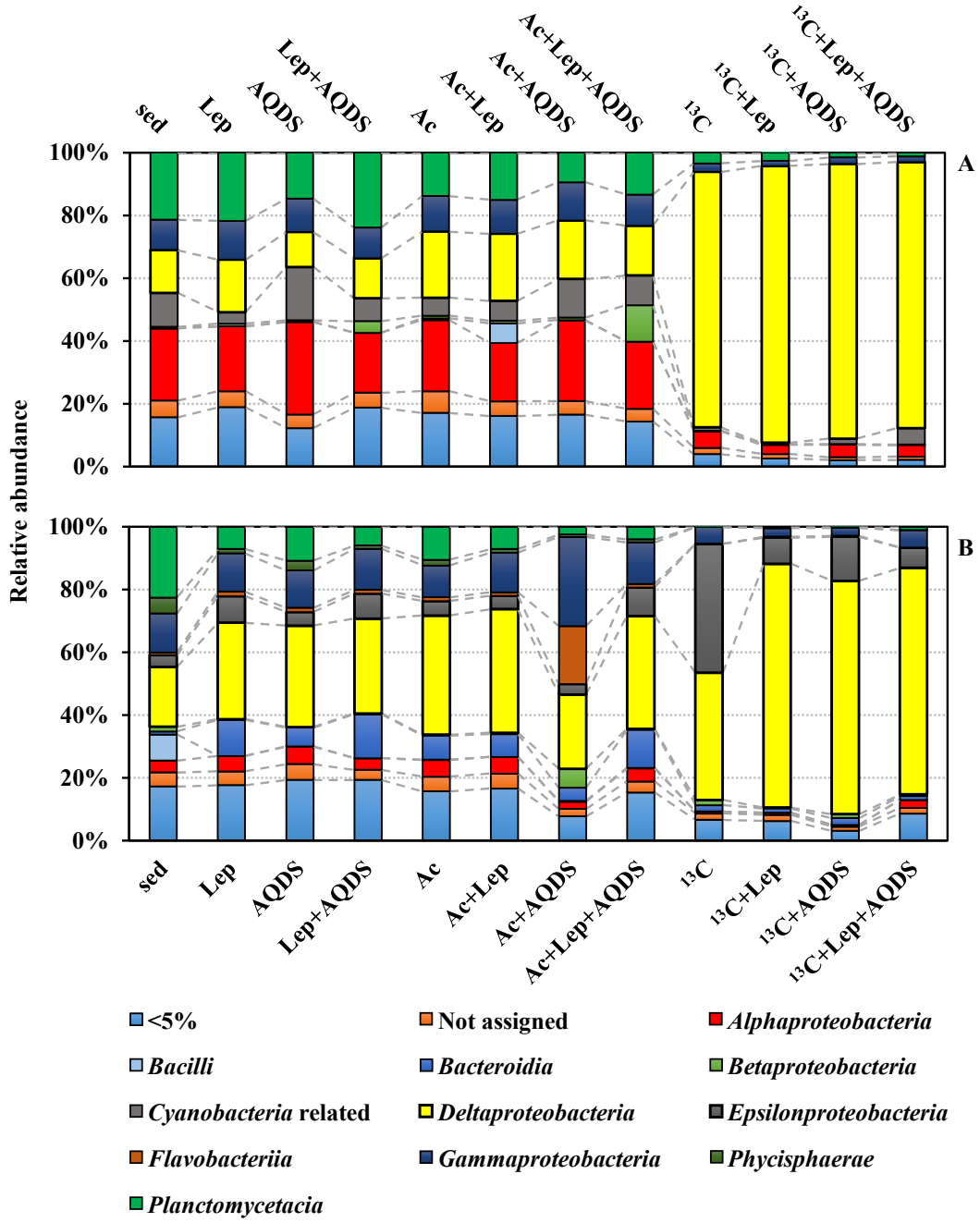

Figure 2. Phylogenetic affiliation (class level) and relative abundance of 16S rRNA sequences retrieved from heavy gradient fractions (~ 1.82 g mL^{-1}). Only sequences with relative class abundance of more than 5 % across all treatments are shown. **A**. Incubations with Dorum tidal flat sediments. **B.** Incubations with Helgoland mud area sediment. Sed- incubations with only sediment slurry. Lep- lepidocrocite. Ac- acetate. ^{13}C- ^{13}C-labeled acetate.

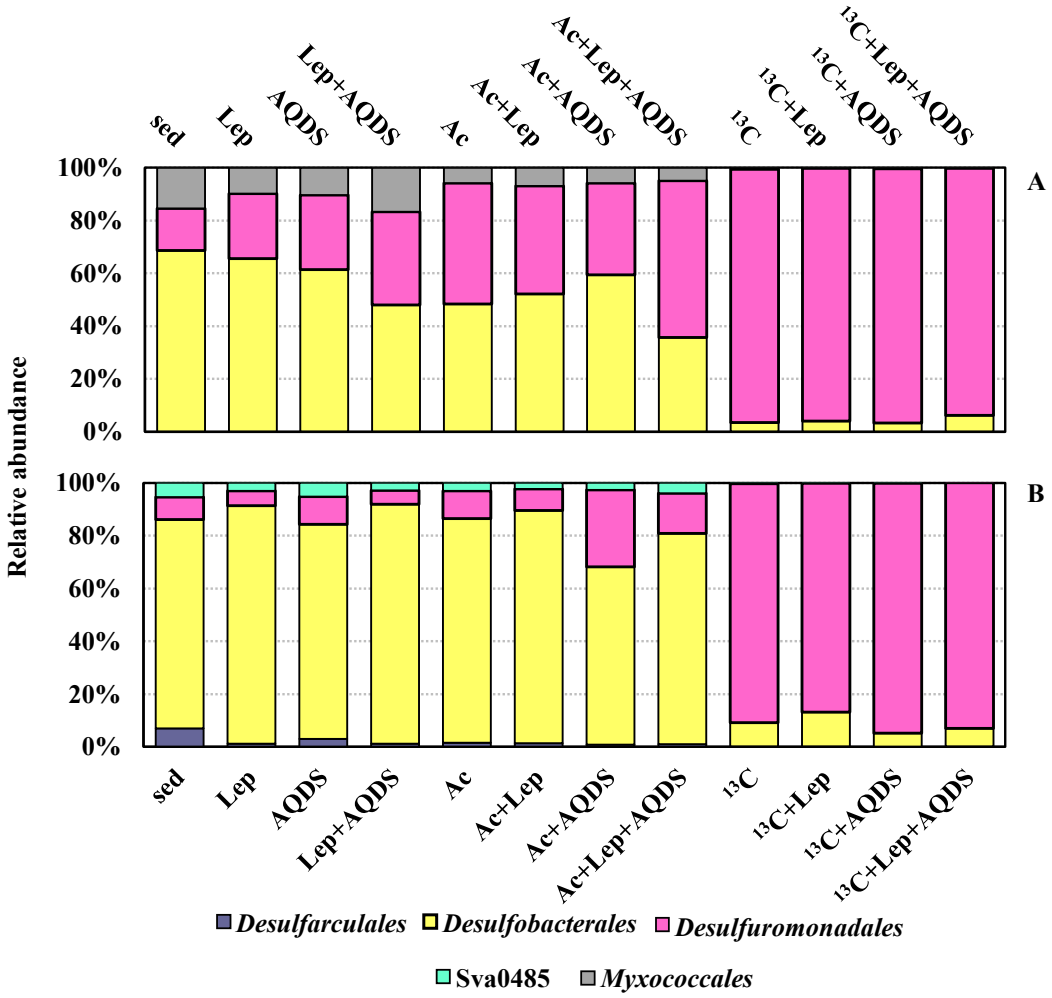

Figure 3. Phylogenetic affiliation (order level) and relative abundance of *Deltaproteobacteria* 16S rRNA sequences retrieved from heavy gradient fractions (~ 1.82 g mL^{-1}). **A**. Incubations with Dorum tidal flat sediments. **B.** Incubations with Helgoland mud area sediment. Sed- incubations with only sediment slurry. Lep- lepidocrocite. Ac- acetate. ^{13}C- ^{13}C-labeled acetate

Tables:

Table 1 Manganese (Mn^{2+}) concentrations (µM) in incubations from both sediment types. Mn^{2+} was measured at the beginning (T_0) and from each treatment at the end of the incubation time (8 days for Dorum sediment, 9 days for Helgoland sediment). ^{13}C-^{13}C-labeled acetate, Lep- lepidocrocite.

	Dorum	**Helgoland**
T_0	199.1	17.38
^{13}C	224.25	20.46
^{13}C+Lep	232.1	20.7
^{13}C+AQDS	222.25	24.85
^{13}C+Lep+AQDS	252.65	24.7

Table 2. Phylogenetic affiliation and relative abundance of 16S rRNA sequences retrieved from heavy gradient fractions (~ 1.82 g mL^{-1}) of Dorum sediment incubations. Only sequences with relative family abundance of more than 1 % across all treatments are shown. ^{13}C- ^{13}C-labeled acetate, Lep- lepidocrocite. Most abundant sequence groups are highlighted in bald face.

	Frequency of sequences (%) of			
Phylogenetic affiliation	**^{13}C**	**^{13}C+Lep**	**^{13}C+AQDS**	**^{13}C+Lep+AQD**
Proteobacteria				
α-Proteobacteria				
Rhizobiales				
Hyphomicrobiaceae	1.9	-	1.3	-
Rhodobacterales				
Rhodobacteraceae	1.9	1.3	1.7	2.1
δ-Proteobacteria				
Desulfobacterales				
Desulfobulbaceae	1.7	2.8	2.4	4.6
Desulfobacteraceae	1	-	-	-
Desulfuromonadales				
Desulfuromonadaceae	6.9	5.5	3.7	6.1
Clone Sva1033	**67.5**	**75.5**	**78.4**	**69.8**
Geobacteraceae	2.4	2.2	-	2.4
Cyanobacteria related	1	-	1.8	5.3
Planctomycetes				
Planctomycetacia				
Planctomycetales				
Planctomycetaceae	3.4	2.7	1.5	1.2
Not assigned	2	1.4	-	1.1
Sum (%) of sequences	89.7	91.4	90.8	92.6

Table 3. Phylogenetic affiliation and relative abundance of 16S rRNA sequences retrieved from heavy gradient fractions (~ 1.82 g mL^{-1}) of Helgoland mud sediment incubations. Only sequences with relative family abundance of more than 1 % across all treatments are shown. ^{13}C- ^{13}C-labeled acetate, Lep- lepidocrocite. Most abundant sequence groups are highlighted in bald face.

	Frequency of sequences (%) of			
Phylogenetic affiliation	**^{13}C**	**^{13}C+Lep**	**^{13}C+AQDS**	**^{13}C+Lep+AQDS**
Proteobacteria				
β-Proteobacteria				
Burkholderiales				
Comamonadaceae	1.6	-	1.2	-
δ-Proteobacteria				
Desulfobacterales				
Desulfobulbaceae	3.1	7.8	2.7	4.5
Desulfobacteraceae	-	2.2	1.2	-
Desulfuromonadales				
Desulfuromonadaceae	10.9	11.8	24.7	18
Clone Sva1033	11.2	**37.6**	**37.7**	**44.1**
Geobacteraceae	14.1	17.1	7.3	4.1
ε-Proteobacteria				
Campylobacterales				
Campylobacteraceae	**40.4**	7.6	12.8	5.4
Helicobacteraceae	-	-	1.1	-
γ-Proteobacteria				
Pseudomonadales				
Pseudomonadaceae	3	-	-	2.8
Bacteroidetes				
Bacteroidia				
Bacteroidales				
Marinilabiaceae	2	1.3	2.2	1.1
BD2-2	1.4	1.1	-	-
SB-1	-	-	-	2.6
BD1-5	2.8	2.9	1.2	1
Planctomycetes				
Planctomycetacia				
Planctomycetales				
Planctomycetaceae	-	-	-	1
Not assigned	2	1.9	1.3	1.9
Sum (%) of sequences	92.5	91.3	93.4	86.5

Supplementary figures and tables:

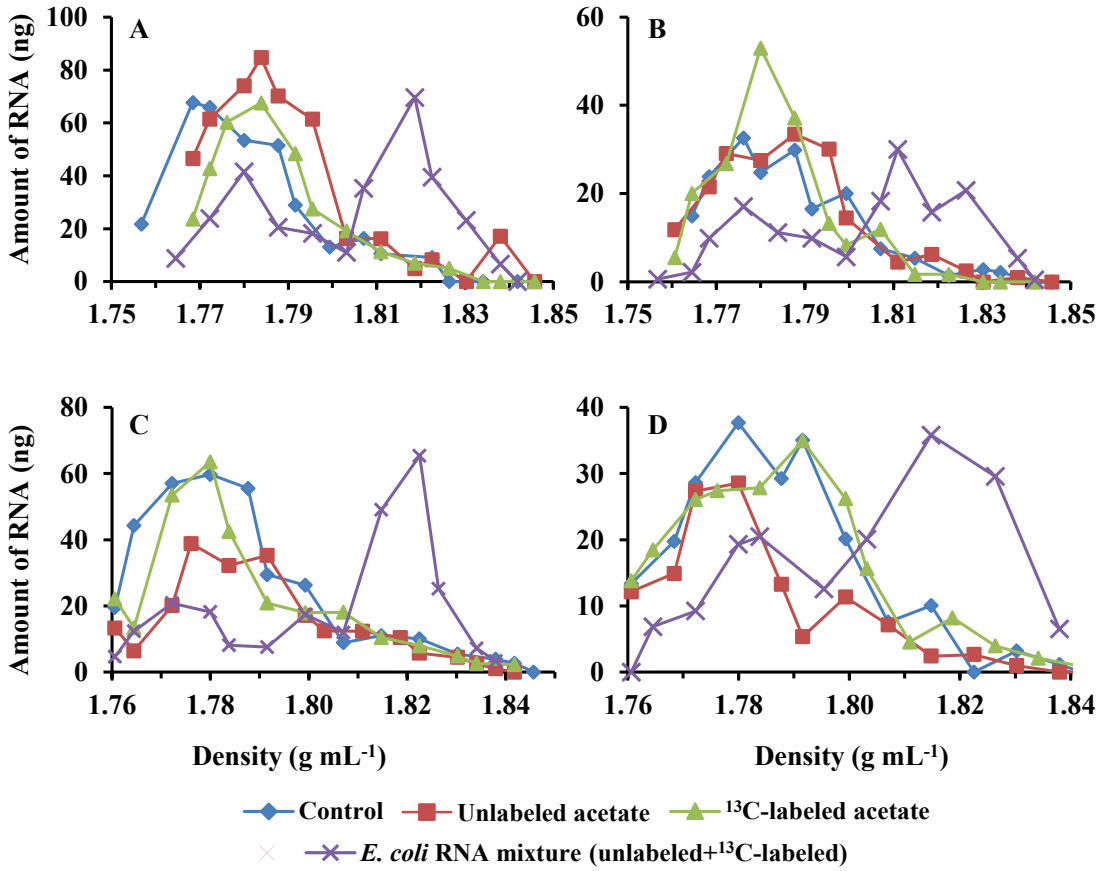

Figure S1. RNA (ng) distribution along density gradient after centrifugation. RNA from Dorum sediment incubations without further addition (**A**). With lepidocrocite (**B**). With AQDS (**C**), and with lepidocrocite and AQDS (**D**). Control- incubations without electron donor addition than stated. For each set, a parallel tube containing *E. coli* RNA was centrifuged and used as marker for identifying heavy gradient fraction (~ 1.82 g mL^{-1}) and light gradient fraction (~ 1.78 g mL^{-1}).

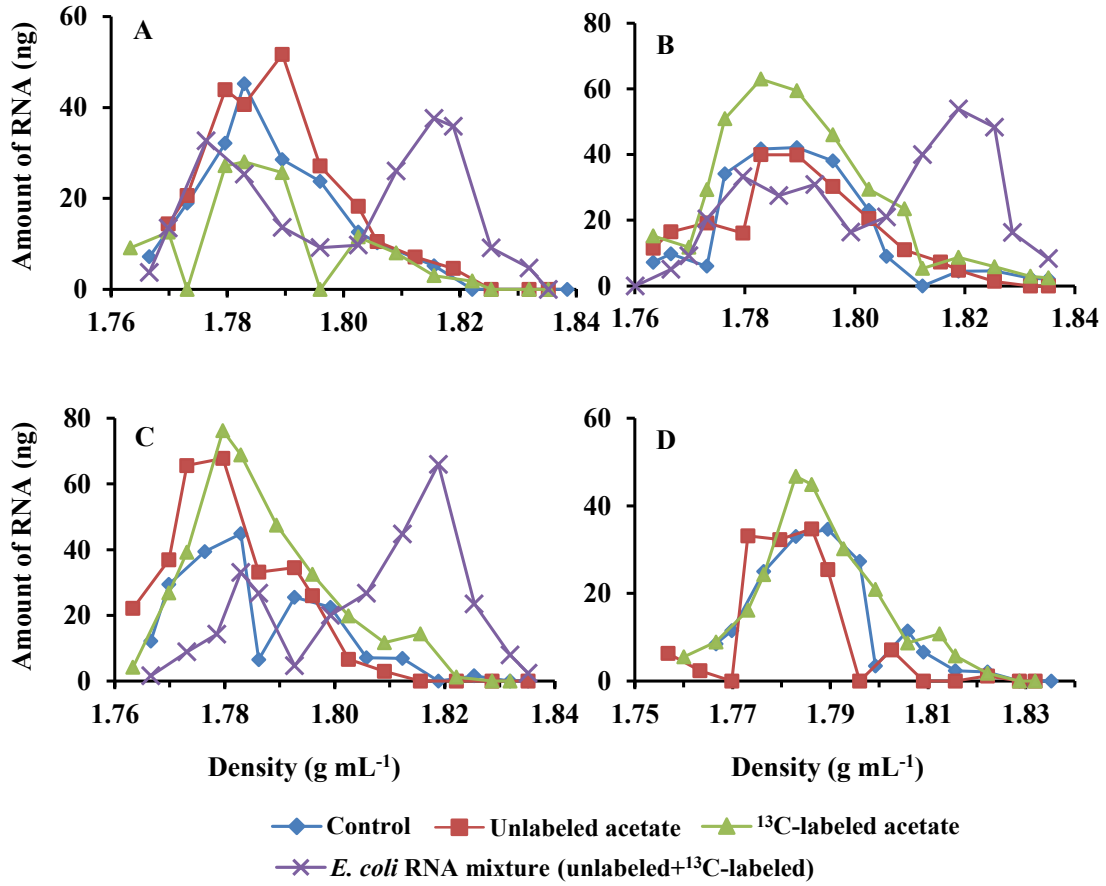

Figure S2. RNA (ng) distribution along density gradient after centrifugation. RNA from Helgoland mud sediment incubations without further addition (**A**). With lepidocrocite (**B**). With AQDS (**C**), and with lepidocrocite and AQDS (**D**). Control- incubations without electron donor addition than stated. For each set, a parallel tube containing *E. coli* RNA was centrifuged and used as marker for identifying heavy gradient fraction (~ 1.82 g mL^{-1}) and light gradient fraction (~ 1.78 g mL^{-1}).

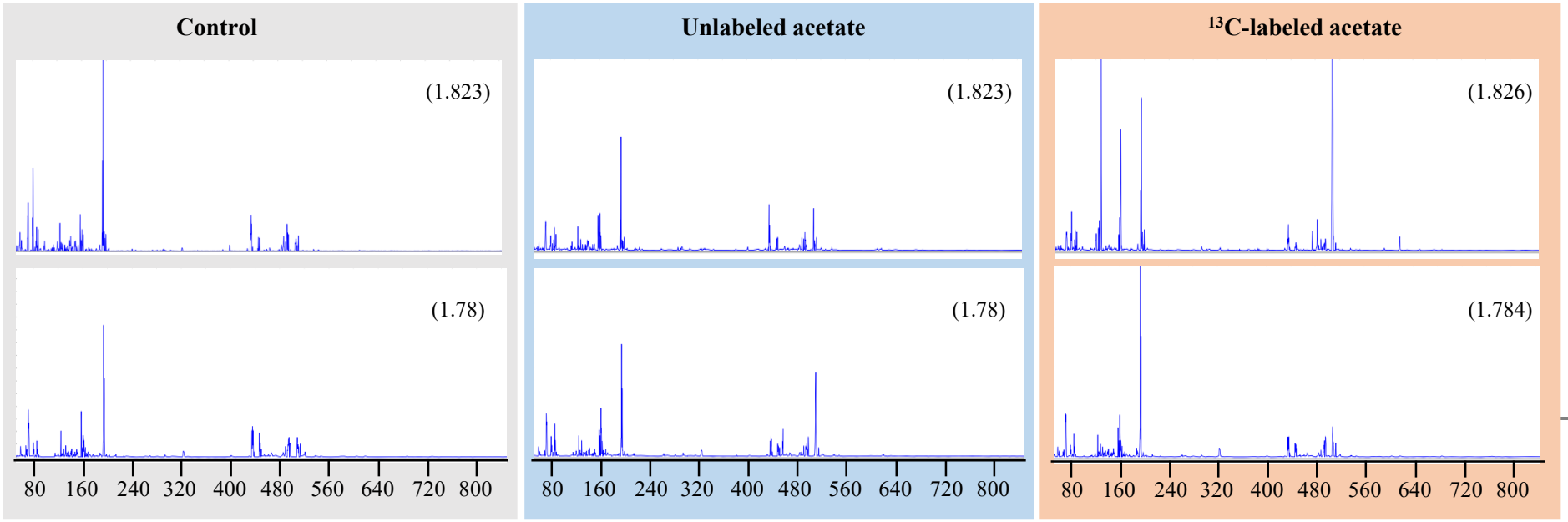

Figure S3. TRFLP finger printing analysis of RNA from heavy (~ 1.82 g mL^{-1}) and light (~ 1.78 g mL^{-1}) gradient fractions from Dorum sediment incubations without further addition than stated. Control- incubations without electron donor addition.

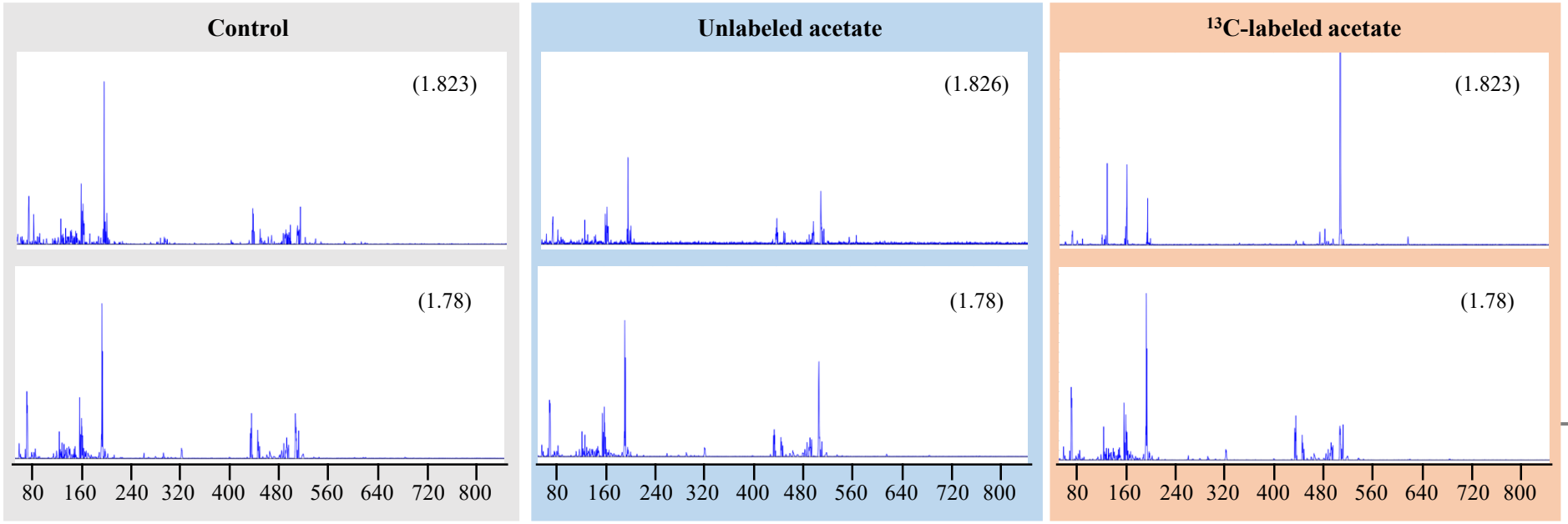

Figure S4. TRFLP finger printing analysis of RNA from heavy (~ 1.82 g mL^{-1}) and light (~ 1.78 g mL^{-1}) gradient fractions from Dorum sediment incubations with lepidocrocite. Control- incubations without electron donor addition.

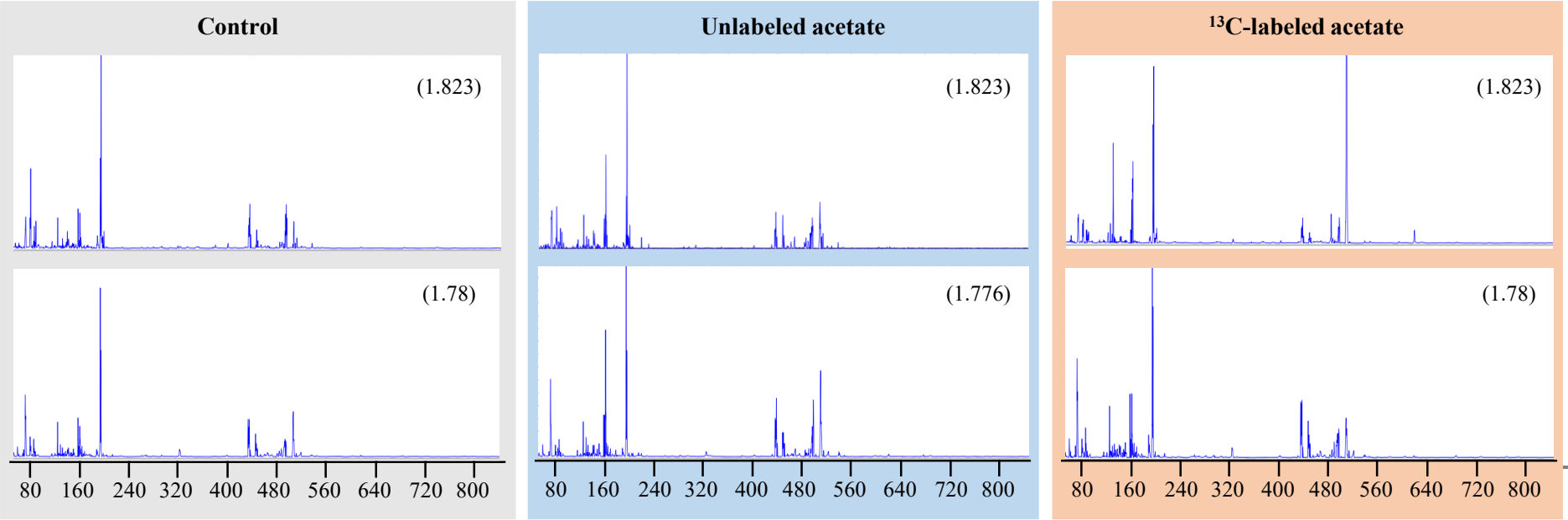

Figure S5. TRFLP finger printing analysis of RNA from heavy (~ 1.82 g mL^{-1}) and light (~ 1.78 g mL^{-1}) gradient fractions from Dorum sediment incubations with AQDS. Control- incubations without electron donor addition.

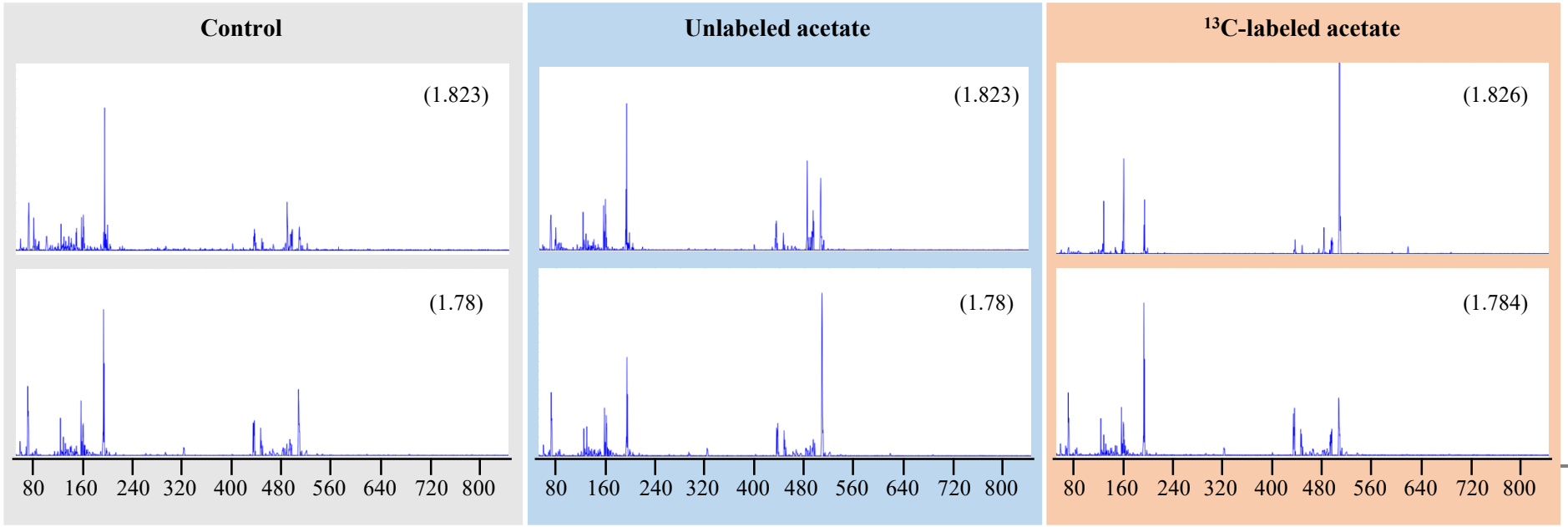

Figure S6. TRFLP finger printing analysis of RNA from heavy (~ 1.82 g mL^{-1}) and light (~ 1.78 g mL^{-1}) gradient fractions from Dorum sediment incubations with lepidocrocite and AQDS. Control- incubations without electron donor addition.

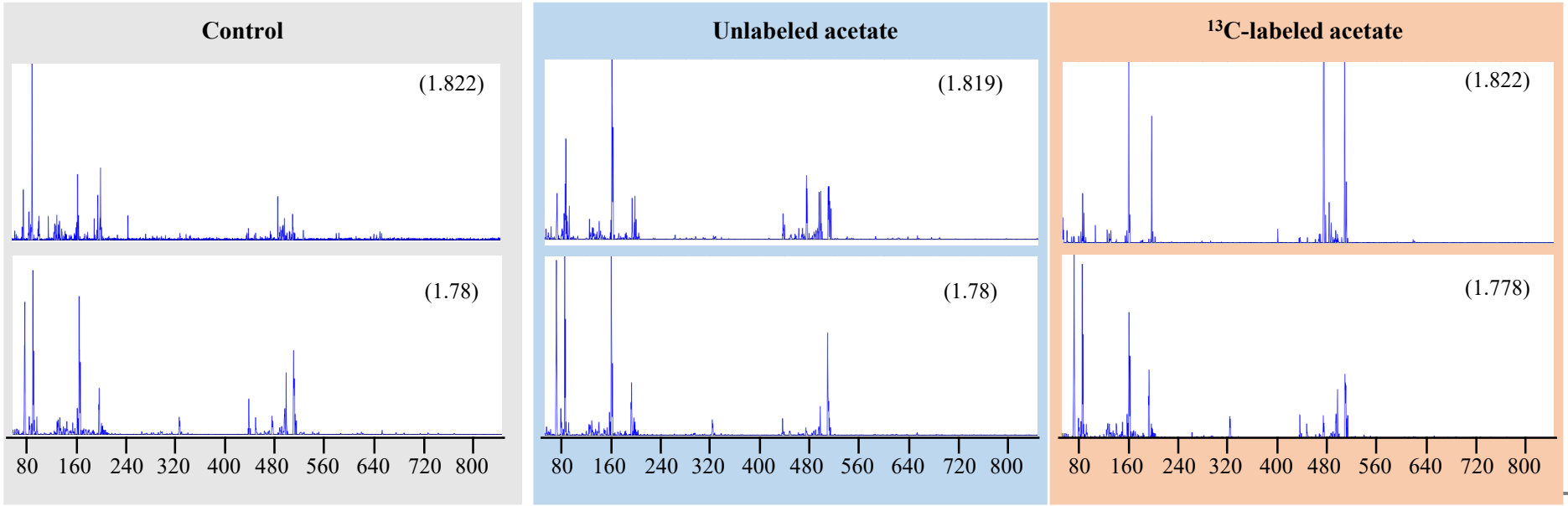

Figure S7. TRFLP finger printing analysis of RNA from heavy (~ 1.82 g mL^{-1}) and light (~ 1.78 g mL^{-1}) gradient fractions from Helgoland mud sediment incubations without further addition than stated. Control- incubations without electron donor addition.

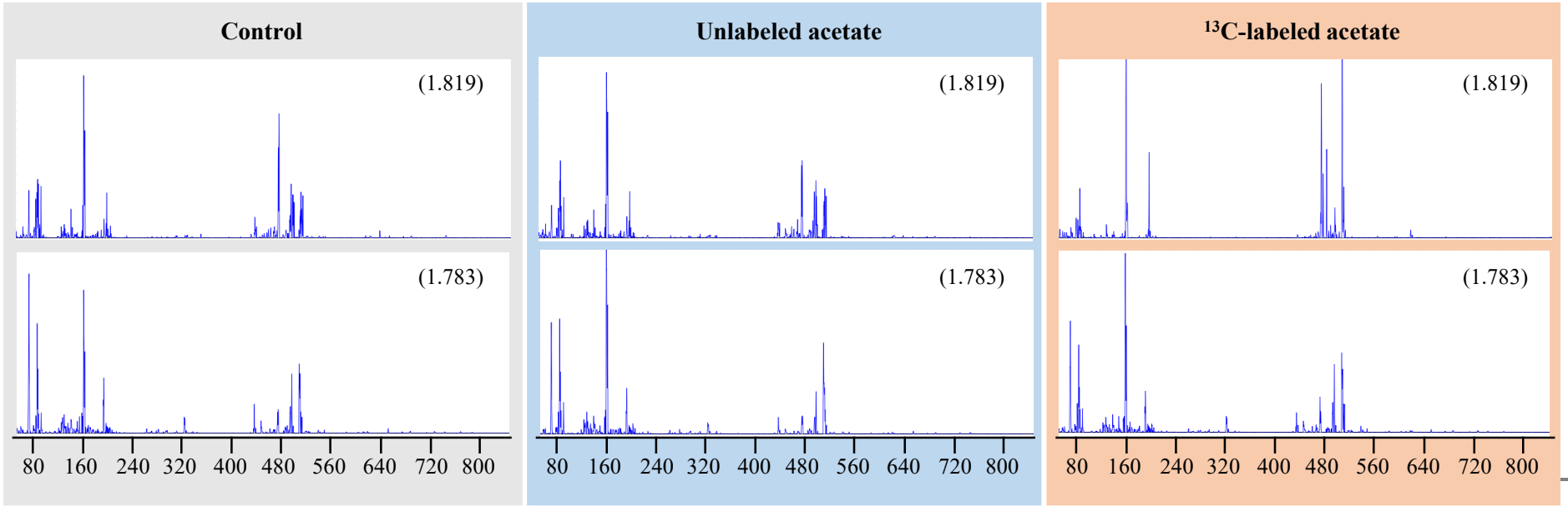

Figure S8. TRFLP finger printing analysis of RNA from heavy (~ 1.82 g mL^{-1}) and light (~ 1.78 g mL^{-1}) gradient fractions from Helgoland mud sediment incubations with lepidocrocite. Control- incubations without electron donor addition.

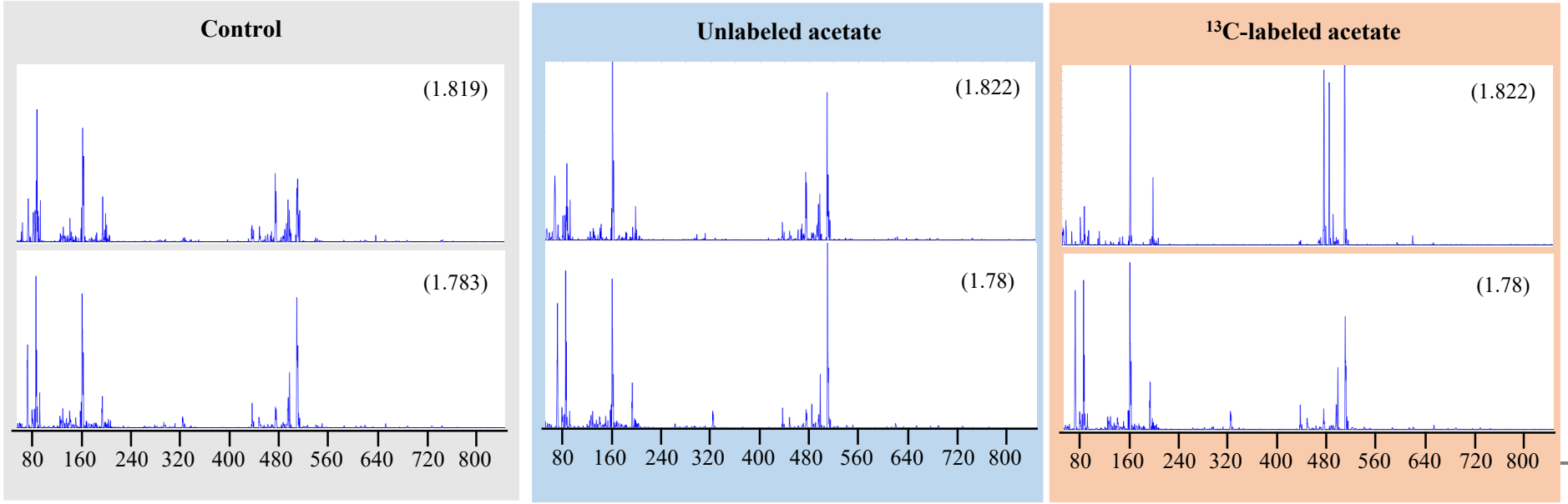

Figure S9. TRFLP finger printing analysis of RNA from heavy (~ 1.82 g mL^{-1}) and light (~ 1.78 g mL^{-1}) gradient fractions from Helgoland mud sediment incubations with AQDS. Control- incubations without electron donor addition

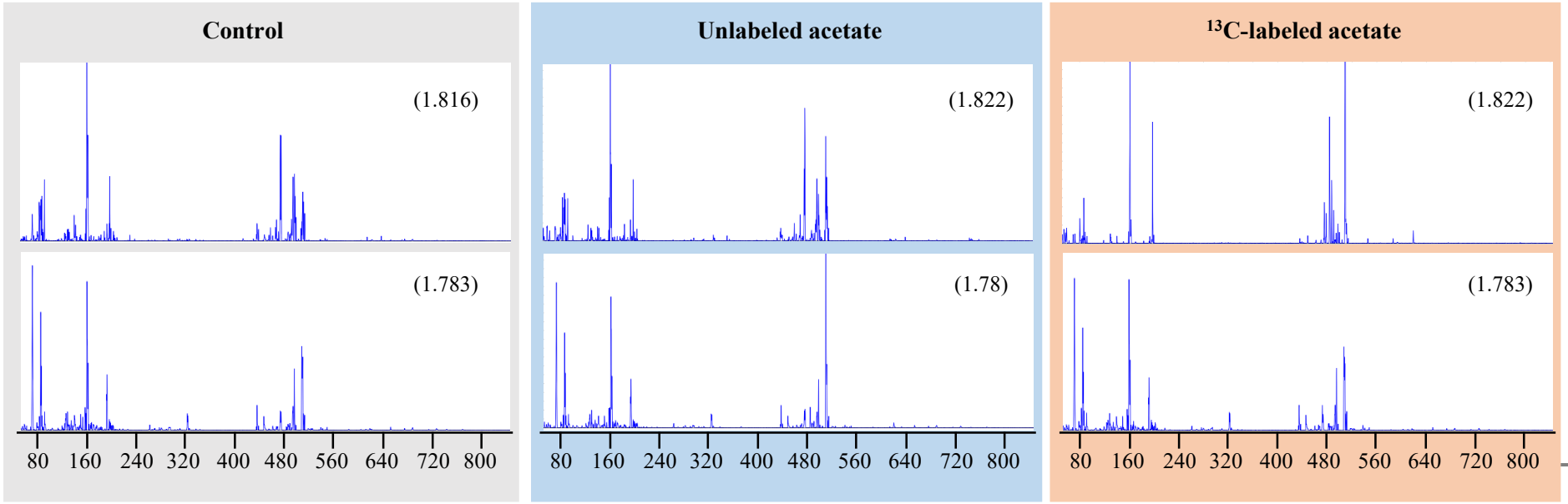

Figure S10. TRFLP finger printing analysis of RNA from heavy (~ 1.82 g mL^{-1}) and light (~ 1.78 g mL^{-1}) gradient fractions from Helgoland mud sediment incubations with lepidocrocite and AQDS. Control- incubations without electron donor addition

Table S1. Statistic summary of sequences data from the SILVA pipeline. Samples names: D- Dorum, sed- sediment, Lep- lepidocrocite, ^{13}C- ^{13}C-labeled acetate, ^{12}C- unlabeled acetate.

Sample Name	Number of sequences	Min. length	Avg. length	Max. length	% Classified	% Rejected
D sed	39889	272	482	542	94.73	0.01
D sed AQDS	40407	273	476	540	95.66	0.01
D sed Lep	35828	272	487	542	94.96	0
D sed Lep AQDS	42436	272	485	542	95.29	0.01
D sed ^{13}C	69596	273	511	540	97.98	0
D sed ^{13}C AQDS	78142	273	514	540	99.1	0
D sed ^{13}C Lep	84224	272	514	541	98.57	0
D sed ^{13}C Lep AQDS	86342	272	511	539	98.91	0
D sed ^{12}C	40173	273	486	542	93.09	0.01
D sed ^{12}C AQDS	41805	272	481	542	95.66	0.01
D sed ^{12}C Lep	39679	273	489	542	95.32	0
D sed ^{12}C Lep AQDS	48519	272	484	540	95.91	0.01
H sed	50940	273	501	542	95.54	0
H sed AQDS	40422	272	501	542	94.94	0
H sed Lep AQDS	38546	274	501	542	96.76	0
H sed Lep	37530	273	500	541	95.59	0.01
H sed ^{12}C Lep	37337	272	503	541	95.29	0
H sed ^{12}C Lep AQDS	44373	272	503	541	96.52	0
H sed ^{12}C	40336	272	503	542	95.27	0
H sed ^{12}C AQDS	45573	273	501	541	97.62	0
H sed ^{13}C	69577	273	499	540	97.97	0
H sed ^{13}C AQDS	71845	272	506	539	98.72	0.01
H sed ^{13}C Lep AQDS	151489	272	508	541	98.14	0
H sed ^{13}C Lep	64788	273	511	541	98.06	0

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Chapter 5

DHVEG-6 archaea couple acetate oxidation to humic compound mediated iron reduction in shallow hydrothermal vent sediment, Dominica Island, Lesser Antilles

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Abstract

Shallow hydrothermal vents around Dominica island (Lesser Antilles) fuel the accumulation of hydrous ferric oxides up to 20 cm sediment thickness upon mixing of Fe^{2+}-rich vent fluids (~ 200 µM) with oxygenated, cold sea water. However, it is unknown which microorganisms utilize these highly enriched iron oxides as terminal electron acceptor in this system. Here, we identified both, iron reducing and humic substance reducing microorganisms by stable isotope probing of RNA (RNA-SIP). The kinetics of iron reduction in surface sediment slurries was followed with and without the humic acid analog anthraquinone disulfonic acid (AQDS) as extracellular electron shuttle in combination with acetate (^{13}C-labeled and unlabeled) as electron donor and carbon source. Without AQDS amendment, *Deferribacteres* related spp. were identified as main acetate utilizers. AQDS addition (with or without acetate) resulted in higher stimulation of iron reduction compared to controls and members of the archaeal *Halobacteriales* group DHVEG-6 were identified as main acetate assimilating microorganisms. Our data show that iron reduction in Dominica's shallow hydrothermal vent system can be strongly stimulated by humic substances, and that uncultivated DHVEG-6 archaea appear to have the ability to utilize acetate when an extracellular electron shuttle is available.

5.1 Introduction

Hydrothermal vent systems are unique environments in which reduced substrates from vent fluids are mixed with oxygenic seawater. Thus, giving opportunities to a variety of redox reactions (electron donors and acceptors) and life style (heterotrophs *vs*. autotrophs) (Amend and Shock, 2001; Tarasov et al., 2005; Price et al., 2013a). Shallow hydrothermal vent systems can be found worldwide related to volcanic activity at depths less than 200 m (Price and Pichler, 2005; Tarasov et al., 2005). Primary productivity in shallow systems can be fueled by chemo- and photosynthesis (Tarasov et al., 2005; Giovannelli et al., 2013). Additionally, since they can be found at areas close to shoreline they are easier to access and to investigate (e.g. Pichler et al., 1999; Price and Pichler, 2005; Handley et al., 2013).

The fluids of the shallow hydrothermal vent system on the southwest section of the island of Dominica (Lesser Antilles) are rich in Fe^{2+} and depleted in SO_4^{2-} relative to the ambient seawater (McCarthy et al., 2005; Gomez-Saez et al., 2015; Kleint et al., 2015). Accumulation of insoluble Fe(III) in the form of hydrous ferric oxides (HFO; 2-line ferrihydrite) around the vent area is considered to be from mixing of Fe^{2+} rich vent fluids with oxygenated seawater (McCarthy et al., 2005; Gomez-Saez et al., 2015). Thus, this environment provides conditions, in which microbial iron reduction can be expected to occur (Emerson, 2009; Handley et al., 2010; Handley et al., 2013).

Dissolved organic matter (DOM) and humic substances (HS) can sorb to iron oxides and to co-precipitate (Rashid, 1985; Livens, 1991; Riedel et al., 2013; Shimizu et al., 2013). This was shown to happen in hydrothermal vent fluids during oxidation of Fe^{2+} and can affect availability of organic matter (Bennett et al., 2011; Gomez-Saez et al., 2015). HS have a strong effect on iron reduction and play an important role in

anaerobic respiration (Lovley et al., 1996; Lovley et al., 1998; Kappler et al., 2004; Lipson et al., 2010; Lipson et al., 2013). HS also enhance electrical current production in marine based microbial fuel cells (Holmes et al., 2004). Microorganisms able to reduce HS have been isolated from marine sediments (Coates et al., 1998). In addition, in North Sea sediment incubations, microbial iron reduction was enhanced up to ~ 4.5 - fold when HS were available (chapter 3). Altogether, this indicates that HS act as shuttle for extracellular electron transport in marine sediments.

DOM in vent fluids at the shallow hydrothermal vent system Dominica and in surface water was found to be of terrestrial origin. HS of terrestrial origin have higher content of aromatic moieties than marine derived HS, resulting in higher electron uptake capacity (Aeschbacher et al., 2010; reviewed in: Piepenbrock and Kappler, 2012). Therefore, HS are expected to play a role in the iron cycle in this environment.

By using stable isotope probing of RNA (RNA-SIP) technique combined with the resolution of high-throughput sequencing, it is possible to link microbial physiology to phylogeny (Manefield et al., 2002; Zemb et al., 2012; Kleindienst et al., 2014; Aoyagi et al., 2015). Identification of the key organisms in marine surface sediments, which can couple the oxidation of acetate to the reduction of iron and HS using RNA-SIP, showed that almost all acetate oxidizers were able to respire iron oxide and humic acid analog anthraquinone disulfonic acid (AQDS) (chapter 4).

In this study, we investigated the effect of HS as electron shuttle molecule on microbial iron reduction in the hydrothermal sediments of the shallow vent system of Dominica Island. In addition, we investigated the active acetate oxidizing iron/HS reducing microorganism population in those sediments. We hypothesize that amendment of AQDS as electron shuttle molecule will strongly stimulate iron reduction

and result in a shift of acetate oxidizers. Sediment slurries were incubated with acetate or ^{13}C-labeled acetate, with or without AQDS. The production of Fe^{2+} was monitored, and the active population was analyzed using RNA-SIP combined with Illumina sequencing.

5.2 Methods

5.2.1 Sediment sampling

Surface sediment samples were collected from an active hydrothermal vent site at the coast of Soufriere- Dominica Island, Lesser Antilles on April 2013 (15.232N, 61.3616W; Fig. 1) from a water depth of 5 m and sediment temperature of 55 °C. At this site, it is possible to identify the activity by streams of gas bubbles discharged from the sediment on shore and underwater. Sediment cores (plexiglass tubes 5.2 cm in diameter, 30 cm long) were retrieved using scuba diving after identifying hot spots with temperature probes as described earlier (Price et al., 2013a; Price et al., 2013b; Gomez-Saez et al., 2015; Kleint et al., 2015). Samples were stored until processing in the lab at 4 °C (9 days). Full geochemical characterization of pore water from this site can be found in Gomez-Saez et al. (2015).

5.2.2 Sediment Incubations

For sediment incubations, slurries were prepared by mixing sediment samples (upper 5 cm) with autoclaved anoxic sulfate-free artificial sea water (ASW) at a 1:1 ratio under continuous flow of N_2 gas. ASW contained (per liter of deionized water) 14.3 g NaCl, 6 g $MgCl_2 \cdot 6H_2O$, 0.8 g $CaCl_2 \cdot 2H_2O$, 0.4 g KCl. Under a stream of N_2,

sediment slurries (15 mL) were transferred into vials and supplemented with sterile anoxic stocks. Vials were sealed with butyl rubber stoppers and incubated for 7 days at 50 °C in the dark. Duplicate incubation vials were made for each time point. Sodium acetate was added as electron donor to a final concentration of ~ 1 mM. For ^{13}C-labeling, 1,2- $^{13}C_2$ 99 % sodium acetate was used to a final concentration of ~ 1 mM (Cambridge Isotope Laboratories, USA). AQDS (AB136728, ABCR, Karlsruhe, Germany) was added to a final concentration of ~ 50 µM as electron shuttle molecule.

5.2.3 Chemical analyses

At each time point, liquid samples were taken as previously described (chapter 3). Fe^{2+} was measured using the ferrozine assay (Stookey, 1970; Hegler et al., 2008; chapter 3).

5.2.4 Nucleic acid extractions

RNA and DNA were co-extracted (chapter 3) from original slurry (T_0) and after homogenizing equal amounts of sediment slurry from the sixth and seventh days of incubation. DNA was digested after saving aliquots for further work and RNA was quantified as described earlier (capter 4).

5.2.5 Terminal restriction fragment length polymorphism (TRFLP) analysis

For TRFLP analysis, 16S rRNA genes were amplified from DNA extracts using bacterial primers 8f-FAM labeled (Turner et al., 1999) and 907r (Muyzer et al., 1995) and digested using *Msp*I (chapter 3). Size separation was performed (Schauer et al., 2010) and peaks were analyzed using TREX software (Culman et al., 2009; chapter 3).

5.2.6 Isopycnic separation and fractionation

Density gradient centrifugation was performed following Lueders et al. (2004) and as described in chapter 4, using 500 – 600 ng of RNA. RNA was precipitated from CsTFA gradient fractions using isopropanol and sodium acetate. Then, a washing step with ethanol, followed by elution of the pellet in 20 µL nuclease free water and quantification of RNA (chapter 4).

Mixture of *Escherichia coli* RNA (^{13}C-labeled and unlabeled 1:1 ratio) was centrifuged in parallel tubes to help identify the "heavy" (~ 1.82 g mL^{-1}) and "light" (~ 1.78 g mL^{-1}) fractions. *E. coli* cells were grown on fully ^{13}C-labeled or unlabeled medium (*E. coli* OD2 ^{13}C labeled- 110201102; *E. coli* OD2- 100002, Silantes, Munich Germany).

5.2.7 Reverse transcription, sequencing and sequence analysis

From each sample, RNA of isotopically "heavy" gradient fractions, corresponding to gradient fractions of fully ^{13}C-labeled *E. coli* RNA, was used to construct cDNA libraries (chapter 4). Total RNA (prior to density separation) from each incubation was also used for cDNA construction. From the original slurry (T_0), DNA was used for sequencing. Amplification was done (chapter 3) using primers set 341f (Herlemann et al., 2011) and 805r (Takahashi et al., 2014) (variable regions V3-V4; chapter 2, Table 2) and the products were analyzed by Illumina's paired end sequencing (primer set 341f/805r) at MR. DNA, Molecular Research LP (Texas, USA) (chapter 3). Joined sequences were depleted of primers and barcodes using split_libraries.py as implemented in QIIME (version 1.8.0; Caporaso et al. (2010)) using default settings (chapter 3). The sequence reads were processed by the NGS analysis pipeline of the

SILVA rRNA gene database project (SILVAngs 1.2) (Quast et al., 2013; chapter 3) using the Silva Incremental Aligner (SINA v1.2.10 for ARB SVN (revision 21008)). OTUs were clustered using cd-hit-est (version 3.1.2; http://www.bioinformatics.org/cd-hit). Classification was performed by a local nucleotide BLAST search against the non-redundant version of the SILVA SSU Ref dataset (release 119.1; http://www.arb-silva.de) using blastn (version 2.2.30+; http://blast.ncbi.nlm.nih.gov/Blast.cgi) with standard settings. Low identity and artificial BLAST hits, which did not exceed the value of 50 from the function "(% sequence identity + % alignment coverage)/2", remained unclassified. Raw sequences can be found on supplemented DVD.

5.2.8 Community analysis

Cluster analysis (paired group algorithm) plots were created with Bray-Curtis similarity coefficient using PAlaeontological STatistics (PAST version 2.17c; http://folk.uio.no/ohammer/past) (Hammer et al., 2001).

5.3 Results

5.3.1 Iron reduction in slurry incubations

During incubation (7 days), Fe^{2+} accumulated in all treatments. In all incubations, highest rates were observed within the first day. Highest rates were determined for incubations amended with acetate + AQDS (~ 1.2 mM Fe^{2+} day^{-1}) and ^{13}C-acetate + AQDS (~ 1 mM Fe^{2+} day^{-1}) (Fig. 2). Amendment of AQDS only resulted in lower rates of iron reduction (~ 0.7 mM Fe^{2+} day^{-1}). For incubations amended only with acetate (labeled or unlabeled), Fe^{2+} formation rates were similar to the un-amended control treatment (~ 0.66 – 0.69 mM day^{-1}) (Fig. 2).

At the end of the incubations, highest stimulation of Fe^{2+} formation was observed in samples amended with AQDS (with or without electron donor; Fig. 2). AQDS addition resulted in a stimulation of 1.2 – 1.3 times compared to controls. Contrastingly, when only acetate was added (labeled or unlabeled) we did not observe elevated Fe^{2+} concentrations, and similar values were estimated for the control and for samples amended with solely acetate at the end of the incubation time.

5.3.2 Bacterial and archaeal affiliations in slurry incubations

The bacterial and archaeal diversity was investigated at the beginning and at the end of the incubations (one week) in slurry samples (Fig. 3). The Number of sequences that were analyzed by the SILVA pipeline ranged ~50,230 – 160,000 per sample (average of ~ 90,700). The average length was ~ 433 base pairs per sequence (Table S1).

At the beginning of the incubations, most sequences were identified as related to Bacteria, whereas less than 0.3 % of the sequences were identified as related to Archaea. Approximately 30 % of all sequences were related to *Chloroflexi*, followed by *Proteobacteria* (25 %), and *Bacteroidetes* (18 %) (T_0, Fig. 3 A). Most of *Chloroflexi* related sequences were related to *Anaerolineae*. Within the *Proteobacteria*, most sequences were related to the *Deltaproteobacteria* (Fig. 3 B). *Halobacteriales* related spp. were found as ~ 55 % of the archaeal sequences. Within the *Halobacteriales*, deep-sea hydrothermal vent group 6 (DHVEG-6) related sequences were the most dominant with ~ 30 % of the total archaeal related sequences.

At the end of the incubations, archaeal related sequences were still 0.04 – 0.8 % of total sequences within all samples, however, DHVEG-6 increased to 80 – 98 % of all archaeal sequences. A shift in the community composition within all treatments was observed, and the most dominant phylum was *Bacteroidetes* (69 – 79 % of all sequences). After which, *Proteobacteria* and *Chloroflexi* were found with similar abundances (5 – 9 % and 4.5 – 8 %, respectively) (Fig. 3 A). The most dominant taxonomic class within all incubations was group SB-1 (*Bacteroidetes*) (60 – 68 %), the second most abundant class in each of the samples had a maximum of 6 % abundance (Fig. 3 B).

5.3.3 Bacterial community ordination

Cluster analysis of 16S rRNA genes TRFLP fingerprints indicates that the microbial community structure was influenced mainly by the addition of an electron shuttle molecule (AQDS). Communities from incubations amended with AQDS clustered together (Fig. 4 A, in red). Communities from incubations amended with acetate clustered separately with communities from un-amended control incubations,

and communities from incubations amended with ^{13}C-labeled acetate were found closer to communities from incubations amended with AQDS, although in a separated branch. A similar pattern was observed in cluster analysis using phylogenetic affiliation on the class level (Fig. 4 B).

5.3.4 Identification of microorganisms capable of acetate assimilation

After 6 – 7 days of incubation (see 5.2.4), RNA was extracted and density separated for identifying actively ^{13}C-acetate incorporating microorganisms. Sequencing data of 16S rRNA from heavy fractions of non-labeled samples (Fig. 5) showed a similar community composition compared to the community composition before isopycnic separation (Fig. 3 A, B). The phylum *Bacteroidetes* was most dominant (40 – 56 % of all sequences) followed by *Chloroflexi* (13 – 23 % of all sequences) and *Proteobacteria* (10.5 – 16.5 % of all sequences) (Fig. 5 A). From the *Bacteroidetes*, the most common class found was the SB-1 group. From the *Chloroflexi*, half of the sequences were related to *Anaerolineae* and half unidentified *Chloroflexi*, and within the *Proteobacteria* most sequences were related to the *Deltaproteobacteria* (Fig. 5 B).

From the sequencing data of 16S rRNA from heavy fractions of samples amended with ^{13}C-labeled acetate, it can be seen that mainly two phylogenetic groups were enriched. Sequences related to the bacterial phylum *Deferribacteres* (represented by *Deferribacter*) and to the archaeal phylum *Euryarchaeota* (represented by the *Halobacteriales* group DHVEG-6) (Fig. 5). When ^{13}C-acetate was amended solely, *Deferribacteres* related sequences were ~ 25 % of the total abundance, and *Halobacteria* related sequences were ~ 6 %. In addition, we found that sequences related to *Deltaproteobacteria* were enriched to ~ 15 % of the total sequences (compared to ~ 9.5 % in the non-labeled acetate incubations). Within the

Deltaproteobacteria, *Geobacteraceae* related sequences had the highest increase in relative abundance. In incubations amended with ^{13}C-labeled acetate and AQDS, sequences related to DHVEG-6 were ~ 24 %, and sequences related to *Deferribacteres* were ~ 12 % of the total sequences.

5.4 Discussion

Potential for iron reduction has been shown in hydrothermal vents (Emerson, 2009; Handley et al., 2010; Handley et al., 2013). Additionally, DOM was found to interact with iron oxide during Fe^{2+} oxidation (Bennett et al., 2011; Gomez-Saez et al., 2015). Nonetheless, there is no information for the potential to use HS as electron shuttle molecule for respiration in hydrothermal vent systems. We found that AQDS addition as electron shuttle molecule strongly stimulated iron reduction in sediment samples from the shallow hydrothermal vents of Dominica Island. In addition, we show, to our knowledge for the first time, that microorganisms related to the archaeal group DHVEG-6 are able to oxidize acetate when AQDS was added to slurry incubations.

Iron reduction rate was stimulated (1.5 – 1.7 fold) as a result of AQDS addition to incubations amended also with acetate within the first day; although low stimulation (1.05 fold) when only AQDS was amended. Overall, AQDS amendment resulted in higher Fe^{2+} concentrations (1.2 – 1.3 fold) in all treatments. This indicates that electron shuttle molecules could be utilized by indigenous microbial populations. On the contrary, the addition of acetate to the incubations did not stimulate iron reduction and can suggest that electron donors were not limiting. Nonetheless, as reduction rates (at the first day) were higher for incubations amended with acetate and AQDS compared to

only AQDS amendment (1.4 – 1.7 fold), it is possible that acetate oxidation only contributed to iron reduction via extra cellular electron shuttling.

When comparing our sequence data from the beginning of the incubation to the end, a shift in microbial diversity can be observed. For $T_{0,}$ DNA was sequenced in order to get an estimation of relative abundance of the different taxa in the initial communities. At the end of the incubations, we sequenced RNA instead. The relative abundance of the sequences provides information about activity regarding ribosome synthesis. Some differences may be due to the use of DNA or RNA. In the original sediment, we found *Chloroflexi* related sequences with highest abundance (Fig. 3 A). *Chloroflexi* spp. can be found in iron rich hydrothermal vent systems (Handley et al., 2010; Kawaichi et al., 2013). They were shown to be able to reduce iron oxides in pure cultures and enrichments, although a specific family affiliation was not clear (Kawaichi et al., 2013; Hori et al., 2015). Thus, we assume that *Chloroflexi* spp. found in our incubations might participate in iron reduction *in-situ*.

Bacteroidetes spp. were found to be active in the incubations resulting as the most dominant sequences, although there was a low abundance at the beginning (< 20 %) (Fig. 3). Members of the *Cytophaga-Flavobacteriia-Bacteroides* (CFB) cluster were found in another shallow hydrothermal vent system as in Milos (Sievert et al., 2000; Giovannelli et al., 2013) suggesting that they take part in organic matter degradation. Additionally, *Bacteroidetes* related spp. were found when acetate was added as electron donor in microbial fuel cell enrichments (Zhang et al., 2011), in most probable number (MPN) incubations for iron reducing microorganisms (chapter 3), and in enrichments of MPN dilutions with iron oxides and acetate + lactate as electron donors (Lin et al.,

2007). We suspect that in our incubations this group was as well involved in organic matter degradation at a high rate.

Community structure in the incubations based on cluster analysis from both 16S rRNA gene based TRFLP profiles (Fig. 4 A) and 16S rRNA sequences (Fig. 4 B) was controlled mainly by the presence of AQDS as electron shuttle. This observation is in contradiction to our previous results from different marine sediments (chapter 3) and points out the specificity of different microorganism groups towards the use of extracellular electron shuttle at this site.

Using sequence analysis of 16S rRNA from the heavy fractions (~ 1.82 g mL^{-1}) of incubations amended with ^{13}C-labeled acetate, we identified the active acetate assimilating community. As a guiding line, groups which had a similar or higher relative abundance in heavy fractions of unlabeled acetate treatments were not considered to assimilate acetate. Production of ^{13}C-labeled CO_2 and subsequent autotrophic uptake might happen and will result in mixed labeled population of heterotroph and autotroph microorganisms. As mainly two groups were found, we assume that this is not the case, and the results represent the active acetate assimilators. In addition, previous observations and assumptions on their life style, as will be explained later support this idea.

We identified *Deferribacteres* related spp. as most predominant acetate assimilators by RNA-SIP (Fig. 5). Members of this phylogenetic group were found in other hydrothermal vent systems and are known to be able to couple acetate oxidation to iron reduction as well as to fix CO_2 (Greene et al., 1997; Miroshnichenko et al., 2003; Takai et al., 2003; Handley et al., 2013; Alauzet and Jumas-Bilak, 2014). Assimilation of ^{13}C-labeled acetate was found to be linked to anaerobic respiratory processes as iron

reduction (Hori et al., 2010). Although we did not find stimulation of iron reduction by acetate (labeled or unlabeled) (Fig. 2), we can speculate that *Deferribacter* related spp., that were found to assimilate acetate in our incubations, might have reduced iron as well.

When incubations were amended with ^{13}C-labeled acetate and AQDS, a strong shift in relative sequence abundances was observed. Sequences related to DHVEG-6 were found as the main acetate assimilating community. DHVEG-6 is a diverse uncultivated, and yet uncharacterized archaeal group detected first in a deep-sea hydrothermal vent system (Takai and Horikoshi, 1999). Sequences related to DHVEG-6 were found in different environments from fresh water to marine and hypersaline mats and in diverse temperature ranges (Sørensen et al., 2004; Kan et al., 2011; Wemheuer et al., 2012; Carmichael et al., 2013). DHVEG-6 were more abundant with algal bloom and as other *Halobacteria* spp. are assumed to actively take part in organic matter degradation (Auguet et al., 2009; Wemheuer et al., 2012). But, there is no direct indication for their metabolic capabilities. To the best of our knowledge, this is the first direct indication for their ability to assimilate acetate. Furthermore, our results imply that they are involved in respiration of extracellular electron shuttle molecules. Thus, they might be contributing to iron reduction via electron shuttling molecules (HS).

5.5 Conclusions

Humic substances were shown to play an important role for iron reduction in the shallow hydrothermal vent system of Dominica. Furthermore, it appears that either acetate is not the main electron donor for iron respiration or that it is not limiting in this system. *Deferribacteres* related species were found to be the main acetate oxidizers. Nonetheless, we could not link them directly to the iron reduction with acetate as electron donor observed in our incubations. As acetate did not stimulate iron reduction, it might be that other electron donors play a role. Therefore, further investigations including different electron donors are needed to understand direct iron reduction kinetics and the microorganisms involved in the process. We found that the archaeal group DHVEG-6 could oxidize acetate in the presence of AQDS, suggesting their ability to respire extra cellular electron shuttle molecules and by that contributing to the overall iron reduction.

Acknowledgements

This study was supported and funded by the University of Bremen and through the DFG Emmy Noether Grant BU 2606/1-1 given to S.I.B. The authors would also like to acknowledge all the members of the Dominica scientific expedition (J. Amend, G. V. Gomez-Saez, C. Kleint, A. Koschinsky, T. Pichler, P. Pop-Ristova, S. M. Sievert and M. Sollich), especially J. A., T. P., S. M. S. and M. S., for retrieving the sediment cores and G.V. G-S., for creating the maps. We would also like to thank the Dominican Department of Fisheries, especially to A. Magloire for granting sampling permission. We are also thankful to A. Madisetti (Izzy) and O. Lugay (Seacat) for logistical support,

transportation and underwater documentation (A. M.). In addition, we thank Edinson Puentes Cala for helping in lab work and measurements, T. Ferdelman, D. Ionescu and C. Reyes for methods discussions and helpful comments. We are also thankful to C. Glöckner and A. Hillmer for all the logistic help.

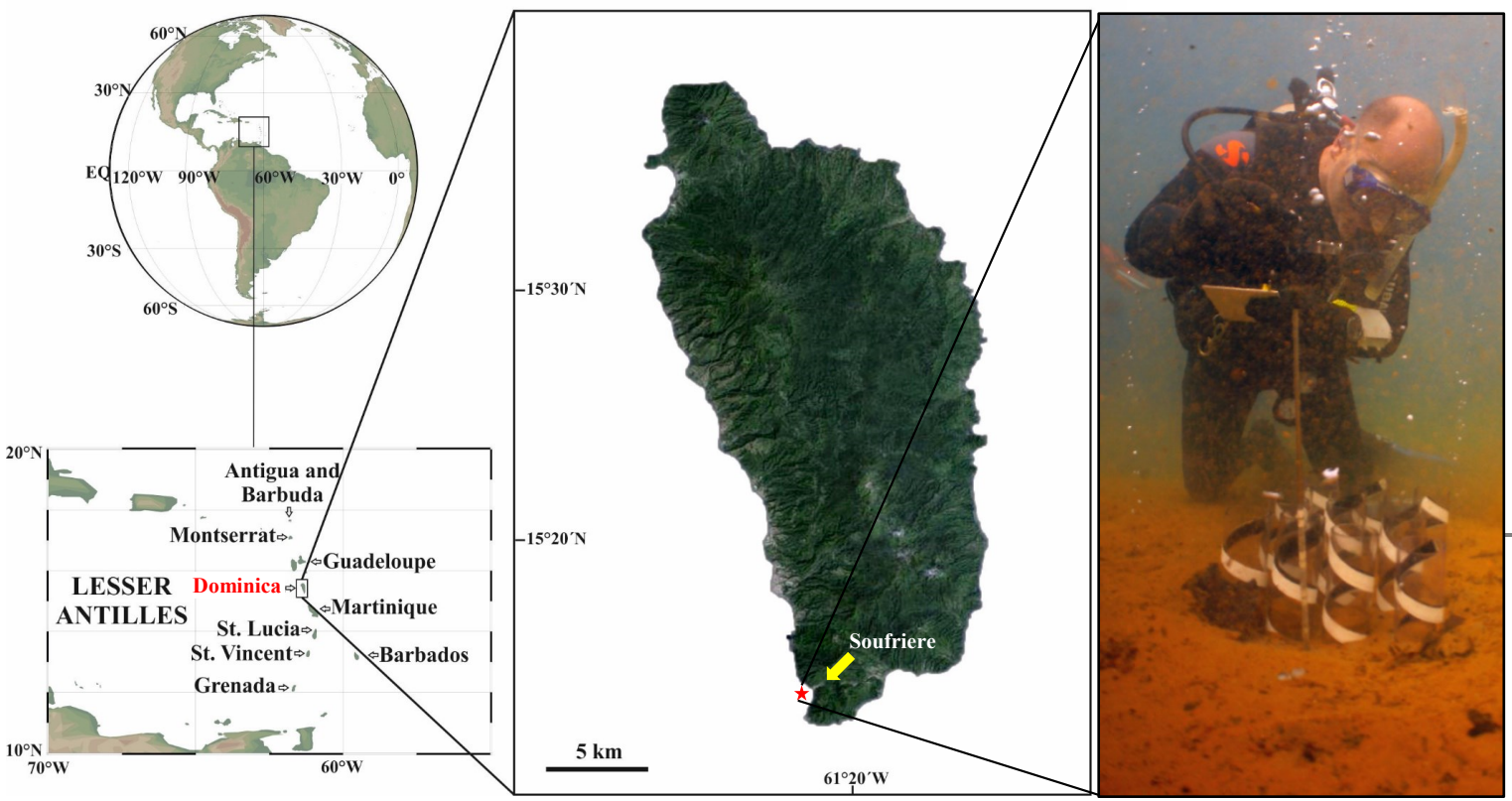

Figure 1. Sampling site at Soufriere bay Dominica, Lesser Antilles (15.232 N, 61.3616 W). Maps were created after Gomez-Saez et al. (2015) using Ocean Data View (R. Schlitzer, http://odv.awi.de) and Google Earth (http://earth.google.com). Submarine photo by courtesy of A. Madisetti; core sampling at the location showing visible bubbling from the sediment and the color of the sediment due to iron precipitation.

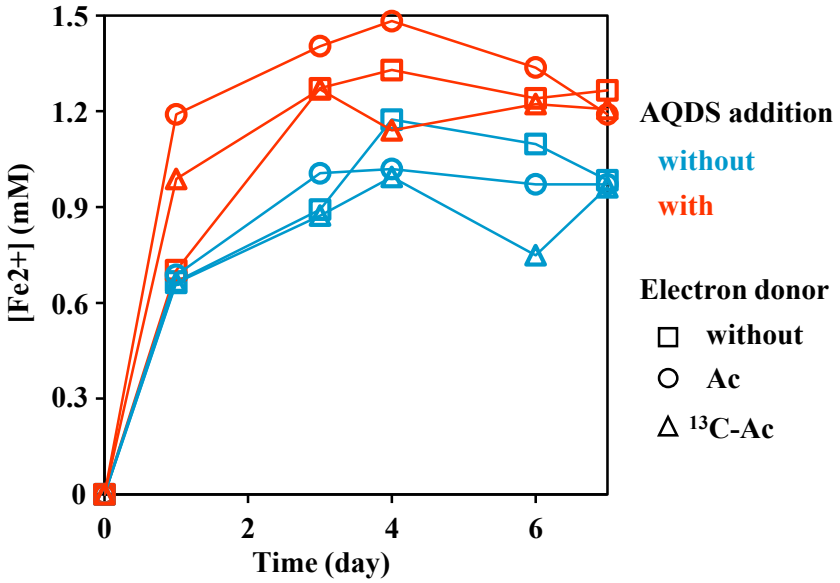

Figure 2. The effect of electron shuttle (AQDS, 50 µM final concentration) and electron donor (acetate, 1 mM final concentration) on Fe^{2+} formation in sediment incubations. All graphs show net formation of Fe^{2+} i.e., the production of Fe^{2+} from which the initial Fe^{2+} concentrations were subtracted. Ac- acetate, ^{13}C-Ac- ^{13}C-labeled acetate. Results represent average n = 2. Error bars are not presented. For each time point- duplicates were sacrificed for measurements.

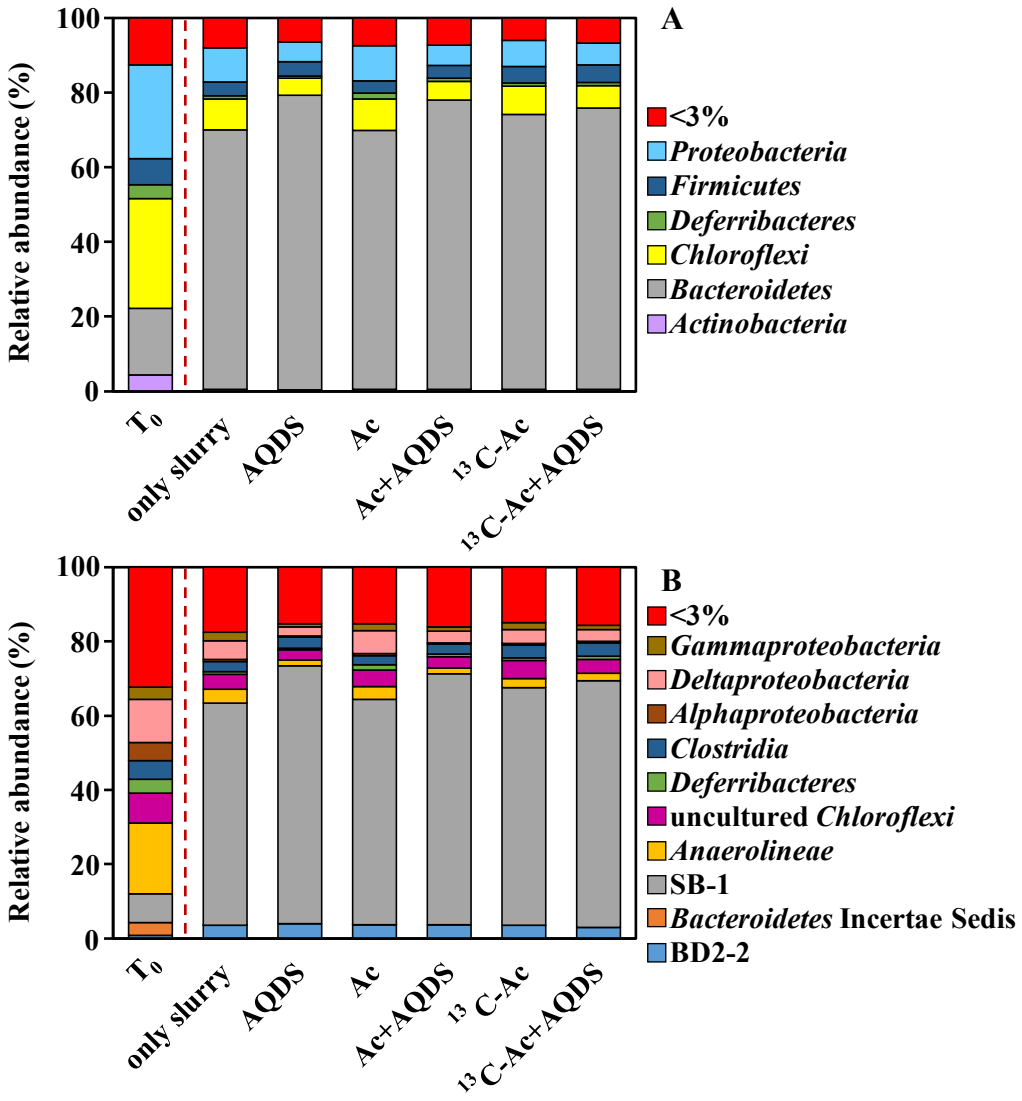

Figure 3. Relative abundance (%) of total 16S rRNA genes at beginning of incubations (T_0; separated by a red dashed line) and 16S rRNA sequences at the end of incubations (before isopycnic separation) with affiliations to different phyla (**A**) and classes (**B**). Phylogenetic groups, which had a relative abundance of less than 3 % in all samples were grouped together. Ac- acetate, ^{13}C-Ac- ^{13}C-labeled acetate, T_0- original sediment prior to incubations.

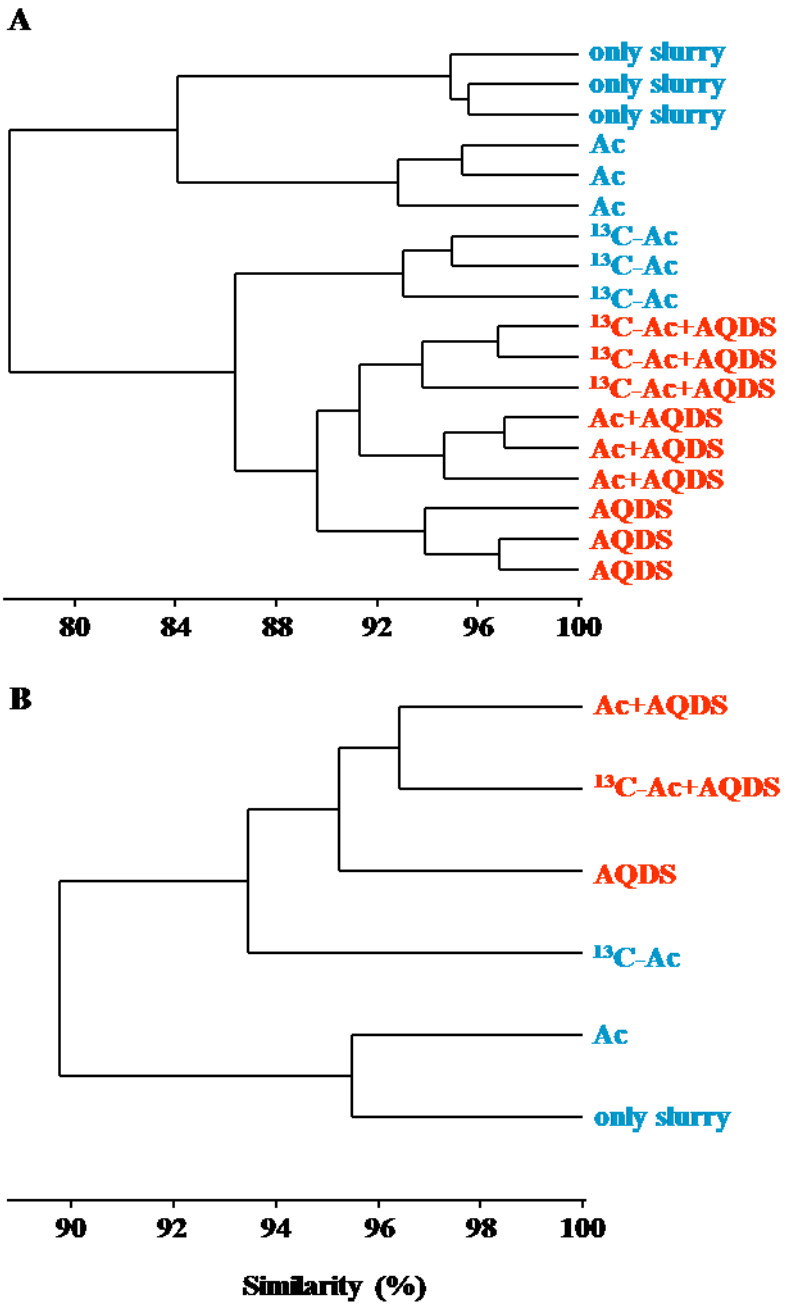

Figure 4. Cluster analysis (based on Bray-Curtis similarity coefficient) of **A.** TRFLP profiles from 16S rRNA genes of the different incubations, technical amplification replicates (n=3) are shown. **B.** based on the phylogenetic affiliation into class level of total 16S rRNA retrieved at the end of the incubation time prior to isopycnic separation. Clustering pattern can be seen based on the addition of AQDS (50 µM final concentration). Ac- acetate, ^{13}C-Ac- ^{13}C-labeled acetate.

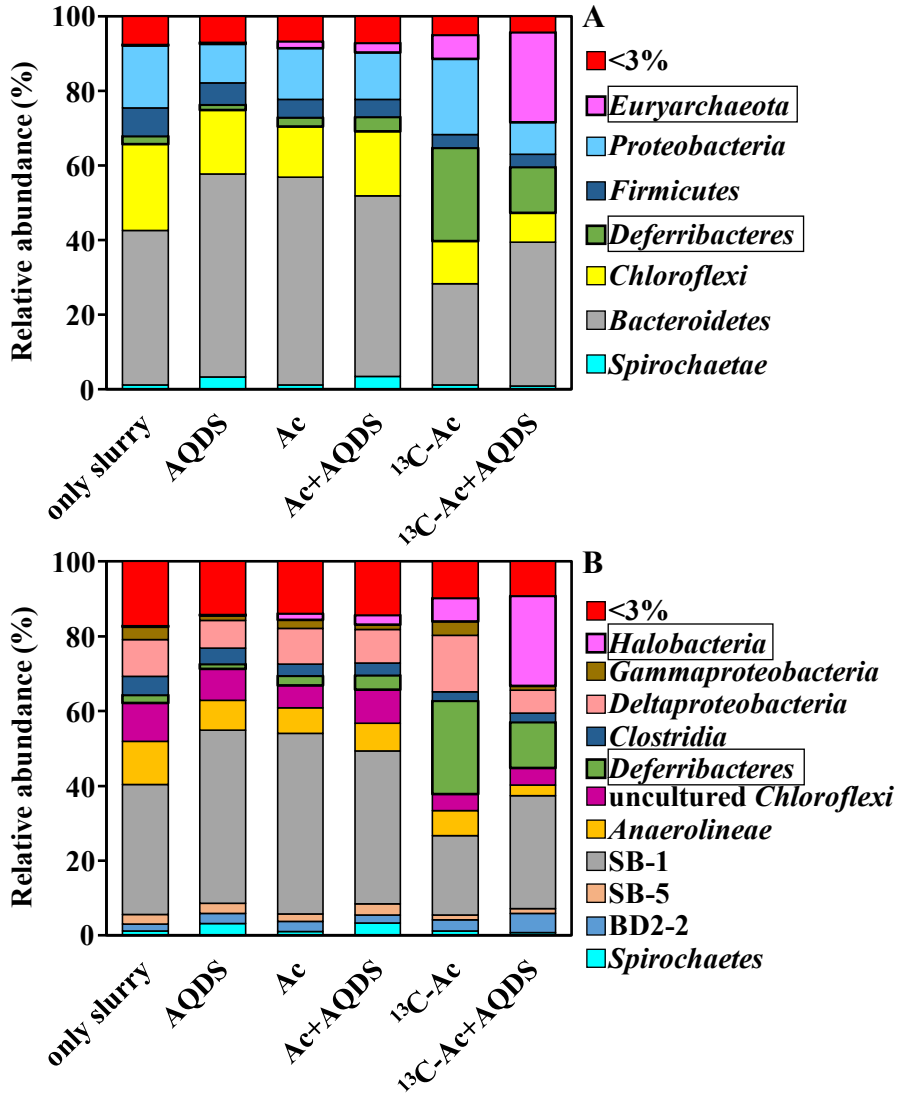

Figure 5. Relative abundance (%) of 16S rRNA sequences retrieved from high density gradients (~ 1.82 g mL^{-1}) of all samples after isopycnic separation. Phylogenetic groups which had a relative abundance of less than 3 % in all samples were grouped together. Major groups which were enriched in the labeled incubations are marked. **A.** Phylogenetic affiliation to different phyla. **B.** Phylogenetic affiliation to different classes. Ac- acetate, ^{13}C-Ac- ^{13}C-labeled acetate.

Supplementary table:

Table S1. Statistic summary of sequences data from the SILVA pipeline.

Sample Name		Number of sequences	Min. length	Avg. length	Max. length	% Classified	% Rejected
16S rRNA genes	T_0	56163	175	429	524	99.99	0.01
16S rRNA before centrifugation	only slurry	159964	175	435	535	99.99	0.01
	AQDS	102847	176	435	533	100	0
	Ac	158070	175	434	539	99.99	0.01
	Ac+AQDS	67223	176	435	517	100	0
	^{13}C	115646	175	435	537	99.99	0.01
	^{13}C+AQDS	84387	175	435	519	99.99	0.01
16S rRNA from high density gradient	only slurry	58076	175	432	519	99.99	0.01
	AQDS	89426	175	434	542	100	0
	Ac	69913	176	434	527	99.99	0.01
	Ac+AQDS	50233	175	433	525	99.99	0.01
	^{13}C	89643	175	434	545	99.99	0.01
	^{13}C+AQDS	77928	175	426	524	99.99	0.01

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Chapter 6 Discussion

Iron oxides are important electron acceptors in marine sediments and account for up to 60 % of local organic matter (OM) mineralization (Jensen et al., 2003) and about 17 % on a global scale (Thamdrup, 2000). The use of humic substances (HS) as electron shuttling molecules is known to highly stimulate iron reduction rates (Lovley et al., 1996) and by that increasing mineralization of organic carbon (C_{org}). The potential to use HS for respiration in soil freshwater systems was previously shown (Kappler et al., 2004; Lipson et al., 2010). HS were also shown to stimulate electrical current production in microbial fuel cells incubated with marine sediments (Holmes et al., 2004b). Nonetheless, there were no direct indications for the ability to use HS as an electron shuttle for iron reduction in marine sediments. Furthermore, not much is known about the identity of microorganisms capable to reduce HS in marine sediments (Coates et al., 1998; Holmes et al., 2004b; Lin et al., 2007).

In this work, I (1) investigated the potential to use HS as electron shuttle molecules for iron reduction in marine sediments, (2) estimated the number of cells which can use HS in specific marine sediment, and (3) identified the microbial community which is capable of coupling acetate oxidation (assimilation) to reduction of iron and HS (anthraquinone-2-4-disulfonic acid; AQDS) in three different locations. The different results are presented and discussed in detail in previous chapters. In this chapter, I will present my findings in a broader perspective and present hypotheses for future research.

6.1 Stimulation of iron reduction and respiration

In chapters 3 and 5, I have shown that addition of a humic acid (HA) analog AQDS stimulated (even more than 4 times) Fe^{2+} formation in slurry incubations. Reduction of AQDS with acetate as electron donor is energetically favorable: CH_3COO^- + 4 AQDS + 4 H_2O → 2 HCO_3^- + 4 AH_2ODS + H^+, with $\Delta G°'$ value of -73 kJ per reaction (Cervantes et al., 2000) and can explain the high stimulation of iron reduction through electron shuttling. In contrast to the high stimulation observed when AQDS was amended (even in low concentrations), addition of low concentrations of natural HA (Aldrich HA) did not have a strong effect on iron reduction (chapter 3). Moreover, this might even have a negative effect on reduction rates due to absorption of humics to iron which blocks the mineral for reduction by direct contact (Piepenbrock et al., 2011).

Sulfate reduction cannot be completely excluded as a pathway of respiration in the incubations due to the presence of sulfate in the slurries (3 – 4.5 mM; chapter 3), and the presence of *Desulfobacterales* related spp. in the incubations (chapter 4), although there are known members which can reduce iron as well as electrodes (Holmes et al., 2004a; Holmes et al., 2004b). Iron oxides can be abiotically reduced by sulfide species, which will end up in precipitation of iron sulfide minerals (Canfield, 1989; Thamdrup et al., 1994; Thamdrup and Canfield, 2000). Additionally, DOM and AQDS can be as well abiotically reduced by sulfide, (Canfield et al., 1992; Heitmann and Blodau, 2006; Aranda-Tamaura et al., 2007; Yu et al., 2015; Yu et al., 2016). Nonetheless, accumulation of Fe^{2+} in all treatments (including controls) suggests that free sulfide is limited and iron reduction proceeds as a result of direct bacterial respiration (Canfield, 1989) or from bacterial reduced AQDS. Additionally, sulfide was measured at random points and was never above 3.5 µM (data not shown). Sequencing results of heavy

fractions from incubations with ^{13}C-labeled acetate showed that *Desulfuromonadales* spp. (composed mainly of *Desulfuromonas* spp.; chapter 4) were involved in acetate oxidation. *Desulfuromonadales* spp. are known iron and sulfur reducers which do not reduce sulfate (Webster et al., 2010; Handley et al., 2013). That further supports the assumption that iron was probably reduced through microbial respiration (or through HS respiration) and not as a result from sulfate reduction.

6.2 Identification of humic and iron reducing microorganisms

Populations related to *Desulfuromonadales* spp. were found as the most active acetate oxidizers in Dorum and Helgoland sediment incubations (chapter 4; Fig. 2, 3; Tables 3, 4), despite the fact that this group's relative abundance in the indigenous community is low in both sites, based on 16S sRNA gene sequencing. (2 %- Dorum, 8 %- Helgoland) (Fig. 1, A, B). Additionally, *Desulfuromonadaceae* spp. sequences were found in all highest dilutions of MPN incubations (chapter 3 Table 3). The findings of members from the order *Desulfuromonadales* (*Desulfuromonadaceae* spp. and *Geobacteraceae* spp.), are in agreement with previous enrichments of acetate oxidizers and AQDS reducers (mainly related to *Geobacter* spp.) from rice field soils, sandy aquifers, and fresh water sediments (Coates et al., 1998; Snoeyenbos-West et al., 2000; Zhou et al., 2014). In addition, *Desulfuromonas* spp. were recovered from marine sediments using AQDS as electron acceptor (Coates et al., 1998). This group seems to be important in humic reduction coupled to acetate oxidation in Wadden and North Sea sediments. Other microorganisms (as sulfate reducing, halorespiring and methanogenic microorganisms) can play a role in humic and iron reduction (Cervantes et al., 2002), and not all can oxidize acetate. The use of specific electron donors is an important factor

in shaping microbial communities in incubations (Lentini et al., 2012), and community can be biased towards *Desulfuromonadales* spp., due to the use of acetate (Piepenbrock et al., 2014). Nonetheless, acetate is an ideal substrate for labeling of RNA when the goal is identification of iron (Hori et al., 2010) and humic reducing microorganisms with RNA-SIP approach. In the absence of other electron acceptors, acetate oxidation to CO_2 is thermodynamically possible only via respiration (e.g. coupled to iron and humic reduction) or through syntrophic acetate oxidizing consortia (Thauer et al., 1977; Schink, 1997; Nüsslein et al., 2001).

Despite the apparent importance of *Desulfuromonadales* spp. in acetate oxidation with iron and HS as electron acceptors in North Sea sediment incubations, they were not found to play a major role in sediment incubations from Dominica (chapter 5). This is surprising, as *Desulfuromonadales* spp. were found in similar abundances in fresh sediment from Helgoland (8 %; Fig. 1B) and Dominica (6 %; Fig. 1C). Additionally, *Desulfuromonadales* spp. were found with the highest abundance in fresh sediment from shallow hydrothermal vents near Santorini Greece, as well as after incubations using acetate as electron donor (Handley et al., 2013). As carbon source (acetate) is an important factor in shaping microbial communities in incubations (Lentini et al., 2012; Piepenbrock et al., 2014), I would have expected to find them as main acetate oxidizers in incubations from Dominica as well. The results from sediment incubations from Dominica (chapter 5) points to the archaeal group DHVEG-6 (*Halobacteriales*) as main acetate oxidizers when AQDS was amended and *Deferribacteraceae* without AQDS amendment. It is important to note that only bacterial primers were used for identifying communities in sediment incubations from Wadden and North Sea (chapters 2, 4), whereas general prokaryotic primers were used for identifying communities in sediment incubations from Dominica (chapters 2, 5). Therefore, there is still lack of information

concerning the role of Archaea in humic reduction in Wadden and North Sea sediments. Members of the group DHVEG-6 were found in diverse environments from fresh water to marine and hypersaline mats (Sørensen et al., 2004; Kan et al., 2011; Wemheuer et al., 2012; Carmichael et al., 2013), and as other *Halobacteria* spp. are assumed to actively take part in organic matter degradation (Auguet et al., 2009; Wemheuer et al., 2012). Moreover, *Halobacteriales* spp. were found in enrichments for iron reducers using hypersaline lake sediments (Emmerich et al., 2012). As there are known Archaea which can respire HS (reviewed in: Martinez et al., 2013), it can be assumed that this uncharacterized group plays an important part in humic reduction coupled to acetate oxidation.

The potential to use HS as electron acceptors can also be seen from our MPN estimations, where AQDS reducing microorganisms were estimated to be ~ 50 times more abundant than iron reducing microorganisms. Those differences are also within the range of other estimations from fresh water systems (Kappler et al., 2004; Piepenbrock et al., 2014). All of the estimations had a high range of confidence levels, but as the numbers are closer to the low end, it might be that the actual numbers are higher. In the highest positive MPN tube (6 times 1:10 dilutions from the fresh sediment) incubated with acetate and AQDS, diverse populations were found with high sequence relative abundances (*Bradyrhizobiaceae* spp. 27 %, *Desulfuromonadales* spp. 20 %, and *Alteromonadaceae* spp. 36 %). I suppose that no other electron donors or acceptor from the sediment was carried over in an extent that can be used for growth in that high dilution.

Desulfuromonadales spp. were found to assimilate acetate into RNA as discussed above. Surprisingly, sequences related to *Bradyrhizobiaceae* spp. and

Alteromonadaceae spp. were not found enriched in heavy gradient fractions from incubations with ^{13}C acetate. Members of the *Alteromonadaceae* were found to reduce chelated Fe(III) (Handley et al., 2009). Other members from the order *Alteromonadales* (*Shewanellaceae*) are known HA reducers (Lovley et al., 1996; Lovley, 2013). Other members (*Colwelliaceae*) were found to assimilate acetate using SIP incubations from marine sediments while another member of the family (*Colwellia* sp.) was found in MPN counts for acetate oxidizing manganese reducing bacteria, but not in SIP incubations (Vandieken et al., 2012; Vandieken and Thamdrup, 2013). This can point for a diversity regarding metabolic capabilities of *Alteromonadales* related spp. Species related to *Rhizobiales* were isolated as anthrahydroquinone 2,6, disulphonate (AHQDS; reduced form of AQDS) oxidizers coupled to nitrate reduction. They were also found to oxidize acetate with nitrate (Coates et al., 2002). It was shown that AQDS reducing microorganisms are capable also of oxidizing AHQDS. It is possible that the *Bradyrhizobiaceae* population found in the MPN tube could couple acetate oxidation to AQDS reduction, but not in slurry incubations. AQDS can serve as interspecies electron transfer (Lovley et al., 1999). It can be that it happened in the incubations. Although, in that case it is unclear what served as terminal electron acceptor for AHQDS oxidizing microorganisms.

6.3 Impact of *in-situ* humic substances on electron shuttling in marine sediments

Addition of AQDS as electron shuttle molecule (to a final concentration of 5 µM) affected differently the Fe^{2+} formation rates at the three sites tested. This can be due to differences regarding the type of endogenous HS as it will be explained below. After one week of incubations, AQDS addition resulted in higher Fe^{2+} formation, which is an indication of an average rate of ~ 3.45 folds (Helgoland sediment incubations; North Sea), ~ 1.25 folds (Dominica sediment incubations) and ~ 1.25 folds (Dorum sediment incubation; Wadden Sea). DOM (and therefore HS) in Helgoland surface sediment was considered to be of marine origin (Oni et al., 2015). In contrast, DOM in hydrothermal fluids and surface water in Dominica was found to be mostly of terrestrial origin (Gomez-Saez et al., 2015). Although we do not know the exact composition of DOM in Dorum sediment, we can assume that it is more similar to terrestrial origin due to the proximity to agriculture area and runoffs of OM. This assumption is also supported by an earlier work in which HA from several marine sediments were analyzed based on absorbance ratio of A2/A4 (Fooken and Liebezeit, 2000). Sediments from coastal area had an A2/A4 ratio of 2.4 – 3.1, where ratio of > 3.4 represents terrestrial sample and 1.9 represents marine sample. Wadden Sea sediment had generally a higher ratio than North Sea sediment and was closer to a terrestrial signal (Fooken and Liebezeit, 2000). HS of terrestrial origin is characterized by higher content of aromatic moieties and higher electron uptake capacities (reviewed in: Piepenbrock and Kappler, 2012). Therefore, it is possible that Helgoland sediment is more limited with respect to quinone-like moieties and thus, addition of AQDS had greater effect.

It was estimated that HS in peatlands with an area of 1 km^2 (and water fluctuations of 0.2 m) store ~ $1.52x10^6$ mol equ, which corresponds to a suppression of methanogenesis of $1.9x10^5$ mol CH_4 $(km^2\ yr)^{-1}$ (Klüpfel et al., 2014). Those estimations are higher than expected from marine sediments, due to higher OM content, as well as a different distribution between the dissolved and the solid phases. The Wadden Sea is the biggest tidal flat area in the world with an area of 10,000 km^2 and characterized with semidiurnal tide (reviewed in: Bosselmann, 2007; Rocker et al., 2012). Therefore, HS can potentially have high impact on electron flow in tidal flats, although those estimations need to be adjusted to *in-situ* conditions.

With this work I have shown that dissolved HS (AQDS) enhanced Fe^{2+} formation rates and probably C_{org} oxidation in incubations with marine sediments. Fluxes of Fe^{2+} from marine continental shelf sediments are estimated ~ 72 Gmol yr^{-1}, which corresponds to ~ 48 % of total flux from marine sediments with high impact from dissimilatory iron reduction (Dale et al., 2015). An increase on precipitation and fluvial input will result in increased input of iron oxide and HS to the continental shelf. Therefore, HS can be an important parameter controlling iron fluxes in the future.

Humic acids can stay soluble in seawater, but, due to their nature of complexing metal, they tend to coagulate with increasing salinity (Rashid, 1985), and there is a probability to find HS in marine sediments in a solid phase. Nevertheless, in other systems solid phase HS were found to act as electron shuttle molecules between microorganisms and iron (Roden et al., 2010). Also, solid phase OM (HS) were found to suppress methane production while respiration of C_{org} continued (Keller and Takagi, 2013). Minerals such as pyrite and magnetite were shown to act on extracellular electron transfer (EET) processes (Kato et al., 2012; Malvankar et al., 2015). Therefore,

it is likely that solid phase HS can act as electron acceptors or as electron shuttling molecules in marine sediments as other conductive minerals.

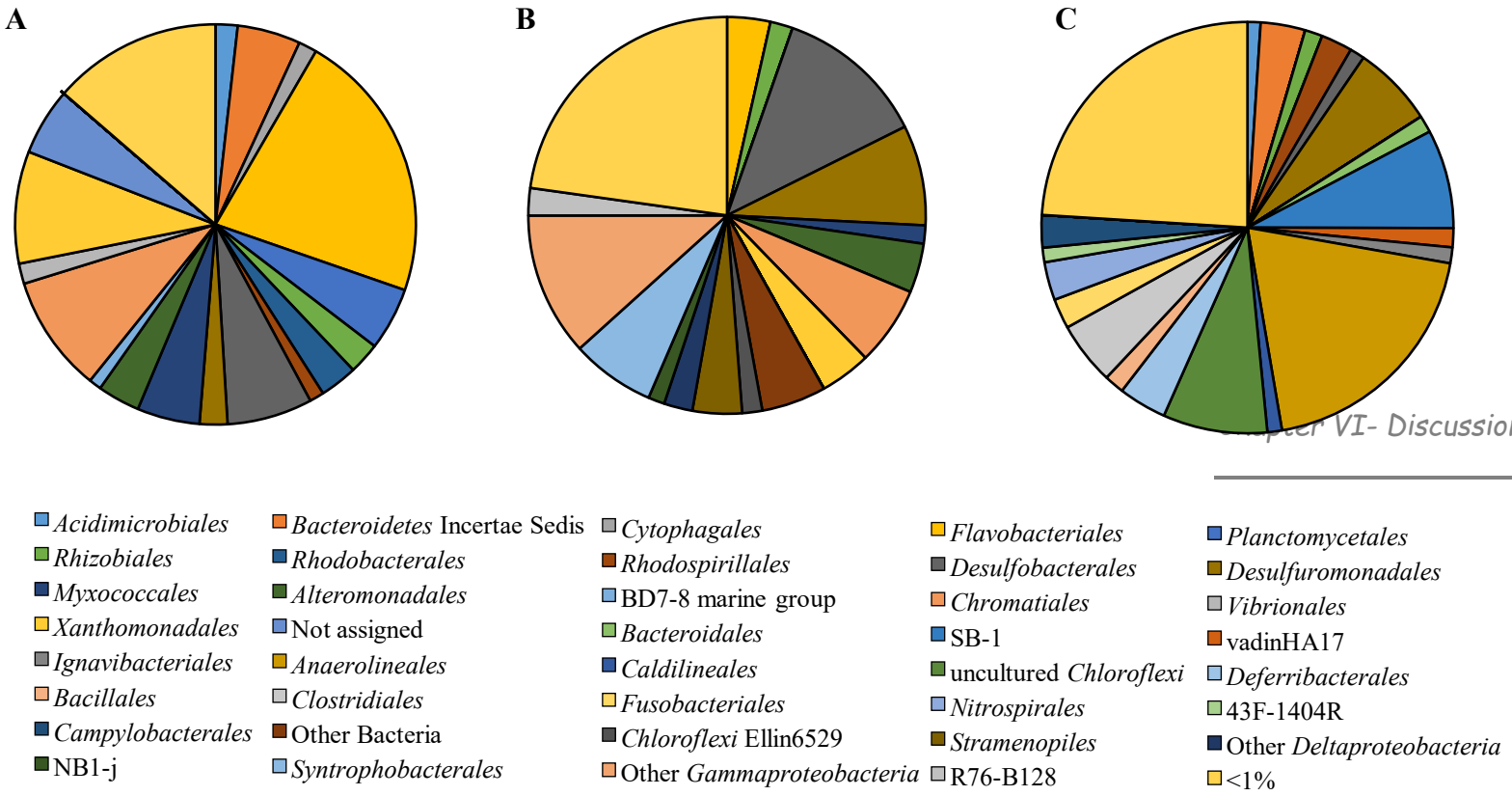

Figure 1. Indigenous microbial population (at order level) from **A**. Dorum, **B**. Helgoland; replotted using data from Oni et al. (2015), and **C**. Dominica.

6.4 Conclusions

My aim in this work was to answer three research questions:

1. Is there a potential for utilizing HS as electron shuttle molecules for iron reduction in marine sediments?
2. Who are the active microorganisms that use HS as electron shuttle/sink in marine sediments?
3. What are the abundances of the active humic and iron reducing populations in marine sediments?

I have shown that marine microorganisms have the ability to use HS as electron acceptor for respiration. Therefore, there is a potential for utilizing HS as electron shuttle molecules in marine sediments. The hypothesis that Fe^{2+} formation rates are higher in HS amended slurry incubations was proven. AQDS, as a HS analog and Aldrich HA stimulated higher Fe^{2+} formation rates in incubations with marine sediments. Moreover, low concentration of AQDS (5 µM) resulted in higher stimulation of Fe^{2+} formation as compared to higher AQDS concentration (50 µM). In contrast, concentration of 1 mM AQDS did not stimulate Fe^{2+} formation. However, as AQDS was found (qualitatively) to be reduced by indication of color changing, it can be concluded that it was reduced, probably coupled to microbial oxidation of C_{org}.

It was found that *Desulfuromonadales* related spp. are the active acetate oxidizers when AQDS is added to sediment incubations from North and Wadden Sea, whereas *Halobacteriales* related spp. are the active acetate oxidizers when AQDS is added to sediment incubations from Dominica. The hypothesis, that oxidation of acetate is mediated by different populations in the absence and presence of HS, was not entirely

correct and seems to be more site dependent. In incubations with Dorum tidal flat sediment, the same population mediated acetate oxidation in the presence and absence of AQDS (as HS analog). It can be concluded that due to the proximity to land, the indigenous acetate oxidizing population is already oriented towards respiration of quinone rich HS, therefore no differences were observed. In incubations with Helgoland sediment, a change in microbial community composition was observed. Thus suggesting that microorganisms capable of HS reduction are a major portion of the total acetate oxidizer community. Finally, addition of AQDS to incubations with sediment from Dominica resulted in differences in microbial populations which can oxidize acetate, suggesting a specificity of the archaeal group DHVEG-6 to couple acetate oxidation to HS reduction.

The third hypothesis, that HS reducing microorganisms are more abundant than iron reducing microorganisms, was proven as well. AQDS reducing microorganisms were found to be ~ 50 times more abundant than iron reducing microorganisms. This result stresses out the high potential of humic respiration in marine sediments. Additionally, it indicates that iron reduction can be an indirect process led by non-iron reducing microorganisms in marine sediments through electron shuttling molecules-HS. Based on this, together with the increase of iron reduction rates, as was shown in slurry incubations, we can conclude that the impact of HS on the iron cycle is probably higher than assumed before.

6.5 Future perspective

The results I have presented in this thesis raised questions that can be answered by future investigation. First, an important aspect would be to check the *in-situ* redox condition of HS, as was shown by Kappler et al. (2004). Quantifying the electron uptake capacity of HS can give indication on the electron flow in the sediment. It could help us to answer if HS are used as electron shuttle or sink and give better estimations of C_{org} mineralization and iron reduction rates. Thus, we could get better understanding of CO_2 emissions from sediments.

During the work, estimations for the activity of HS were done using the HA analog AQDS. In sediments, diverse types of HS with different characteristics and redox potentials, amount of quinone moieties and absorptions to minerals, can be found. Moreover, HS can be found in solid phase compared to AQDS, which is in solution. As a result, incubation experiments can provide higher rates. In order to better understand the role on natural OM in EET and the ability of marine microorganisms to use it, incubation experiments should be done with real HS. As a start, known characterized HA can be used (in addition to Aldrich HA). Secondly, following characterization of natural HS from marine sediments, they should be used in sediment incubations. Incubations can be done in a similar manner to those presented in this study. By combining incubations with different ^{13}C labeled substrates, we get a better understanding of the active communities.

MPN incubations with acetate and AQDS as terminal electron acceptor resulted in interesting observations regarding the microbial community. Populations, which based on RNA-SIP identification are not assimilating acetate, were found in MPN incubations with acetate as sole carbon source and electron donor electron. Additionally, microbial

populations related to AHQDS oxidizers were found in the MPN incubations, even though they did not have any other electron acceptor besides AQDS. It would be interesting to investigate the metabolic capabilities of those populations.

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Acknowledgements

First of all, I would like to thank my supervisor **Prof. Dr. Michael W. Friedrich** for providing me the opportunity to work on this fascinating topic. Your help and guidance for the last years have been invaluable and I am truly grateful for that. Thank you for using your expertise to review my writings and therefore helping me improve them. I have learned a lot from you, I consider myself lucky to have been working with such knowledgeable and experienced scientist.

Dr. Solveig I. Bühring has been an incredible teacher. I thank you first for agreeing to be the second reviewer of this work. I also want to thank you for the invitation to join your sampling campaign to Dominica. It was an incredible adventure scientifically as well as personally. During this expedition, I also had the chance to learn from your knowledge and experience, and ever since I considered you a mentor. I truly hope we can keep working together in the future.

I would like to thank **Prof. Dr. Ulrich Fischer** and **Prof. Dr. Kai Bischof** for being the examiners of my defense.

Prof. Dr. Karl-Heinz Blotevogel, thank you for being part of my PhD committee and always bringing fruitful discussion. I feel like I have shared so much with you. Whether it was for scientifically or personal advice or just to share a good laugh, you always had time for me and I am truly thankful. I will always be grateful for having you around during the difficult times of the PhD, not for nothing do we call you „Grandpa Heinz“. You comforted me in times of stress and like a good grandpa you provided cookies all year long, not to mention the summer beers, and the winter Feuerzangenbowle. Thank you.

Dr. Carolina Reyes, my office and lab mate. You are an amazing scientist and I have learned so much from you, thanks for sharing your knowledge and expertise with me. You are also a very good friend who I truly appreciate. Thanks for all the good times we spent together in the lab/office and in “real life”. Keep smiling, never be ‘cara de limón’, but most important of all don’t forget to obey the ferret.

Ajinkya Kulkarni, thank you for who you are. Don’t forget that “you still need a tray” and no matter what you do “may the force be with you”.

I would like to thank **Dr. Tim Ferdelman** for being in my thesis committee. Also because during the last four years I always felt like I could knock on your door or send you an email any time, and I always got response from you. You are not only a great scientist but also a great teacher and mentor.

Dr. Alban Ramette, thank you for being in my thesis committee and for your help when I was lost in statistics. You are a great scientist, and I hope that in your new job they appreciate you as much as we do here in Bremen.

My colleagues, **Oluwatobi Oni**, **Tim Richter-Heitmann** and **Yin Xiuran,** it was great to work with you for so long. I think we had some mutual helping, discussing and improving going on, at least I feel that you have definitely help with my work in any way you could.

Cedric Hahn, thank you for representing me in the examination committee. Good luck with your scientific career.

Charlotte Wilms, I am thankful for all technical assistance. After some dark times, you brought the light and order to our labs.

Tina Stickan, our amazing secretary. Thank you for helping with all formalities and administrations.

Dr. Tetsuro Miyatake, thank you for teaching and helping me. You always had a smile in your face and you were always willing to help. I will never forget you, and all the people that had the chance to work with you will always remember you. May you rest in peace.

I would like to thank the **International Max Planck Research School for Marine Microbiology (MarMic) and Universität Bremen** for funding of my work.

Martin Nowak and Mechanische Werkstatt, these are the anonymous heroes, without them we could not be doing our science. Thank you.

Dr. Christiane Glöckner, Anita Tingberg and Algrid Hillmer for administrative and moral support. You are the wonder women of the MPI. Thanks for everything.

Bernd Stickfort, the hero behind the books. Thank you for helping me (and all other scientists in the MPI) to find any manuscript and book needed.

Prof. Dr. Friedrich Widdel and Prof. Dr. Jens Harder, I appreciate the time that you took to discuss data with me. Thanks for sharing your knowledge with me.

Edinson Puentes Cala and Oliver Jäckle, my lab rotation students. You both did a great job. Thanks for giving me the chance to teach you something and give a little bit back to the system. I wish you best of luck for your future as scientists.

Besides all the support I had as a scientist, there are so many people that helped me go through the last years. For someone coming from such a warm place like Eilat it is not so easy to get used to cold and rainy Bremen. However, it does help to have good friends like **Judith Zimmermann (Zimmerfrau), Rodrigo Mora Lugo, Lizbeth Sayavedra Camacho, Daniel Meier, Mario Schimak, Adrien Assie, Max and Jenny Rubin Blum and Gonzalo (Gozalito) Gómez Sáez.**

Mina and Danny, how can I thank you enough? It would take the whole length of the thesis and more. But shortly, you are my family, without you I would have never met the love of my life, and I could have not done this 6+ years here without you. Thanks for everything you do. Thanks for **Duan and Eva-Mai**, whom I am lucky to call my family. Thank you.

My parents and family back in Israel- I love you.

אמא, אבא, סבתא בלה, סיון, איציק, דניאל ואדר. איך אתחיל? תודה על שתמיד הייתם שם, תמכתם והאמנתם בי שאצליח. לא הייתי מסוגל לעבור לפה ולסיים את התואר בלעדיכם. אני באמת בר מזל שנולדתי לכזאת משפחה. המרחק מקשה אך תמיד הצלחתם לגרום לי להרגיש שאנחנו ביחד. אני מקווה שגרמתי לכם גאווה רבה. אני אוהב אתכם ומתגעגע מאוד.

To my wife, the love of my life, **Beppy**, you were always there for me. I am nothing without you and everything with you. I cannot imagine doing this without you by my side. Thank you for everything you are. I am yours forever. Te amo mucho.

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