Selective effects of transient oxygen and nitrate exposure on sulfate reducing/fermentative consortia

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الحمد لله الذي هدانا لهذا وما كنّا لِنَهْتَدِيَ لولا أن هدانا الله

Aller Preis gebührt Gott, der uns hierzu rechtgeleitet hat; denn wir hätten gewiß nicht den rechten Pfad gefunden, wenn Gott uns nicht rechtgeleitet hätte.

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

وَاللَّهُ خَلَقَ كُلَّ دَابَّةٍ مِنْ مَاءٍ ۖ فَمِنْهُمْ مَنْ يَمْشِي عَلَىٰ بَطْنِهِ وَمِنْهُمْ مَنْ يَمْشِي عَلَىٰ رِجْلَيْنِ وَمِنْهُمْ مَنْ يَمْشِي عَلَىٰ أَرْبَعٍ ۚ يَخْلُقُ اللَّهُ مَا يَشَاءُ ۚ إِنَّ اللَّهَ عَلَىٰ كُلِّ شَيْءٍ قَدِيرٌ

{النّور 45}

IM NAMEN GOTTES, DES ALLERGNÄDIGSTEN, DES GNADENSPENDERS:

Und Gottt ist es, der alle Tiere aus Wasser erschaffen hat; und (er hat gewollt, daß) unter ihnen solche sind, die auf ihren Bäuchen kriechen, und solche, die auf zwei Beinen gehen, und solche , die auf vieren gehen. Gott erschafft, was Er will: denn, wahrlich, Gott hat die Macht, alles zu wollen.

{an-Nur (Das Licht) 45}

Übersetzung
Mohammad Asad

Abstract

The activity and diversity of prokaryotes is one of the keys to understand element cycling in our environment. Many microbes couple the oxidation of carbon compounds with the reduction of inorganic compounds such as oxygen, nitrogen, manganese, iron and sulfate. The sulfur cycle is one of the most important elements cycles, because of the high abundance of sulfate in the marine environment and the rich speciation of sulfur compounds at different redox states. The most stable and abundant form of sulfur is sulfate which is found in sea water at a high concentration of 28mM. About 50% of the remineralization of organic carbon substrates was suggested to be coupled to sulfate reduction.

Sulfate reducers couple the oxidation of organic carbon compounds or hydrogen with the reduction of sulfate to sulfide. Their ability to oxidize organic carbon compounds is known to be mostly limited to those compounds that are produced by fermentative bacteria. However, only few studies directly address the ecological relationship between fermentative and sulfate reducing bacteria.

This thesis addresses precisely this point. Consortia of sulfate reducing and fermentative bacteria were enriched in long term continuous culture incubations inoculated with biomass extracted form the top sediment layers of the intertidal flat Janssand in the German Wadden Sea. The cultures were provided with a marine medium that contained, in addition to sulfate, seven different amino acids, glucose and acetate in a ratio that mimicked the composition of decaying biomass (50% protein, 30% polymeric sugars, 20% lipids) in terms of its monomers. Chemical and metagenomic analysis were used to analyze the activity and community composition of

the selected consortium. Most cultures were performed under stable, sulfate reducing conditions (Chapter 2). Chapter 3 addresses the effect of transient exposure to oxygen and nitrate on the enrichment of consortia of fermentative/sulfate reducing bacteria.

Under all conditions investigated, the enriched sulfate reducers belonged to the Deltaproteobacteria, dominated by *Desulfovibrio* and *Desulfotignum* populations. The enriched fermentative bacteria were mainly affiliated with Firmicutes, followed by Spirochaetales. The enrichment and phylogenomic characterization of "*Candidatus* Thammenomicrobium ektimisum", a fermentative representative of the candidate division Hyd24-12 is described in Chapter 4.

The results presented in chapters 2 and 3 suggest that hydrogen and acetate were the main fermentation products. Metagenomic, transcriptomic and stoichiometric modeling of microbial metabolism suggested that the sulfate reducers displayed at least partially autotrophic growth by assimilating carbon dioxide, despite the supply of copious carbon sources to the cultures. Transient exposure to oxygen did not result in a strong selective effect, and neither the fermentative nor the sulfate reducing populations showed a strong transcriptional response to exposure to oxygen. Transient availability of nitrate led to the enrichment of a different population of Deltaproteobacteria, affiliated with Desulfuromonadales, in two replicate experiments. This population was apparently incapable of sulfate reduction but performed ammonification of nitrate to ammonia.

Overall, the results presented in this thesis provide new insight in the selective pressure exerted by dynamic environmental conditions on sulfate reducing/fermentative consortia.

Zusammenfassung

Die Aktivität und die Diversität von Mikroorganismen sind mit die wichtigsten Schlüssel um die Umwelt zu verstehen. In Stoffkreisläufen koppeln Bakterien die Oxidation kohlenstoffhaltiger Substanzen an die Reduktion anorganischer Ionen wie Sauerstoff, Nitrat, Magnesium, Eisen und Sulfat und folgen dabei dem Reduktionspotential dieser Substanzen. Der bedeutendste dieser Stoffkreisläufe ist der Schwefelkreislauf, weil in der Umwelt die Schwefelkonzentration hoch ist und Schwefel einen großen Bereich von Reduktionszuständen einnehmen kann. Im marinen Bereich ist Sulfat die stabilste und häufigste Form des Schwefels und erreicht im Meerwasser eine Konzentration von 28mM. Etwa 50% der Remineralisierung kohlenstoffhaltiger Substanzen wird an die bakterielle Sulfatreduktion gekoppelt.

Die bakterielle Sulfatreduktion reduziert Sulfat zu Sulfid durch die Oxidation kohlenstoffhaltiger Substanzen oder Wasserstoff. Häufig wird die Fähigkeit von sulfatreduzierenden Bakterien Kohlenstoff abzubauen durch bakterielle Fermentationsprodukte limitiert. Doch bisher gibt es nur wenige direkte Untersuchungen der ökologischen Beziehung zwischen fermentierenden und sulfatreduzierenden Bakterien.

Im Rahmen dieser Doktorarbeit wurde das Konsortium sulfatreduzierender und fermentierender Bakterien untersucht. Dieses Konsortium wurde in Langzeitinkubationen mit Biomasse, die aus den obersten Sedimentschichten der Gezeitenzone Janssand im deutschen Wattenmeer extrahiert wurde, im Chemostat angereichert. Das Inkubationsmedium bestand aus Meersalz, Sulfat, einer Mischung aus sieben Aminosäuren, Glukose und Acetat, wobei der jeweilige Anteil dem von zerfallender

Biomasse in Bezug auf die Monomere entsprach (50% Eiweiße, 30% Zuckerpolymere, 20% Lipide).

Mittels chemischer Methoden und Metagenomik wurde die Aktivität und Zusammensetzung des Konsortiums analysiert. Die meisten Kulturen wurden unter stabilen sulfatreduzierenden Bedingungen kultiviert und untersucht (Kapitel 2). Der Einfluss den Sauerstoff im Wechsel mit Nitrat auf die sulfatreduzierenden Bakterien ausübte, wurde beobachtet und im Kapitel 3 beschrieben.

In allen Experimenten gehörten die angereicherten sulfatreduzierenden Bakterien zur Klasse der Deltaproteobakterien, wobei *Desulfovibrio* und *Desulfotignum* die Population dominierten. Die angereicherten fermentierenden Bakterien wurden größtenteils den Firmicutes zugeordnet, gefolgt von Spirochaetales. Die Anreicherung und phylogenetische Charakterisierung von "*Candidatus* Thammenomicrobium ektimisum", einem Vertreter der „candidate division Hyd24-12" wurde im Kapitel 4 beschrieben.

Die Ergebnisse, die im Kapitel 2 und 3 vorgestellt werden, legen nahe, dass Wasserstoff und Acetat die Hauptprodukte der Fermentation waren. Metagenomik, Transkriptomik und stöchiometrische Modellierung des mikrobiellen Metabolismus lassen vermuten, dass sulfatreduzierende Bakterien zumindest teils autotrophes Wachstum aufwiesen und zwar durch die Assimilation von Kohlendioxid, auch wenn der Anreicherungskultur Kohlenstoff nur in gebundener Form zugegeben wurde. Die wechselnde Zugabe von Sauerstoff hatte keinen selektiven Prozess zur Folge, und weder die fermentierende noch die sulfatreduzierende Population reagierten transkriptomisch auf die Sauerstoffexposition. Die wechselnde Verfügbarkeit von Nitrat hingegen führte in zwei

Versuchsreplikaten zur Anreicherung einer weiteren Ordnung von Deltaproteobakterien, welche den Desulfuromonadales angehörte. Diese Population kann offensichtlich kein Sulfat reduzieren, dafür Nitrat zu Ammonium ammonifizieren.

Zusammengefasst präsentieren die Ergebnisse dieser Thesis neue Einblicke in den Selektionsdruck, der in dynamischen Umweltbedingungen auf das Konsortium von sulfatreduzierenden und fermentierenden Bakterien einwirkt.

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Chapter 1

General Introduction

Chapter one

1. General Introduction

1.1 Sulfur cycle and the role of sulfate reduction

Sulfur is one of the most abundant elements on the earth; it is represented in nature in its solid phase as (pyrite) FeS_2 or (gypsum) $CaSO_4$ in rocks and sediments and as sulfate in its liquid phase in seawater (Muyzer and Stams, 2008). The reduction and the oxidation of the sulfur species form the sulfur cycle (Rabu*s et al*., 2013). There are several factors which make the sulfur cycle complex; first, the wide range of species at different oxidation states (the most oxidized form is +6 and the most reduced form is -2). Second, sulfur can react both chemically and biologically. The sulfur cycle is linked to the cycles of other elements like carbon and oxygen (Muyzer and Stams, 2008).

An essential part of the sulfur cycle is sulfate reduction. Sulfate is the most thermodynamically stable form of sulfur and is highly abundant in seawater (Henrichs and Reeburgh, 1987; Skyring, 1987; Jørgensen, 1987 ; Widdel, 1988; Muyzer and Stams, 2008; Rabu*s et al*., 2013). Most of the sulfate-reducing bacteria (SRB) have been isolated from aquatic environments (Rabu*s et al*., 2013).

Sulfate-reducing bacteria reduce sulfate (as an electron acceptor) completely to sulfide. This process is coupled to the oxidation of an electron donor which could be hydrogen or organic carbon (Muyzer and Stams, 2008; Rabu*s et al*., 2013). In anoxic environments like sediments and oil brines a significant portion of the sulfide produced by SRB is re-oxidized by abiotic reactions or biologically to sulfur compounds of intermediate oxidation states and eventually sulfate (Zerkl*e et al*., 2009). Chemotrophic and phototrophic sulfur oxidation besides sulfur compound disproportionation form the

biological sulfur oxidation pathways (Zerkl*e et al*., 2009). During sulfate reduction the intermediates of the 8-electron transferring steps from sulfate to sulfide are normally not transferred to the environment (Rabu*s et al*., 2013). Only in two cases, minor concentrations of excreted sulfite or thiosulfate by *Desulfovibrio desulfuricans* were reported (Vainshtein *et al*., 1980 ; Fitz and Cypionka, 1989). In marine sediments, about 50% of the total carbon remineralization was estimated to be performed by SRB (Jørgensen, 1982),. Therefore, the sulfur cycle, especially sulfate reduction, plays an important role in the marine environment.

1.2 Sulfate reducing bacteria and carbon substrates

Based on the ability to degrade organic compounds, SRB are divided into two groups: incomplete degraders which produce acetate as an end-product, and complete degraders which produce carbon dioxide (Muyzer and Stams, 2008; Rabu*s et al*., 2013). Many organic compounds are known to be used as electron donor by SRB including fatty acids, alcohols, amino acids, methylated N- and S- compounds, sugars, and aromatic and hydrocarbon compounds (Rabu*s et al*., 2013). Gittel *et. al*., 2008 showed by direct cultivation with dilute (>a million-fold) intertidal flat sediment that the indigenous SRB used lactate, acetate, pyruvate, formate, fumarate, ethanol and hydrogen. Although SRB are commonly considered to be outcompeted for sugars by faster growing fermentative microorganisms, sugar utilization (e.g. fructose) has been reported for SRB cultures (Klemps *et al.*, 1985). Amino acids are also utilized by SRB especially in marine systems (Rabus *et a*l., 2013). In general, polymeric organic compounds, such as starch, cellulose, proteins, nucleic acids (DNA and RNA) and fats are not found as a direct substrates for

SRB. Thus, in nature, SRB are dependent on other microorganisms which are able to simplify these polymeric substrates in fermentation processes and form products which can be used as substrates by SRB (Muyzer and Stams, 2008) (Fig 1.) However, the consortium of fermentative and SRB has rarely been investigated directly: in the second chapter of this thesis (Manuscript 1) the oxidation of organic matter by a consortium of fermentative and sulfate-reducing bacteria was investigated. These consortia were enriched in long term continuous culture incubations supplied with a marine medium with sulfate, seven different aminoacids, glucose and acetate in a ratio that mimicked the composition of decaying biomass (50% protein, 30% polymeric sugars, 20% lipids) in terms of its monomers. The enrichment was inoculated with a sample from a marine intertidal sediment.

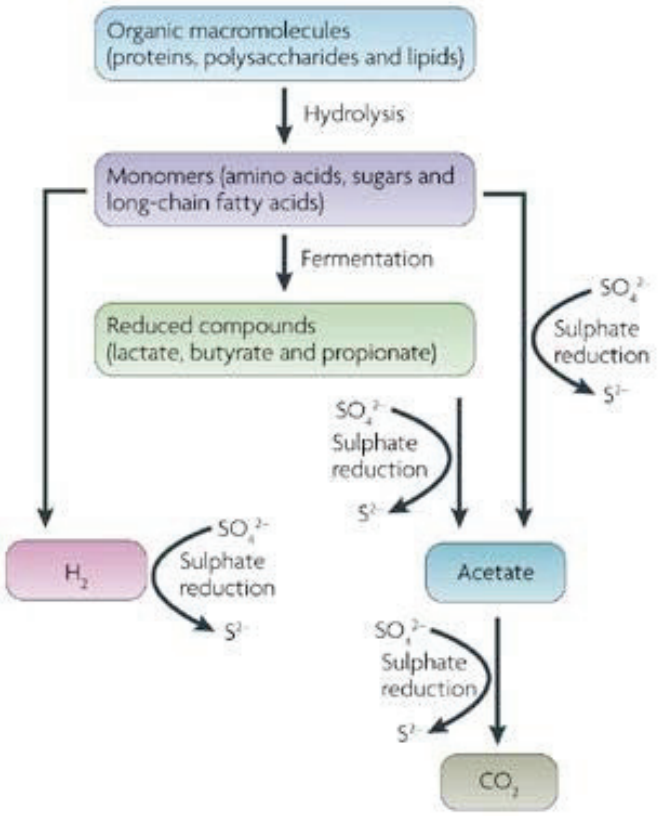

Fig. 1:The position of the sulfate reduction in term of the selection of the carbon substrates. First ; Organic Macromolecules (Proteins, polysaccharides and lipids are hydrolyzed, second ; Monomers (amino acids, sugars and long chains fatty acids) are fermented, third; reduced compounds such as (lactate, propionate and butyrate) are oxidized by the sulfate reducing bacteria. Source : (Muyzer and Stams, 2008).

1.3 Costal areas and Sampling site Janssand

The coastal areas are known to receive high inputs of nutrients from land and rivers which leads to high primary production (Gattuso *et al.*, 1998). Due to that these areas are one of the most active environments in the biosphere (Gattuso *et al.*, 1998) . In comparison with open ocean areas, tidal flat sediments characterized by steep geochemical gradients which may achieve a typical deep microbial communities even at few centimeters depth (Wilms *et al.*, 2006; Gittel *et al.*, 2008). In these areas about 50% of the primary production reached to the top surface and then quickly remineralized by the surface organisms (Wollast, 1991 ; Gittel *et al.*, 2008). Furthermore, tidal flat sediments are also influenced by permanently changing environmental conditions like temperature and light intensity, bioturbation, air exposure, and hydrodynamic features like currents (Gittel, 2007) and are likely to have high biodiversity.

Janssand is an intertidal sand flat in the backbarrier area of the Island of Spiekerog in the German Wadden Sea (Billerbeck *et al.*, 2006). Janssand (13 km^2) (Fig 2) is known to be inundated with ~ 2 m of seawater for (6–8) h during each semidiurnal tidal cycle, beside being exposed to air during the low tide about (6-8) h (Billerbeck *et al.*, 2006; Gao *et al.*, 2009) . About 3-35% of the total mineralization in an intertidal sand flat was attributed to the activity of SRB (Billerbeck *et al.*, 2006) which were present throughout the whole sediment (Llobet-Brossa *et al.*, 2002; Gittel *et al.*, 2008). Nevertheless, the activity of the SRB is found at its highest level in the sediment layers directly beneath the maximum depth of oxygen penetration indicating a clear correlation with the presence of oxygen (Gittel, 2007). A description of SRB in Janssand intertidal flats is mentioned in the introduction of the second chapter (Manuscript 1).

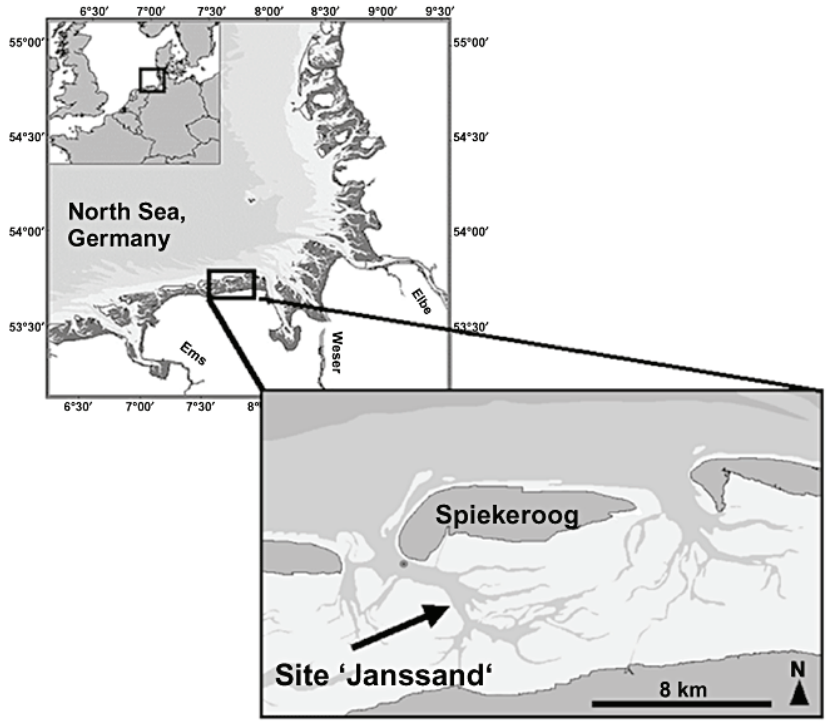

Fig. 2: The location of the sampling site Janssand in the German Wadden Sea. Source (Gittel *et al.*, 2008).

1.4 *The order of the sulfate reduction in the the Redox tower*

Sulfate-reducing bacteria are obligatory anaerobic bacteria (Muyzer and Stams, 2008). Fenchel and Jørgensen, 1977 introduced the concept of the redox cascade of the terminal electron acceptors, which explains the order of the electron acceptors used by the microbes coupled to the oxidation of the organic matter. It was based on the observation that the activity of the SRB occurs in the deeper sediment layers after the depletion of O_2, manganese oxides, nitrate, iron oxides (Fenchel and Jørgensen, 1977; Froelich *et al.*, 1979; Schulz and Zabel, 2000). Nevertheless, SRB were also found through in all layers of the sediments , also those exposed to oxygen (Llobet-Bross*a et al*., 2002; Gittel *et al*., 2008).

. The tolerance and the survival of anaerobic bacteria like SRB under the presence of oxygen is ecologically relevant and biochemically interesting topic (Rabus *et al.*, 2013). It can help to understand different microbial responses that take place in nature like the exposure of anaerobic microorganisms to oxygen in intertidal flats and the turning of the oxic environments to anoxic in soils or oligotrophic zones due to flooding or eutrophication (Rabus *et al.*, 2013) . The understanding of the effect of oxygen on the anaerobic bacteria include if it is an outcompeting player or if it has a harmful or toxic affects on the cells (Rabus *et al.*, 2013).

Sulfate reducing bacteria showed different reactions and strategies to the presence of oxygen: some showed growth even if the sulfate was absent, they apparently avoid the contact with oxygen by using thiosulfate obtained from the oxidation of the sulfidic media by the oxygen from the oxic head space (Widdel, 1980; Cypionka *et al.*, 1985; Rabus *et al.*, 2013). This mediating sulfur cycle can help to reduce the harm effect of the oxygen by using it in oxidation of sulfide which will be produced again by the reduction of thiosulfate (Rabus *et al.*, 2013). Such a mediating cycle might also present in the interaction oxic – anoxic zone with the presence of electron donors (Rabus *et al.*, 2013). Some cases proved that SRB can also perform the direct utilization of Oxygen coupled with the oxidation of Hydrogen in order to enable significant proton translocation (Dilling and Cypionka, 1990; Dannenberg *et al.*, 1992) , nevertheless no aerobic growth of SRB been observed (Rabus *et al.*, 2013). The mechanism of the utilization of O_2 is not fully understood (Rabus *et al.*, 2013).

Another electron acceptor that can compete with sulfate is nitrate. Nitrate has a higher redox potential and presents SRB with the same potential problems as oxygen. It was found that some SRB species like *Desulfovibrio* (Keith and Herbert, 1983; McCready *et al*., 1983; Mitchell *et al*., 1986; Seitz and Cypionka, 1986) , *Desulfobulbus propionicus* (Widdel and Pfennig, 1982), and *Desulfobacterium catecholicum* (Szewzyk and Pfennig, 1987; Moura *et al*., 1997) showed nitrate reducing activities (Rabus *et al*., 2013). The nitrate reduction activity of *Desulfovibrio desulfuricans* was also found to be repressed by sulfide (Dalsgaard and Bak, 1994; Rabus *et al*., 2013). The final product of nitrate reduction by SRB was found to be ammonium and not nitrogen (Rabus *et al*., 2013).

The third chapter of this thesis is investigating the effect of periodic oxygen and nitrogen cycling on the SRB. Three different conditions were investigated: one with tidal exposure to oxygen, one with tidal exposure to nitrate and one without tidal cycling. Replicate cultures were performed for each condition.

1.5 the phylogeny of the sulfate reducing bacteria

(Muyzer and Stams, 2008) reviewed the phylogeny of SRB. They have been grouped into seven lineages based on comparative analysis of the 16S rRNA (Fig. 3). The phylogenetic tree showed that five of these lineages are within Bacteria and two are affiliated to Archaea. Deltaproteobacteria include most of characterized SRB. While three genera *Desulfotomaculum*, *Desulfosporosinus* and *Desulfosporomusa* are Gram-positive and are affiliated with Clostridiales. The remaining three bacterial lineages are affiliated with Nitrospirae (*Thermodesulfovibrio* genus), Thermodesulfobacteria

(*Thermodesulfobacterium* genus) and Thermodesulfobiaceae (*Thermodesulfobium* genus) (Mori *et al.*, 2003) which are thermophilic. Sulfate reducing prokaryotes within the Archaea, belong to the genus *Archaeoglobus* in the Euryarchaeota, and to the genera *Thermocladium* (Itoh *et al.*, 1998) and *Caldirvirga* (Itoh *et al.*, 1999) in the Crenarchaeota. An up to date overview of the phylogeny of sulfate and sulfur reducers is provided by Rabus et al (2013).

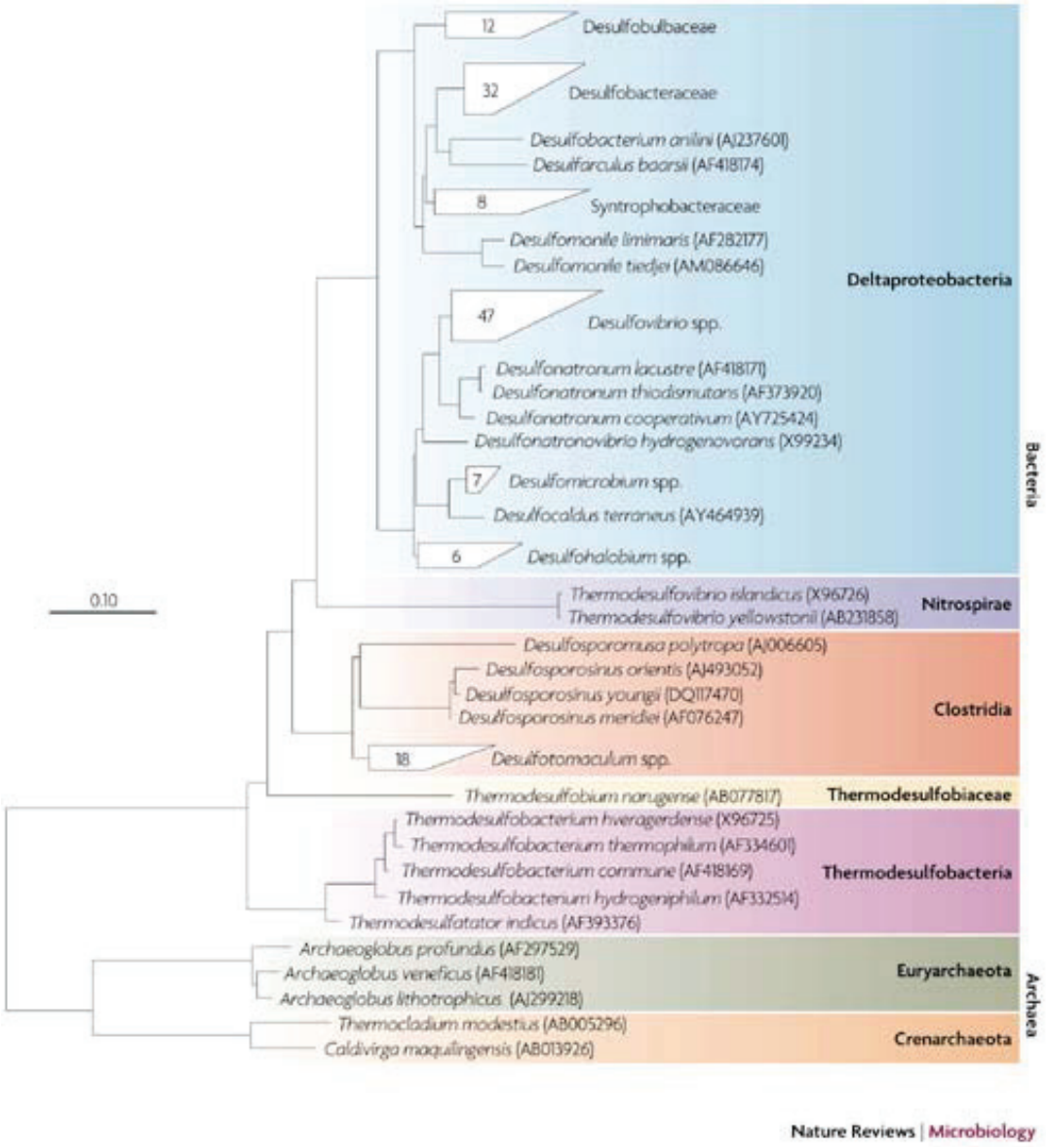

Figure 3 Phylogentic tree showing the seven lineage of sulfate reducing bacteria. calculated with ARB software (Ludwig *et al.*, 2004) using sequences, which were obtained from the SILVA (Pruesse *et al.*, 2007) small subunit (SSU) rRNA database (version 03 08 22). The number within the collapsed clusters refers to the number of different species within a particular group. The scale bar represents 10% sequence difference. Source : (Muyzer and Stams, 2008)

1.6 The role of metagenomics and transcriptomics in this study

For studying the diversity and the metabolic potential of environmental microbes, metagenomics has become the prevalent approach (Teeling and Gloeckner, 2012). In metagenomics, phylogenetic or functional marker genes such as 16S rRNA and dsrAB are often used. The work described in this thesis makes use of shotgun metagenomics, followed by assembly and binning of the assembled contigs. Contigs were binned based on a compositional feature (tetranucleotide frequencies) (Strous *et al.*, 2012). Compared to the marker gene approach, this has the advantage that a more or less complete provisional whole genome sequence is generated for each of the populations. This way, complete insight can be gained on what substrates are used by each population, especially if also transcriptomic information is acquired as was done in chapters 3 and 4 .

Chapter 4 describes the enrichment of the first representative of a candidate phylum known as Hyd24-12 in the SILVA taxonomy (Quas*t et al*., 2013). This phylum- level clade has been detected previously in methane-rich anoxic environments (Quast *et al.*, 2013). The clade Hyd24-12 so far lacks a cultured representative and its metabolic capabilities and ecological niche remained mysterious. . In chapter 4 we present the Hyd24-12 selected in a sulfate reducing continuous culture inoculated with sediments from the Jansand tidal flat. The metagenomics approaches yielded a provisional whole genome sequence as well as transcripts providing some insights in its metabolism.

2. Aims and Objectives

This thesis is divided into three chapters, in which we use continuous culture (chemostat) incubations to investigate the selective effect of environmental conditions on SRB/fermenter consortia.

The chapters are;

1. **Enrichment of a consortium of uncultured Clostridiales and a *Desulfovibrio* sp. from a marine tidal flat converting glucose, amino acids, and acetate in sulfate-reducing conditions;** the aim of this chapter is to investigate the oxidation of organic matter by a consortium of fermentative and sulfate-reducing bacteria.

2. **Selective effects of transient oxygen and nitrate exposure on sulfate reducing/fermentative consortia;** the aim of this chapter is to study the effect of tidal cycling with oxygen and nitrate on sulfate reduction activity of microbial communities from the surface layer of an intertidal flat (Janssand in German Wadden Sea).

3. **Hyd24-12: Insights into a novel bacterial phylum with widespread occurrence in anaerobic, organic-rich ecosystems;** the aim of this chapter is to characterize a microbial SRB/fermenter consortium that included a representative of the novel phylum Hyd24-12.

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Chapter 2

Manuscript 1

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Enrichment of a consortium of uncultured Clostridiales and a *Desulfovibrio* sp. from a marine tidal flat converting glucose, amino acids, and acetate in sulfate-reducing conditions

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Contribution: The structure of the experimental approach, was laid out by M.S. and J.S.G. The set up of the chemostat, the experimental procedure, the chemical analysis in addition to the DNA extraction were carried out by Z.A.B. The extraction of RNA and the Genomic library were done by H.E.T. S.E.R. provided the phylogenic tree. M.S. provided the metagenomic analysis. The manuscript was written by all co-authors.

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Enrichment of a consortium of uncultured Clostridiales and a *Desulfovibrio* sp. from a marine tidal flat converting glucose, amino acids, and acetate in sulfate-reducing conditions

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Summary

This study investigates the spontaneous assembly of microbial consortia responsible for degradation of monomers of decaying biomass in marine sulfate-reducing conditions. A microbial community extracted from the upper 2 cm of a marine intertidal flat in the German Wadden Sea was enriched with a mixture of glucose, amino acids and acetate in two parallel 100-day continuous culture incubations. Metagenomics analysis supported by fluorescence in situ hybridisation, showed that consortia of Clostridiales and sulfate-reducing bacteria (10 - 14% of microbial community) formed in both cultures. Binning of assembled contigs from 454 sequencing reads showed the presence of two bins representing uncultured Clostridiales populations and a population related to *Desulfovibrio* for culture 1. In culture 2, a fourth bin was assigned to *Desulfobacter* (2% of microbial community). Gene analysis indicated potential for glucose utilization in the two Clostridiales bins, with different glucose uptake mechanisms. Genes encoding degradation of different amino acids were present in the Clostridiales bins and in the Desulfovibrio bin. The bins for sulfate reducers indicated the capacity to use fermentation products like lactate, ethanol, formate and hydrogen. Stoichiometric modelling of microbial metabolism showed that the ratio Clostridiales : sulfate-reducing bacteria could be explained by exchange of low-energy fermentation products.

Introduction

In marine sediments, the biogeochemical sulfur cycle is very important. In a study with marine sediments from 0-200 m water depth it has been estimated that approximately 50% of the total organic carbon mineralization resulted from the activity of sulfate-reducing bacteria

(Jørgensen, 1982). Recently, the physiology of sulfate-reducing bacteria has been described in an extensive review (Rabus *et al.*, 2013). Sulfate-reducing bacteria use sulfate as the terminal electron acceptor and reduce it completely to sulfide using molecular hydrogen or organic compounds as electron donor. Commonly, sulfate reducers are divided into two groups based on their ability to degrade organic compounds: incomplete degraders that produce acetate as an end-product, and complete degraders that produce carbon dioxide (Muyzer and Stams, 2008; Rabus *et al.*, 2013). The organic compounds that are known to be used as electron donor by sulfate-reducing bacteria include fatty acids, alcohols, amino acids, methylated N- and S- compounds, sugars, and aromatic and hydrocarbon compounds (Rabus *et al.*, 2013). Direct cultivation with dilute (>one million-fold) intertidal flat sediment samples showed that the indigenous sulfate reducers used lactate, acetate, pyruvate, formate, fumarate, ethanol, and hydrogen (Gittel *et al.*, 2008). The use of amino acids as electron donor for growth has been observed mostly for marine strains (Rabus *et al.*, 2013). Sugar utilization has been reported for some sulfate-reducing bacteria, which showed the ability to utilize fructose (Klemps *et al.*, 1985). Nevertheless, sulfate-reducing bacteria that use sugars are commonly considered to be outcompeted by faster growing fermentative microorganisms (Rabus *et al.*, 2013).

The current understanding of the physiology of sulfate-reducing bacteria suggests that for the degradation of organic matter, a syntrophic consortium of fermentative and sulfate-reducing bacteria is required. However, this concept has rarely been investigated directly: Studies that address sulfate reduction generally provide one or more of the abovementioned substrates as electron donor/carbon source and most studies that address fermentative microorganisms do not provide sulfate as electron acceptor.

In the present study we address the oxidation of organic matter by a consortium of fermentative and sulfate-reducing bacteria. These consortia were enriched in long-term continuous culture incubations supplied with a marine medium with sulfate that contained

seven different aminoacids, glucose and acetate in a ratio that represent the composition of decaying biomass (50% protein, 30% polymeric sugars, 20% lipids) in terms of its monomers. The enrichment was inoculated with a sample from a marine intertidal sediment. Marine intertidal sediments are important for organic matter degradation because of their large surface area and high microbial activity (Jansen *et al.*, 2009).

Coastal ecosystems are considered as one of the most geochemically and biologically active areas in the biosphere, as they receive high inputs of nutrients from land and rivers resulting in high primary production (Gattuso *et al.*, 1998). A large fraction of primary production will subsequently decompose into dissolved organic matter (Williams, 1981), which is taken up by bacteria (Azam and Malfatti, 2007). Tidal flats are influenced by permanently changing environmental conditions like temperature and light intensity, bioturbation, air exposure, and hydrodynamic features like currents (Gittel, 2007) and are likely to have high biodiversity.

About 3-35% of the total mineralization in an intertidal sand flat was attributed to sulfate reduction (Billerbeck *et al.*, 2006) and sulfate-reducing bacteria were present throughout the whole sediment (Llobet-Brossa *et al.*, 2002; Gittel *et al.*, 2008). Using catalyzed reporter deposition - fluorescence in situ hybridization (CARD-FISH), relatives of the *Desulfosarcina, Desulfobacteraceae*, *Desulfobulbaceaea* and *Desulfovibro* were detected in surface layers (0-5 cm) of muddy and sandy sediments (Llobet-Brossa *et al.*, 2002; Mußmann *et al.*, 2005a; Mußmann *et al.*, 2005b; Gittel *et al.*, 2008). In addition, fosmids obtained from Wadden Sea sediment containing clusters of genes involved in dissimilatory sulfate reduction suggest the presence of a yet unidentified sulfate-reducing clade (Mußmann *et al.*, 2005b). In deeper tidal flat sediments, cultivation studies of sulfate-reducing bacteria showed the presence of Deltaproteobacteria and also *Desulfosporosinus* and *Desulfotomaculum* species within the *Firmicutes* (Gittel *et al.*, 2008). Recent 16S rRNA gene pyrotag surveys of sandy coastal

sediments including our sampling site confirmed that *Desulfobacteraceae* related to the *Desulfosarcina/Desulfococcus* group account for the majority of SRB in this sediment type, whereas sulfate-reducing bacteria related to the *Desulfovibrionaceae* and *Firmicutes* were hardly detectable (Marc Mußmann, personal communication).

In the present study, consortia of fermentative and sulfate-reducing bacteria were enriched in long-term (100 day) continuous cultivation incubations supplied with monomers of decaying biomass in a marine medium, to observe what type of consortia would spontaneously assemble from a tidal flat sediment inoculum. Continuous cultivation enabled enrichment at stable and low substrate concentrations and by sparging with argon also the sulfide concentration was maintained at a relatively low level.

Experimental Procedures

Sampling site and inoculum for enrichment experiments

Sediment was sampled from the intertidal back-barrier flat Janssand in the German Wadden Sea (53.73515 ’N, 007.69913’E) in March, 2011. From the upper part of the flat, the top 2 cm of sandy sediment was collected with a flat trowel. After transport of the sediment to the laboratory, an equal volume of artificial seawater (Red Sea Salt, 33.4 g/l; http://www.redseafish.com) was added to the sediment and stirred vigorously. The sediment was allowed to settle briefly, after which the liquid was transferred into 1 l glass bottles that were closed with rubber stoppers and of which the headspace was exchanged with argon. The liquid was kept at 4°C for 6-7 days and then used as inoculum.

Continuous culture setup and medium

Two continuous culture experiments were started following the same procedure, on two consecutive days. A glass vessel was filled with 2.6 l inoculum. The headspace (3.2 l) was kept anoxic by a continuous supply of argon (10 ml min^{-1}) using a mass flow controller (Alicat Scientific). The liquid was continuously mixed by pumping the gas phase (1.2 l min^{-1}) through a sintered glass membrane in the bottom of the chemostat. The off-gas was bubbled through a water lock to create a small overpressure. The oxidation-reduction potential (ORP) and pH were monitored using electrodes (Mettler-Toledo) inserted into the liquid. The pH of the reactor liquid was kept at 7.8 by addition of 1 M HCl. A detailed description of the setup is provided by (Hanke *et al.*, 2014).

Medium was provided and effluent removed at a rate of approx. 0.9-1 l day^{-1}, resulting in a dilution rate of 0.34-0.4 day^{-1}. The medium consisted of Red Sea Salt artificial seawater (33.4 g l^{-1}) which contains 28 mM sulfate, amended with in total 20 mM organic C. The organic carbon consisted of 1.1 mM D-glucose, 1.7 mM acetic acid and a mixture of amino acids (mM, L-glutamic acid 0.38, L-aspartic acid 0.65, L-alanine 0.85, L-serine 0.46, L-tyrosine 0.099, L-histidine 0.035, L-methionine 0.088). In addition, 0.2 mM Na-phosphate, 17 µM FeSO4, 0.2 ml l^{-1} trace element solution (mg.l^{-1}: $ZnCl_2$ 69, $MnCl_2.4\ H_2O$ 100, H_3BO_3 60, $CoCl_2.6\ H_2O$ 120, $CuCl_2.2\ H_2O$ 10, $NiCl_2.6\ H_2O$ 25, $Na_2MoO_4.2\ H_2O$ 25, $AlCl_3.6\ H_2O$ 25 in 0.1% HCl) and 0.2 ml l^{-1} Se/W solution (mg l^{-1}: $Na_2SeO_3.5\ H_2O$ 6, $Na_2WO_4.2\ H_2O$ 8 in 0.04% NaOH) were added. The pH of the medium was adjusted to pH 4 using HCl.

Subsamples from the culture liquid were taken for analysis of reduced sulfur compounds, short chain fatty acids, and biomass. For sulfide, samples were fixed with 5% Zn-acetate solution and stored until analysis. For thiosulfate, Zn-acetate fixed culture samples were centrifuged and the liquid kept for analysis. For sulfur and cell protein analysis, 4-6 ml of

culture sample was centrifuged, the pellet washed with 1 ml 0.5 M NaCl and stored at -20°C until analysis. Supernatant was stored at -20°C for analysis of glucose and short chain fatty acids. In addition, the release of sulfide to the gas phase was measured by leading the off-gas through 10 ml of 5% Zn-acetate solution and sampling this solution after 5 to 30 minutes.

Analytical methods

Sulfide fixed with Zn was measured colorimetrically according to Cline (1969). The thiosulfate concentration in the culture liquid was determined using the method by Kelly (1969). Elemental sulfur was extracted from washed pellets of culture liquid using acetone and continuous mixing for 16-20 hrs. Sulfur in acetone was analyzed by cyanolysis and colorimetric detection (Sörbo, 1957). The remaining cell pellets were washed with 0.5 M NaCl and incubated in 1 M NaOH at 46°C for 30 min, after which the protein concentration was measured according to Lowry *et al.* (1951).

Glucose and short chain fatty acids were separated with HPLC system (Sykam GmbH) using 5 mM H_2SO_4 as eluent at a flow rate of 0.6 ml min^{-1} and quantified using refractive index or absorbance at 210 nm.

Catalyzed reporter deposition - fluorescence in situ hybridization (CARD-FISH)

Culture samples were fixed with 2% formaldehyde at room temperature for 1.5 h or overnight at 4°C, washed 3 times with PBS and stored in PBS:ethanol 1:1 at -20°C until use. A subsample was diluted in PBS, sonicated and filtered over 0.2 µm pore-sized polycarbonate filters. CARD-FISH was carried out according to Pernthaler *et al.* (2002) at a hybridization temperature of 46°C and using the probes listed in Table S3.

DNA extraction and metagenomic sequencing

For both continuous culture incubations, on day 87, cell biomass of 10 ml culture liquid was collected by centrifugation and stored at -20°C until extraction. DNA was extracted according to (Zhou *et al.*, 1996) following incubation in extraction buffer with lysozyme (2.5 mg ml^{-1}), RNAse (0.1 mg ml^{-1}) and mutanolysin (100 U ml^{-1}) at 37°C for 30 min. Per sample, 500 ng of purified DNA was used for the preparation of sequencing libraries according to the "Rapid Library Preparation Method Manual" (October 2009/Rev. January 2010) provided by Roche. A GS FLX Titanium sequencing run was performed with each of the libraries loaded on half a sequencing picotiterplate.

In silico computational procedures

Assembly of the 454 sequencing reads was done using the Newbler assembler (version 2.6) with the default assembly settings for genomic DNA. Assembled contigs were binned based on tetranucleotide compositions combined with interpolated Markov models (IMMs) with the Metawatt binner (Strous *et al.*, 2012); briefly, four bins were created based on IMMs trained with tetranucleotide bins "Culture-2_low_bin_0", "Culture-2_low_bin_1", "Culture-2_medium_bin_8" and "Culture-1_low_bin_1". Per contig sequencing coverage was estimated by mapping the reads to the assembled contigs with bowtie2 (Langmead and Salzberg, 2012) and coverage and bin size were used to estimate the abundance of each binned population. Transfer-RNAs were identified with Aragorn (Laslett and Canback, 2004). Genome completeness was estimated for each bin by representation of 139 conserved genes as described by (Campbell *et al.*, 2013). The contigs of each bin were annotated separately

with Prokka (http://vicbioinformatics.com) and RAST (http://rast.nmpdr.org). Full length 16S rRNA gene sequences were obtained by searching the assembled contigs with a custom hidden Markov model (Eddy, 2011) trained with representative 16S rRNA gene sequences from the SILVA database (Quast *et al.*, 2013).

Data submission

The 454 sequencing data sets and the assembled contigs are available as metagenome sequencing project in the NCBI database (http://www.ncbi.nlm.nih.gov/bioproject) under BioProject PRJNA246767; BioSample SAMN02769580 for culture 1 and BioSample SAMN02769581 for culture 2. The raw reads were submitted to the Sequence Read Archive (SRA) database (http://www.ncbi.nlm.nih.gov/Traces/sra), with accession numbers SRX541001 for culture 1 and SRX541002 for culture 2. Assembled contigs are available as whole genome shotgun project in the DDBJ/EMBL/GenBank databases under the accession numbers JMSU00000000 for culture 1 and JMSV00000000 for culture 2. The versions described in this paper are JMSU01000000 and JMSV01000000.

Phylogenetic tree calculation

Phylogenetic affiliation was analyzed using the software package ARB (Ludwig *et al.*, 2004) based on the non-redundant SILVA small subunit reference database release 115 (July 2013) (Quast *et al.*, 2013). 16S rRNA sequences were screened for chimeras using the software Mallard (Ashelford *et al.*, 2006), they were aligned using SINA (Pruesse *et al.*, 2012) and the alignment was manually optimized according to the secondary structure. The 16S rRNA phylogenetic tree was calculated using a maximum-likelihood algorithm (PHYML), a

positional variability filter excluding highly variable regions and 100 bootstraps. For tree calculation, only nearly full-length sequences (>1300 bases) were considered.

Stoichiometric modelling

Stoichiometric modelling of microbial metabolism was performed according to Rodríguez *et al.* (2008). Briefly, the biomass yield of fermentation was estimated with the substrates provided to the chemostats and (a) hydrogen, (b) acetate, (c) butyrate and (d) lactate as products. The biomass yields of sulfate reduction were estimated with (a) the substrates provided to the chemostats, (b) hydrogen, (c) acetate, (d) butyrate and (e) lactate as the substrates. For calculation of Gibbs free energies of reaction, a temperature of 20°C was used and a concentration of 200 μM was used for all organic carbon compounds. A sensitivity analysis was performed for the hydrogen concentration (between 10 and 1000 μM).

Results and Discussion

Two continuous culture incubations were inoculated with microbial biomass extracted from the upper 2 cm of a marine intertidal flat. Fresh marine medium containing sulfate, glucose, seven different amino acids and acetate was continuously supplied and removed at a dilution rate of 0.4 per day. Argon gas was also continuously supplied and the cultures were mixed by recycling the argon gas from the headspace, to keep the sulfide concentration in the culture as low as possible. The presence of sulfide was detected after 7 days, and in both continuous cultures the production of sulfide was very similar (Fig. 1 and Fig. S1). The concentration of produced sulfide in the culture liquid increased steadily to approximately 3.4 mM after 50 days. Quantification of sulfide in the gas leaving the culture showed that

approximately 1.7 mmol sulfide day^{-1} escaped to the gas phase. The total sulfide production was 5.2 mmol day^{-1} for the period from day 50 to 96. Other sulfur species were also measured; thiosulfate concentrations in the culture liquid were below 20 μM and the concentration of elemental sulfur ranged from 15 to 40 μM.

< Fig. 1 >

The organic substrates that were supplied were completely converted. During the first 30 days of the culture, acetate was present at a concentration of approximately 4 mM, but after day 38 no acetate was detected (Fig. S2). Fermentation products were detected in the culture liquid, like formate, succinate and butyrate. Concentrations of these compounds were in the range of 0.1 mM for formate and succinate, whereas for butyrate concentrations up to 1.6 mM were observed.

The biomass concentration was assayed as protein present in the culture liquid. From day 18 onwards, the protein concentration was approximately 27 mg l^{-1} (Fig. S3). Assuming a protein content of the biomass of 45%, this equals a biomass production of about 2.5 mmol C l^{-1}.

After 87 days of incubation, samples were taken from both cultures for metagenomic analysis. 454- sequencing yielded 257 Mb of raw sequencing data (average read length 360 nt) for culture 1 and 280 Mb of raw sequencing data (average read length 399 nt) for culture 2, that were assembled into 1364 contigs (N50=53 kb) for culture 1 and 6265 contigs (N50=4.4 kb) for culture 2 (Table S1). The contigs were binned using multivariate statistics of tetranucleotide frequencies followed by Interpolated Markov Modelling of four bins that represented the four most abundant populations detected in the metagenomes of both cultures. Two bins (A and B) were associated with populations affiliated with the order Clostridiales, and two bins were associated with populations affiliated with the class Deltaproteobacteria. Bin C represented a population affiliated with *Desulfovibrio*, and bin D a population affiliated

with the genus *Desulfobacter*. All sequence data was binned into these 4 bins; Figure S4 shows the taxonomic distribution of best blast hits for each of the four bins.

< Table 1 >

The properties of the bins, bin size, sequencing coverage and detection of conserved single copy genes, were calculated for the two cultures (Table 1). From the bin size and the sequencing coverage, the relative abundance of the population associated with each bin was estimated. Based on the metagenome, in culture 1, the microbial community was dominated by Clostridiales bin A and B (together 85.9% of the community) and by Desulfovibrio (bin C; 14%). Based on the large size of bin A (13.4 Mb) and the presence of a duplicated set of conserved single copy genes, this bin contained sequence data of at least two different populations, both affiliated with the Clostridiales. Bin C (Desulfovibrio*)* appears to contain little redundant information, with a genome size of 4.3 Mb and an almost perfect set of conserved single copy genes. The genome of the close relative *Desulfovibrio salexigens* is 4.3 Mb large and contains 89 tRNAs (http://biocyc.org/DSAL52622) compared to 108 tRNAs in bin C. In culture 1, bin D (Desulfobacter) was almost undetectable. Culture 2 was dominated by two populations of Clostridiales (bin B, 73% and bin A, 13%). The sulfate-reducing populations consisted of Desulfovibrio (bin C, 12%) and Desulfobacter (bin D, 2%). In this case, bins A and B each appeared to represent single populations, whereas bin size and the number of conserved single copy genes for bin C indicated some redundancy (Table 1).

< Fig. 2 >

From the metagenomes, four 16S rRNA sequences (>1419 bp) were assembled into contigs (Table S2). Three sequences were affiliated with the order Clostridiales (Fig. 2). Two of these were detected in culture 1, represented bin A, and clustered among the *Lachnospiraceae* and *Clostridiaceae*. The third sequence was detected in culture 2, represented bin B, and belonged to the *Christensenellaceae.* Close relatives of the bin A 16S

rRNA sequences included clone sequences of uncultured bacteria from (disseased) coral tissue (99-98%) (Sekar *et al.*, 2008; Bayer *et al.*, 2013). Cultured relatives included an isolate from hypersaline sediment (98%), *Vallitalea pronyensis* and *V. guaymasensis* (97%) (Lakhal *et al.*, 2013) and *Clostridium* species (≤96%). The 16S rRNA gene sequence representing bin B grouped in a cluster with uncultured clones from the intestinal tract of a sea urchin (96%), coastal environments (≤95%) and from enrichment cultures with contaminants (≤92%). The closest cultured relatives to bin B, with 88% 16S rRNA gene sequence identity, were *Catabacter hongkongensis* and *Christensenella minuta*, which were isolated from human blood cultures and human faeces respectively (Lau *et al.*, 2007; Morotomi *et al.*, 2012). The fourth 16S rRNA gene sequence that was obtained represented bin D (Desulfobacter). This sequence had highest sequence identity (95%) with an uncultured clone from marine sediment and with *Desulfobacter hydrogenophilus*, isolated from anaerobic marine sediment (Widdel, 1987). An additional 16S rRNA sequence, for Desulfovibrio bin C, was assembled manually from partial 16S sequences present in contigs from both cultures. The closest relative of the population of bin C was *Desulfovibrio salexigens*, with 98% 16S rRNA gene sequence identity.

< Fig. 3 >

Fluorescence in situ hybridization (CARD-FISH) microscopy was used to validate the relative abundances of Clostridiales and sulfate-reducing Deltaproteobacteria as inferred from the metagenomes. Small and also very large cells hybridized with Delta495a-c probe (Fig. 3 a,c). The small cells may belong to bin C (Desulfovibrio) whereas the large cells might represent the low abundance of Desulfobacter bin D. Cells hybridizing with probe CLO864 in culture 1 were mostly quite long straight rods (Fig. 3b), whereas in culture 2 mostly very long, thin cells hybridised with probe CLO864 (Fig. 3d). Cells reacted with probe CLO864 did not hybridise uniformly. Microscopic observation indicated that Deltaproteobacteria

accounted for approximately 10% of the overall microbial community and Clostridiales for the remainder.These results were consistent with the metagenomic estimates (14% and 86% respectively). It is likely that in the cultures the Clostridiales populations fermented the supplied glucose and amino acids, while the sulfate-reducing bacteria used the fermentation products as electron donor for the reduction of sulfate. To get more insight into the metabolic interactions of fermenters and sulfate-reducers we investigated the metabolic potential of the bins.

Clostridiales bins A and B both encoded an Emden-Meyerhof-Parnas pathway for the conversion of glucose-6-phosphate to pyruvate, with only one enzymatic step not detected in bin A. The two populations appeared to use different strategies for glucose uptake: Bin A contained genes that encoded a multiple sugar ABC transporter, and a gene putatively encoding hexokinase which catalyzes the conversion of glucose to glucose-6-phosphate. In bin B, a phosphotransferase system was encoded for the uptake of glucose and conversion to glucose-6-phosphate.

The two Clostridiales bins also provided evidence for metabolism of the amino acids supplied with the medium. Amino acid ABC transporters were encoded in both bins, and genes involved in amino acid degradation could be detected for aspartate, glutamate, serine and histidine in bin A, and for aspartate, glutamate, serine and alanine in bin B. Fumarate, 2-oxoglutarate and oxaloacetate were identified as possible intermediates or end-products of the pathways encoded. Aspartate transaminase putatively catalyzed the conversion of aspartate with 2-oxoglutarate to glutamate and oxaloacetate. Glutamate metabolism may have proceeded via deamination to 2-oxoglutarate (bin A), or via N-acetyl-L-glutamate and the urea cycle to fumarate (bin B). In both bin A and B, a putative serine ammonia lyase was encoded for the deamination of serine to pyruvate. Serine could also be converted to glycine, which may subsequently have been decarboxylated using the glycine cleavage system. In bin

A, the capacity for conversion of histidine to glutamate was detected. However, the genes coding for the four enzymatic steps involved in this conversion (Kaminskas *et al.*, 1970) were absent in bin B.

For the processing of pyruvate from glycolysis to the end-products of fermentation, genes encoding enzymes for the production of a range of different compounds were present in bin A. These include production of lactate (lactate dehydrogenase), formate (pyruvate formate lyase), and hydrogen (Fe-hydrogenase and NAD^+ reducing NiFe hydrogenase) and possibly also acetate and ethanol (pyruvate decarboxylase not detected) as well as butyrate (genes coding for 1 out of 6 enzymatic steps not detected). The closest cultured relatives of bin A are *Vallitalea pronyensis* and *V. guaymasensis*, for which the end-products of glucose fermentation were acetate, hydrogen and CO_2, as well as ethanol for *V. pronyensis* (Lakhal *et al.*, 2013; Ben Aissa *et al.*, 2014).

In bin B, genes encoding metabolic pathways were present for the production of acetoin (acetolactate decarboxylase and acetolactate synthase), lactate (lactate dehydrogenase), formate (pyruvate formate lyase), hydrogen (Fe-hydrogenase and NAD^+ reducing NiFe hydrogenase) and possibly ethanol (alcohol dehydrogenase; pyruvate decarboxylase not detected).

Bin C (Desulfovibrio) contained a range of genes putatively involved in energy metabolism that are very similar compared to the well-studied species *Desulfovibrio vulgaris* (Heidelberg *et al.*, 2004) . These include genes required for the utilization of lactate (lactate permease and lactate dehydrogenase FMN dependent), pyruvate (pyruvate:ferredoxin oxidoreductase), formate (formate dehydrogenase), ethanol (alcohol dehydrogenase) and hydrogen. Genes putatively coding for periplasmic Fe-hydrogenase, NiFe hydrogenase and NiFeSe hydrogenase were present, as well as genes coding for a membrane bound energy conserving (Ech) hydrogenase. In addition, putative genes that code for carbon monoxide

dehydrogenase (CooS) and a carbon monoxide sensing transcriptional regulator (CooA) were detected, suggesting there may also be a hydrogenase that uses electrons produced from carbon monoxide oxidation (Coo-hydrogenase). Studies with *Desulfovibrio vulgaris* have shown that the different hydrogenases have different affinity and activity for hydrogen (Fauque *et al.*, 1988; Valente *et al.*, 2005; Van Haaster *et al.*, 2005), and that expression of the hydrogenases depends on the availability of trace metals (Fe, Ni, Se) (Valente *et al.*, 2006). Moreover, hydrogenase gene transcription responded strongly to the electron donor provided (Caffrey *et al.*, 2007; Pereira *et al.*, 2008). During growth on lactate or pyruvate, reducing equivalents released in the oxidation to acetate may be cycled via hydrogen, formate or carbon monoxide (Caffrey *et al.*, 2007; Pereira *et al.*, 2008).

In bin C, genes were also present that code for amino acid uptake using ABC transport and amino acid metabolism of aspartate, glutamate, alanine and serine. Aspartate could have been metabolised by aspartate ammonia lyase to pyruvate or converted by aspartate transaminase with 2-oxoglutarate to glutamate and oxaloacetate. Genes were detected putatively coding for alanine aminotransferase for conversion of alanine with 2-oxoglutarate to pyruvate and glutamate, glutamate dehydrogenase catalyzing the deamination of glutamate to 2-oxoglutarate, and serine ammonia lyase for conversion of serine to pyruvate. Serine conversion to glycine and the glycine cleavage system were also putatively encoded in bin C. Several different species of *Desulfovibrio* have been described in the literature that are able to use single amino acids as electron donor for sulfate reduction or as substrate for fermentation. The amino acids that were used by the different *Desulfovibrio* species are alanine, serine, glycine, aspartate, cysteine, methionine, threonine, leucine, isoleucine and valine (Stams *et al.*, 1985; van der Maarel *et al.*, 1996; Baena *et al.*, 1998; Takii *et al.*, 2008).

Bin C (Desulfovibrio) contained genes involved in sulfate reduction to sulfide. Genes were detected that putatively encode sulfate adenylyltransferase (*sat*), adenylyl-sulfate

reductase (*aprAB*) and dissimilatory sulfite reduction (*dsrAB*), and well as subunits of the membrane-bound complexes DsrMKJOP, Hmc, Rnf and Qmo.

The Desulfobacter population was represented by a small, incomplete bin (1.3 Mb) with short contigs (N50=1055 nt; binned contigs for culture 2; Table 1). In this bin genes putatively coding for adenylyl-sulfate reductase (*aprAB*) were detected. Also genes were detected that are required for the utilization of formate, ethanol and hydrogen, as well as acetate, such as genes coding for acetyl coenzymeA synthetase and multiple steps of the citric acid cycle. The related pure culture *Desulfobacter hydrogenoformans* grows on acetate, pyruvate and ethanol and also autotrophically with H_2 and CO_2 (Widdel, 1987). Acetate was degraded by this species via the citric acid cycle (Schauder *et al.*, 1987).

The annotation of the genes encoded by each of the four bins was consistent with a fermentative role for the Clostridiales populations coupled to sulfate reduction by the deltaproteobacterial populations. However, it remained unclear which substrates were used as electron donor for sulfate reduction. To get more insight in the nature of the fermentation products that might have been exchanged between the Clostridiales populations and the sulfate reducers we performed stoichiometric modelling of microbial metabolism. In this approach, the biomass yield (mol biomass produced/mol substrate consumed) is predicted with a generalized equation for the energy dissipation that occurs during microbial anabolism (Rodríguez *et al.*, 2008). Basically, the energy dissipated during cell growth is a function of the substrates used. Less energy is dissipated with high-quality substrates such as glucose and amino acids, because the cells need to perform less work to grow with these substrates, and more energy is dissipated with low-quality substrates such as carbon dioxide, acetate or formate. In the context of the present study, if the Clostridiales populations would “degrade” all the high- quality substrates provided in the medium into lower quality products like lactate or butyrate, this would lead to a lower biomass yield for the sulfate reducers and a lower

relative abundance for these organisms. Because the total amount of biomass produced was measured experimentally and the relative abundance of both ecological guilds was determined by metagenomic analysis and FISH, stoichiometric modelling might constrain the possibilities as to what fermentation products were exchanged.

< Fig. 4 >

Three different scenarios were explored by modelling: (a) direct use of the provided high-quality substrates (glucose and amino acids) by the sulfate reducers (no fermentation); (b) exchange of lactate and or butyrate between the fermentative and the sulfate-reducing populations; (c) exchange of acetate, formate and/or hydrogen (complete fermentation). For all scenarios the overall biomass yield and the relative abundances of fermentative and sulfate-reducing populations were estimated (Fig. 4). It appeared that the experimental results were best explained by the scenario with complete fermentation (exchange of acetate, formate or hydrogen).

It can be argued that stoichiometric modelling does not provide direct information about the use of fermentation products by the sulfate reducers and that the metagenomic analysis also supports a role for other products of mixed acid fermentation (e.g. lactate). However, the metagenomic analysis only provides information about the capabilities of the different populations. Even with transcriptomic or proteomic evidence it would be difficult to draw firm conclusions. In the future, the use of isotopically labelled substrates combined with metabolic approaches could provide more direct evidence. With the available data, stoichiometric modelling constrained the possibilities as best as possible and suggested an important role for acetate, formate and hydrogen.

Utilization of formate and hydrogen by the sulfate-reducing bacteria is supported by the metagenomic evidence. However, the fate of acetate that was supplied to the cultures or may have been produced by the Clostridiales is not clear. In culture 2, the Desulfobacter

population may have used part of the acetate as electron donor. In both cultures, it is highly likely that acetate was used as C-source for growth. However, the amount of acetate supplied exceeded the observed C-biomass production. Perhaps, acetate may have been transformed into other fermentation products (e.g. butyrate) by the Clostridiales populations.

The 100-day incubation with stable and substrate-limited conditions did not lead to the enrichment of sulfate-reducing populations that were abundant on the tidal flat (Mußmann *et al.*, 2005a; Marc Mußmann, personal communication). A possible explanation is that the dilution rate was too high, leading to a selection for bacteria with a doubling time of at most 1.7 days. It is also possible that the intense contact of the culture liquid with the argon gas, which was implemented to remove sulfide from the culture, also created an effective sink for hydrogen (H_2). This may have "pulled" the fermentative pathways further towards completion and may have prevented the formation of effective consortia that exchanged more valuable substrates (such as lactate, butyrate). Indeed the amount of sulfide produced (5.2 mmol/day) was lower than expected based on stoichiometric modelling (approximately 8 mmol/day). Loss of hydrogen via the gas may explain this difference.

In conclusion, we applied continuous culture cultivation to enrich consortia of sulfate reducing and fermentative bacteria. Stable and substrate-limiting conditions were maintained for 100 days. During this time, the microbial community grew continuously at a relatively long doubling time of 1.7 days. Metagenomic sequencing showed that the culture was dominated by a consortium of different Clostridiales and a Desulfovibrio population. Relative abundances of the two consortium members were confirmed with CARD-FISH and stoichiometric modelling suggested that acetate, formate and hydrogen were the main intermediates that were exchanged. Even though the enriched populations were not abundant in situ, future studies might modify the approach to enable the enrichment of presently uncultured fermentative or sulfate-reducing populations.

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Table 1. Properties of four binned populations present in the metagenomes of sulfate-reducing cultures 1 and 2.

bin	A	B	C	D
Taxonomic assignment	Clostridiales	Clostridiales	Desulfovibrio	Desulfobacter
Culture 1				
Bin size (Mb)	13.4	0.4	4.3	0.03
Contigs	420	87	821	36
N50 length (kb)	76.7	15.4	12.0	0.7
GC (%)	33.8±4.6	30.6±5.2	43.9±3.9	52.8±7.3
Sequencing coverage (×)	14.9	15.8	7.9	4.7
tRNAs	135	2	108	0
Conserved single copy genes[a]	259/139 (125)	4/139 (0)	134/139 (5)	0/139 (0)
Abundance (%)	83.1	2.8	14.0	0.1
Culture 2				
Bin size (Mb)	4.3	4.0	4.8	1.3
Contigs	1623	475	2832	1335
N50 length (kb)	4.2	48.9	2.7	1.1
GC (%)	35.1±2.5	33.5±3.4	44.2±4.2	48.6±4.3
Sequencing coverage (×)	7.2	43.5	5.9	3.9
tRNAs	30	36	108	15
Conserved single copy genes	136/139 (10)	136/139 (12)	162/139 (42)	28/139 (1)
Abundance (%)	13.1	72.8	12.0	2.1

[a]Detection of a set of 139 conserved single copy genes. The number of genes detected multiple times (indicating the presence of multiple genomes in the bin) is indicated in parentheses.

Figure captions

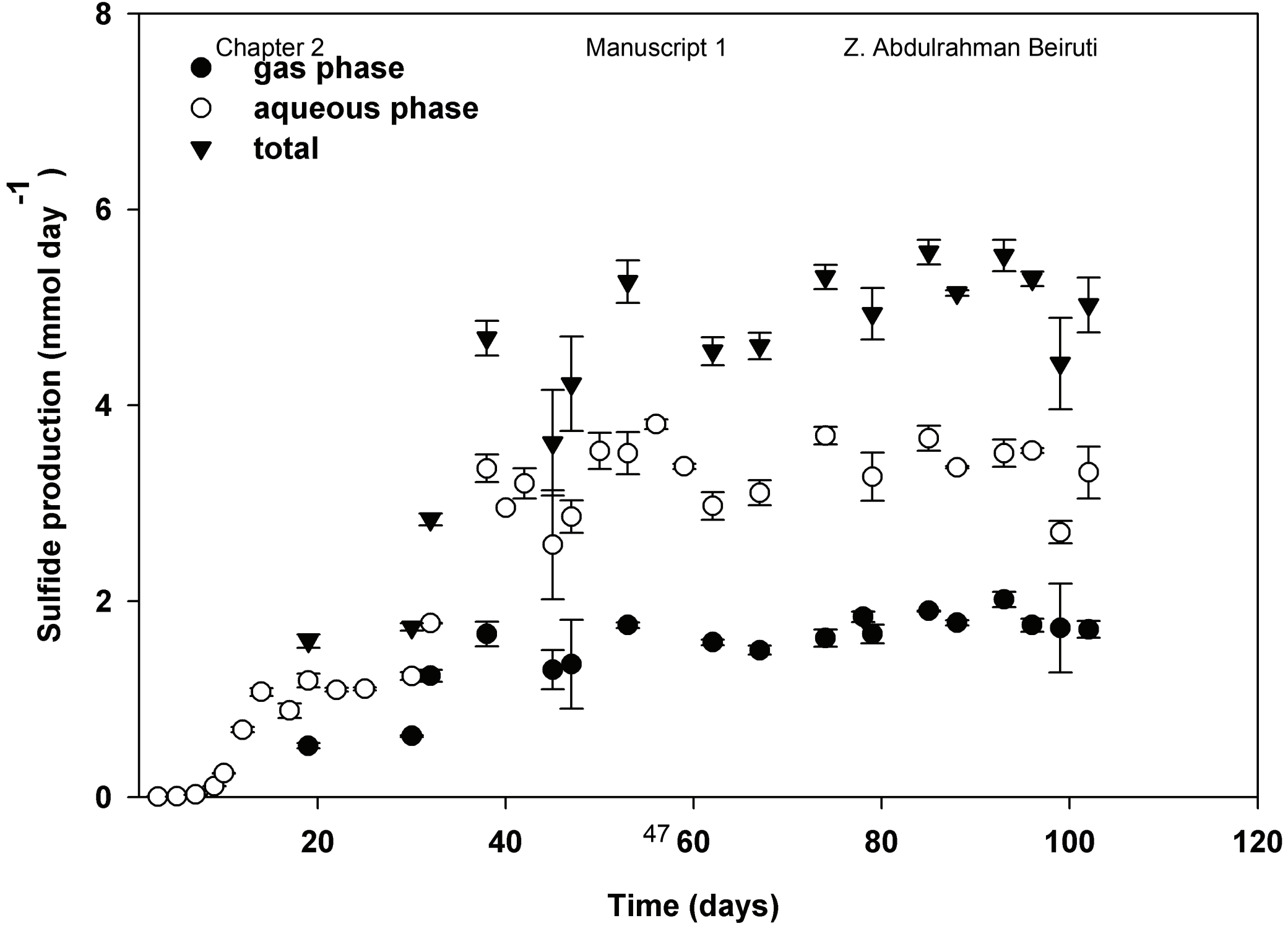
Fig. 1. Total sulfide production in culture 1. Sulfide production was calculated from the sulfide concentration in the culture liquid and from sulfide that escaped to the gas phase and was trapped with zinc acetate.

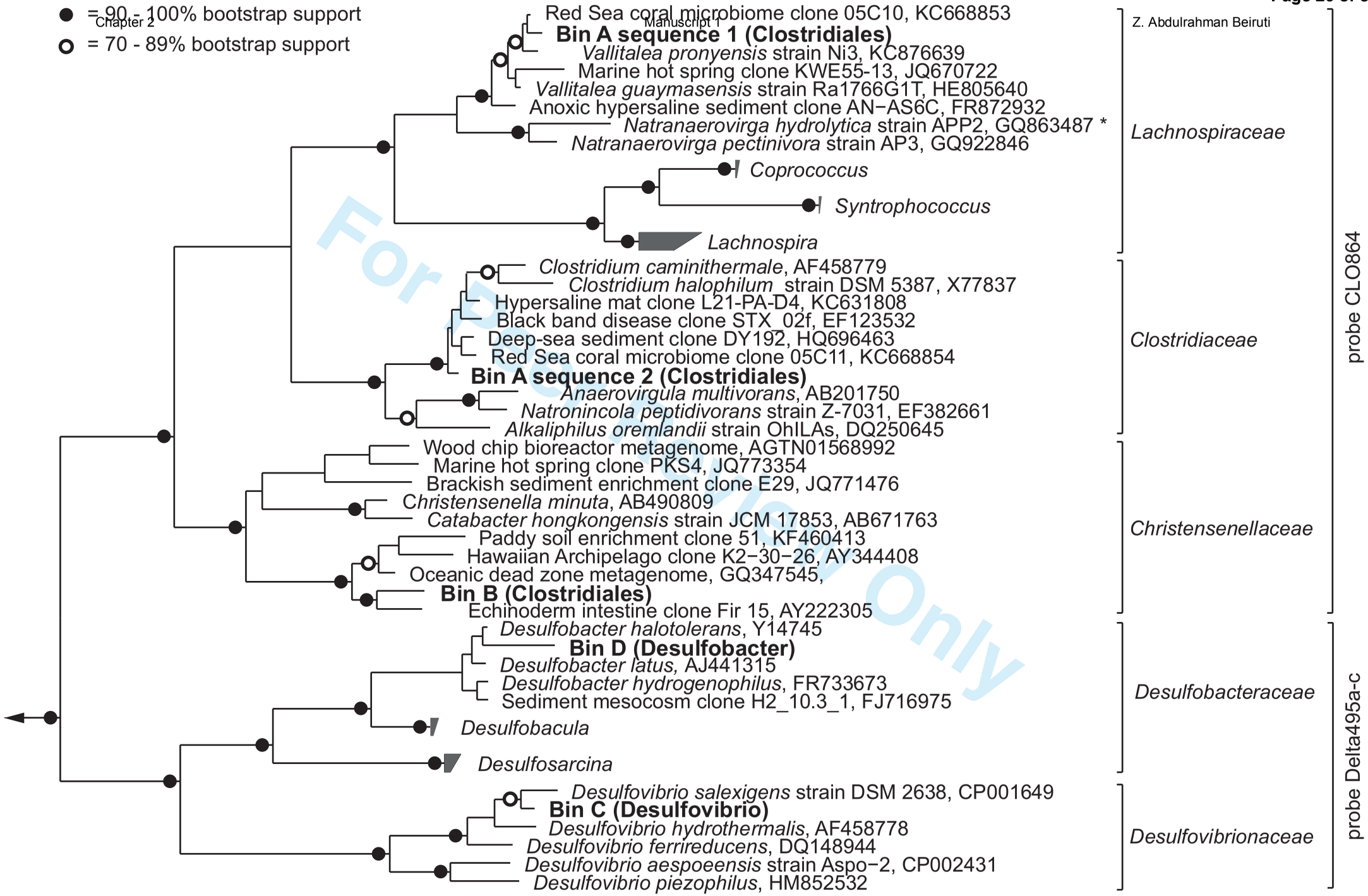
Fig. 2. Phylogenetic affiliation of the bins based on 16S rRNA gene sequences. The phylogenetic tree was calculated using nearly full-length sequences (>1300 bases), a maximum likelihood algorithm and 100 replications. Taxonomic classification was based on ARB-SILVA. The indicated oligonucleotide probes match perfectly to all respective organisms, except for one case marked by an asterisk. Here the probe sequence has one mismatch to the target sequence. The scale bar represents 10% estimated sequence divergence.

Fig. 3. Epifluorescence microscopy images (CARD-FISH) of the microbial consortia. Images are for culture 1 (a,b) and culture 2 (c,d). Green: cells that hybridize with probe Delta495a-c (a,c), and with probe CLO864 (b,d). Blue: DAPI- stained cells. The scale bar corresponds to 2 μm.

Fig. 4. Biomass production predicted by stoichiometric modelling of microbial metabolism. Biomass production in total and by sulfate-reducing microorganisms only is shown. Calculations were made for three scenarios: (left) direct utilization of substrates by sulfate-reducing microorganisms, (middle) fermentation of high quality substrates (glucose and amino acids) to lactate and butyrate that are used as substrates for sulfate reducers, (right)

complete fermentation and exchange of H_2, formate and acetate with sulfate-reducing bacteria.


Chapter 2
Manuscript 1
Z. Abdulrahman Beiruti
gas phase
aqueous phase
total
Sulfide production (mmol day-1)
Time (days)
0
2
4
6
8
20
40
60
80
100
120



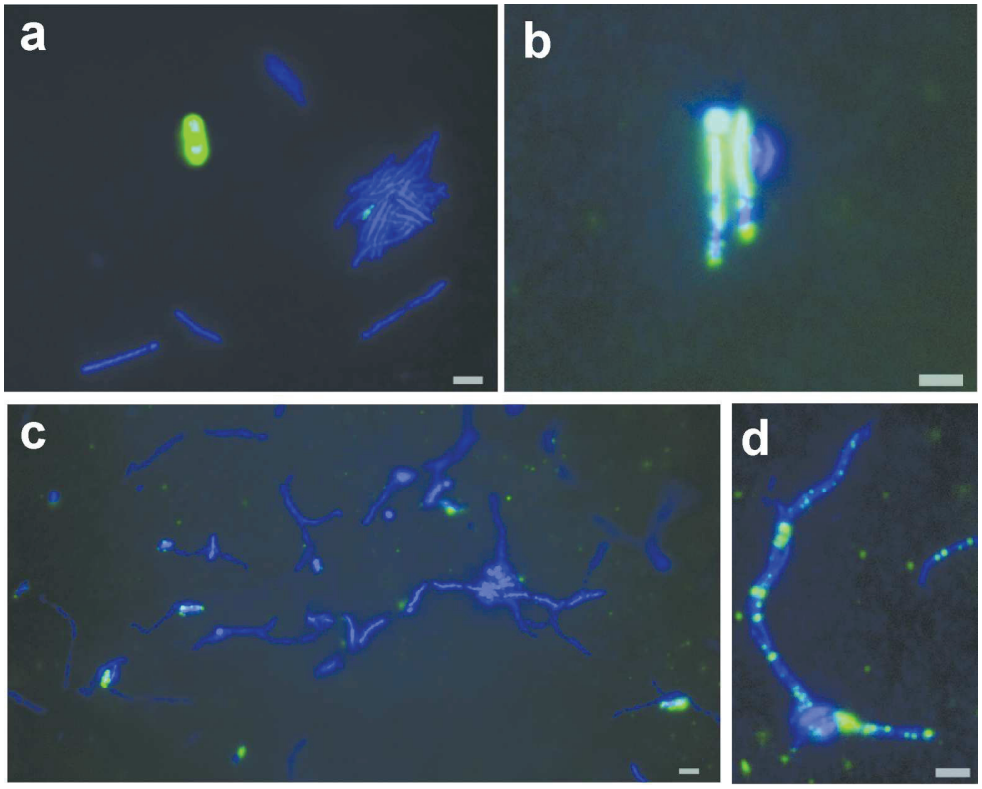
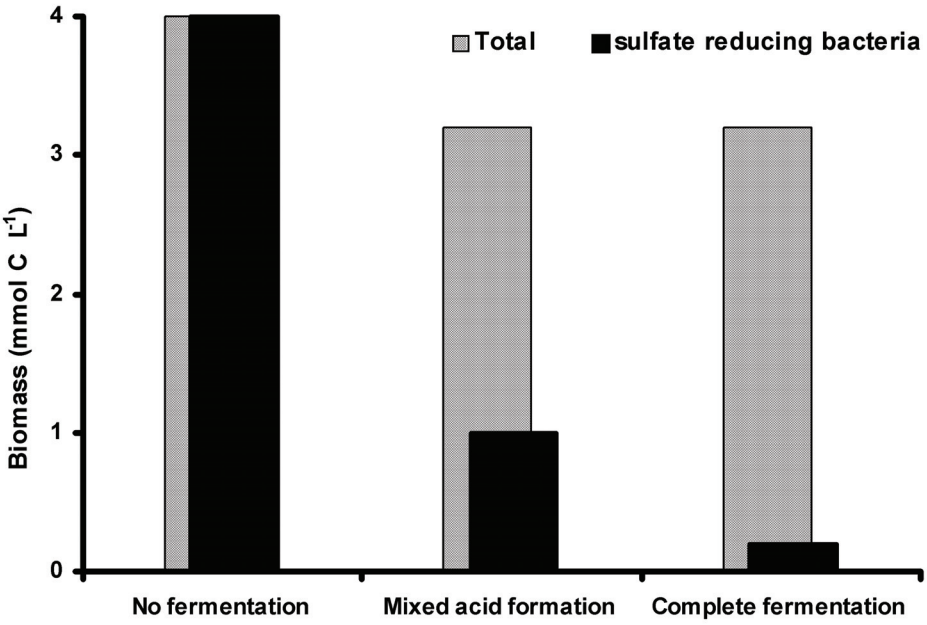


Fig. 3. Epifluorescence microscopy images (CARD-FISH) of the microbial consortia. Images are for culture 1 (a,b) and culture 2 (c,d). Green: cells that hybridize with probe Delta495a-c (a,c), and with probe CLO864 (b,d). Blue: DAPI- stained cells. The scale bar corresponds to 2 µm.
164x131mm (300 x 300 DPI)


Total
sulfate reducing bacteria
Biomass (mmol C L-1)
4
3
2
1
0
No fermentation
Mixed acid formation
Complete fermentation

Supporting Information

Enrichment of a consortium of uncultured Clostridiales and a *Desulfovibrio* sp. from a marine tidal flat converting glucose, amino acids, and acetate in sulfate-reducing conditions

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Table S1. Sequencing and assembly data for metagenomes from cultures 1 and 2.

	Culture 1	Culture 2
Sequence data in reads (Mb)	257	280
Number of reads	714521	702296
Assemble contigs	1364	6265
Total assembled data (Mb)	18.2	14.5
Longest contig (kb)	257.9	144.7
N50 length (kb)	53.3	4.4
Sequencing coverage (×)	13.3	16.5

Table S2. Phylogenetic affiliation of retrieved 16S rRNA gene sequences

Bin	size (bp)	Closest relative using blastn	Accession number	SeqID (%)	Origin
A	1419	Uncultured bacterium clone 05C10	KC668853.1	99	Coral *Stylophora pistillata*, Red Sea, Israel
		Vallitalea pronyensis, DSM 25904^T	KC876639.1	97	Marine alkaline hydrothermal chimney, New Caledonia
A	1423	*Clostridium* sp. L21-PA-D4	KC631808.1	98	Hypersaline microbial mat, Kiribati
B	1484	Uncultured Gram-positive bacterium clone Fir 15	AY222305.2	96	Intestinal Caecum of *Echinocardium cordatum*
		Catabacter hongkongensis, JCM 17853	AB671763.1	88	Human blood
C	1556	*Desulfovibrio salexigens*, DSM 2638^T	NR_102801.1	98	Mud, British Guyana.
D	1558	Uncultured bacterium clone H2_10.3_1	FJ716975.1	95	Marine sediment, Cullercoats, U.K.
		Desulfobacter hydrogenophilus, DSM 3380^T	FR733673.1	95	Marine mud, Venice, Italy

Table S3. Probes used for CARD-FISH identification of different phylogenetic groups.

Name	Probe sequence (5' – 3')	Targeted phylogenetic group	FA[a] (%)	Reference
NON338	ACTCCTACGGGAGGCAGC	negative control	35	(Wallner *et al.*, 1993)
EUB338-I[b] EUB338-II EUB338-III	GCTGCCTCCCGTAGGAGT GCAGCCACCCGTAGGTGT GCTGCCACCCGTAGGTGT	most Bacteria Planctomycetales Verrucomicrobiales	35	(Amann, 1990 ; Daims *et al.*, 1999)
Delta495a[c] Delta495b Delta495c	AGTTAGCCGGTGCTTCCT AGTTAGCCGGCGCTTCCT AATTAGCCGGTGCTTCCT	most Deltaproteobacteria	25	(Loy *et al.*, 2002; Lücker *et al.*, 2007)
CLO864	TTCCTCCTAATATCTACGCA	members of the orders Clostridiales and Selenomonadales[d]	30	This study, modified after (Küsel, 1999)

[a] Formamide concentration in CARD-FISH hybridization buffer
[b] EUB338-I,II,III mixed
[c] Delta495a,b,c mixed with unlabeled competitors AGTTAGCCGGTGCTTCTT, AGTTAGCCGGCGCTTCKT and AATTAGCCGGTGCTTCTT
[d] Probe CLO864 targets members of the families *Christensenellaceae, Clostridiaceae*, *Eubacteriaceae*, *Lachnospiraceae, Ruminococcaceae*, *Peptostreptococcaceae* and *Peptococcaceae* of the order Clostridiales.

Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R., and Stahl, D.A. (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol* **56**: 1919-1925.

Daims, H., Brühl, A., Amann, R., Schleifer, K.H., and Wagner, M. (1999) The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: Development and evaluation of a more comprehensive probe set. *Syst Appl Microbiol.* **22**: 434-444.

Küsel, K., Pinkart, H.C., Drake, H.L., and Devereux, R. (1999) Acetogenic and sulfate-reducing bacteria inhabiting the rhizoplane and deep cortex cells of the sea grass *Halodule wrightii. Appl Environ Microbiol* **65**: 5117-5123.

Loy, A., Lehner, A., Lee, N., Adamczyk, J., Meier, H., Ernst, J., Schleifer, K.H., and Wagner, M. (2002) Oligonucleotide microarray for 16S rRNA gene-based detection of all recognized lineages of sulfate-reducing prokaryotes in the environment. *Appl Environ Microbiol* **68**: 5064-5081.

Lücker, S., Steger, D., Kjeldsen, K.U., MacGregor, B.J., Wagner, M., and Loy, A. (2007) Improved 16S rRNA-targeted probe set for analysis of sulfate-reducing bacteria by fluorescence in situ hybridization *J Microbiol Methods* **69**: 523-528.

Wallner, G., Amann, R., and Beisker, W. (1993) Optimizing fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. *Cytometry* **14**: 136-143.

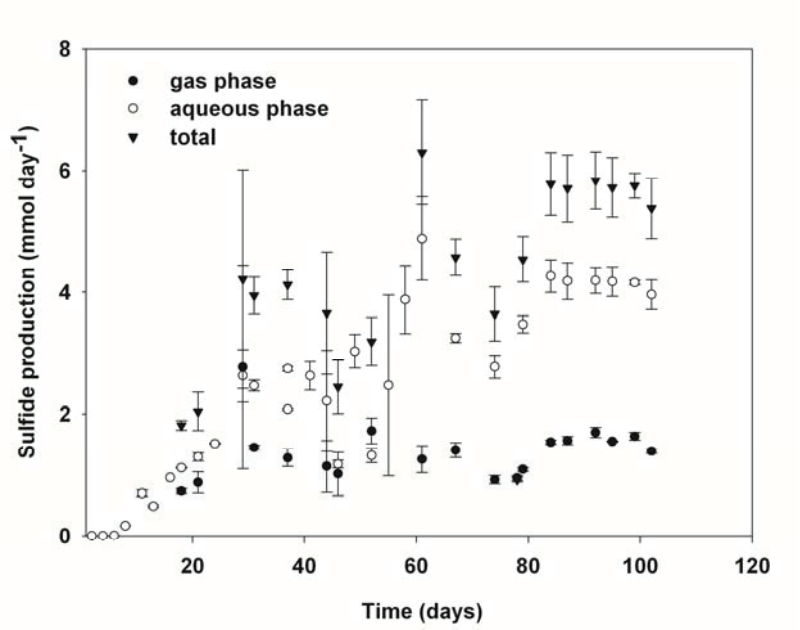

Fig. S1. Total sulfide production in culture 2. Sulfide production was calculated from the sulfide concentration in the culture liquid and from sulfide that escaped to the gas phase and was trapped with zinc acetate.

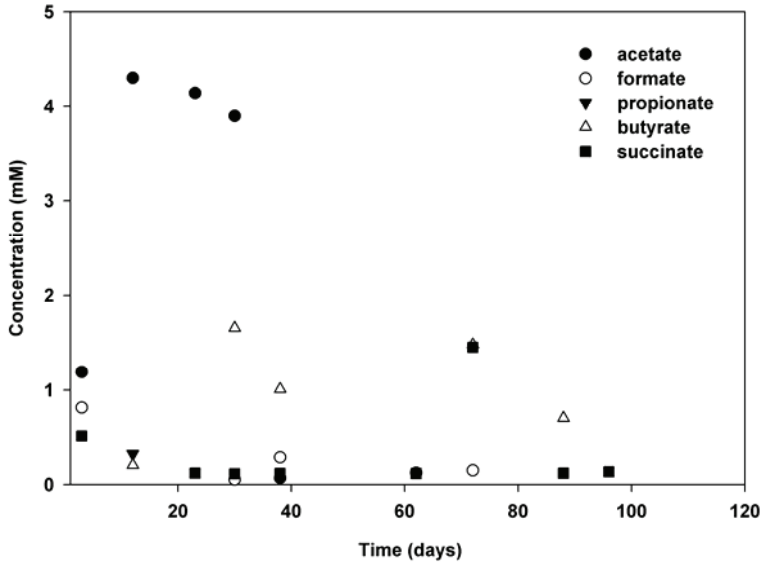

Fig. S2. Short chain fatty acids concentration in the culture liquid of culture 1.

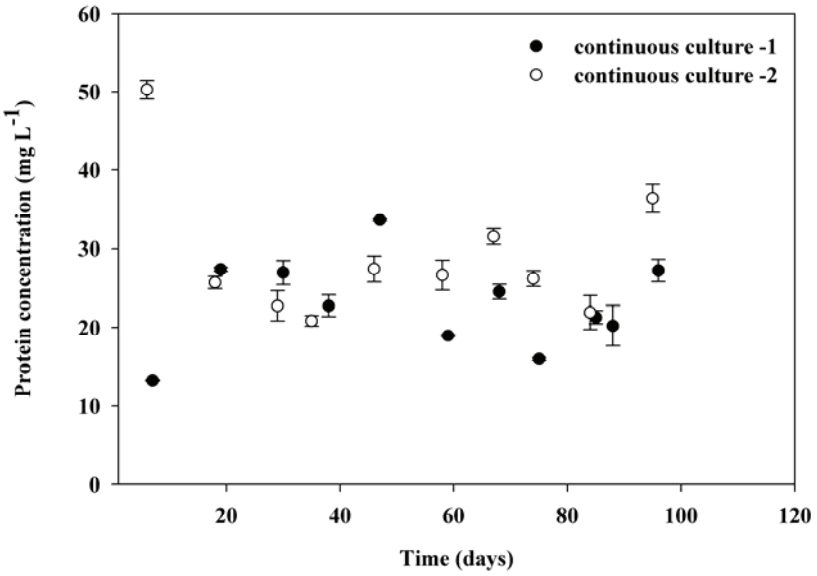

Fig. S3. Total protein concentration in the culture liquid of culture 1 and 2.

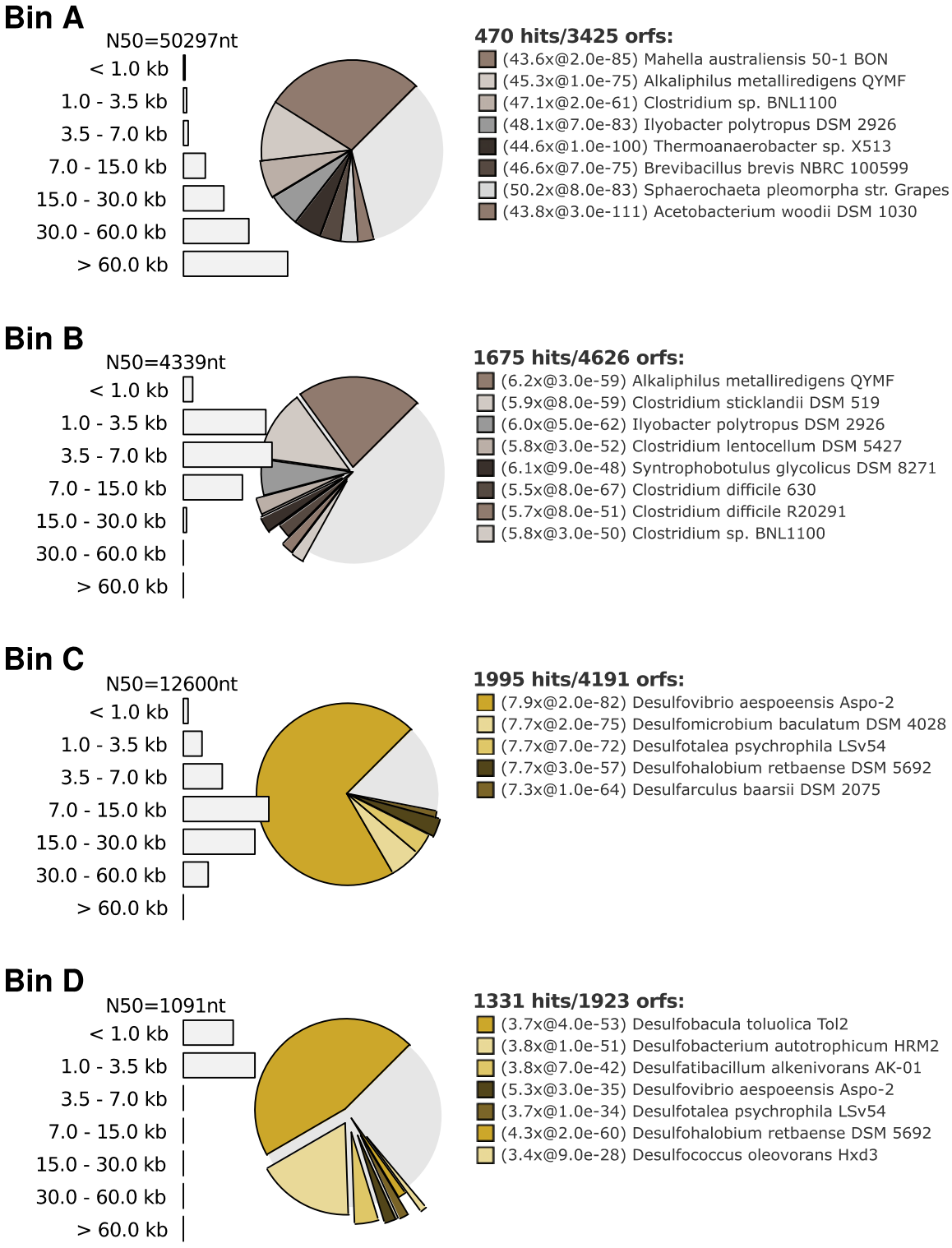

Fig. S4. Contig size distribution and taxonomic distribution of best blast hits of the open reading frames encoded on contigs comprising each of the four bins. Data for bin A, B, and D are for culture 2 and data for bin C are for culture 1 (see also Table 1).

Chapter 3

Manuscript 2

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Manuscript 2

Selective effects of transient oxygen and nitrate exposure on sulfate reducing/fermentative consortia

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Contribution: The structure of the experimental approach, was laid out by M.S. and J.S.G. The set up of the chemostat, the experimental procedure, the chemical analysis in addition to the DNA extraction were carried out by Z.A.B. The extraction of RNA and the preparation of Genomic library were done by H.E.T. M.S provided the metagenomic and trascriptomic analysis. The manuscript was written by all co-authors.

In preparation, ready for submission

Selective effects of transient oxygen and nitrate exposure on sulfate reducing/fermentative consortia

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Abstract

Benthic marine sulfate reducing bacteria are frequently exposed to oxygen or nitrate and. various coping strategies of sulfate bacteria to oxidative stress are known from pure culture studies. Here we investigated the selective effect of exposure to oxygen and nitrate on marine sulfate reducing/fermentative consortia. Six continuous cultures were inoculated with cells extracted from the top 2 cm of a tidal sediment and maintained for 350 days at constant conditions. Oxygen was supplied to 2 cultures twice daily, nitrate to 2 cultures and the remaining 2 cultures received no additions. Addition of oxygen and nitrate led to lower net sulfide production rates. After 300 days, all cultures were characterized with metagenomics and transcriptomics. A population related to *Desulfotignum balticum* was the dominant sulfate reducer in all cultures and fermentation was mainly performed by Firmicutes and Spirochaeta. Transcriptomics analysis suggested that *D. balticum* assimilated CO_2 as its main carbon source despite a copious supply of organic compounds. Nitrate was shown to strongly affect the outcome of selection, whereas the effects of oxygen were more subtle. Transcriptomics showed no apparent effect of the presence of oxygen or nitrate on gene activities of sulfate reducers. Overall, we concluded that in marine sediments the effects of transient exposure to oxygen may not exert a strong selective pressure on sulfate reducing communities.

Introduction

In marine coastal environments, about up to 50% of the pelagic primary production reaches the sediment surface of tidal flat sediments. This results in high input of organic

matter which can be rapidly re-mineralized by the microorganisms in the upper centimeters of the sediment (Wollast, 1991 ; Gittel *et al.*, 2008). Intertidal areas constitute an interesting environment to study the interaction of element's cycles, as there is a high input of organic matters beside the availability of the different electron acceptors like sulfate and nitrate, and the tidal activity that results in the exposure of sediments to oxygen (Wollast, 1991 ; Fenchel *et al.*, 1998; Gittel *et al.*, 2008).Among the electron acceptors, sulfate has the highest concentration (28 mM) (Fenchel *et al.*, 1998)and it has been estimated that about 50% of the total organic carbon mineralization in shallow sediments (<200 m water depth) is coupled to sulfate-reduction (Jørgensen, 1982). For intertidal flats, it was estimated that 3-35% of the total mineralization is coupled to sulfate reduction (Billerbeck *et al.*, 2006). Availability of oxygen leads to a lower relative importance of sulfate reduction because the different electron acceptors are known to be consumed in a thermodynamically determined order (the redox cascade) in which oxygen is used first, followed by nitrate, manganese and iron oxides then sulfate (Froelich *et al.*, 1979). According to this concept, sulfate reduction only occurs in the deeper layers after the depletion of the other above mentioned electron acceptors (Froelich *et al.*, 1979; Schulz and Zabel, 2000). Sulfate reducing bacteria are often strict anaerobes and couple the oxidation of molecular hydrogen or organic compounds to the complete reduction of sulfate to hydrogen sulfide (Muyzer and Stams, 2008; Rabus *et al.*, 2013). Nevertheless, sulfate reducing bacteria were detected throughout the whole sediment of the intertidal flat , including the aerobic and denitrifying zones (Llobet-Brossa *et al.*, 2002; Gittel *et al.*, 2008)

Another electron acceptor that can compete with sulfate is nitrate, which is also above sulfate in the redox tower. It was found that some sulfate reducers like *Desulfovibrio desulfuricans* are able to grow with hydrogen coupled to ammonification of nitrate or nitrite to ammonia (Dalsgaard and Bak, 1994).

In the present study we investigated the effect of tidal cycling with oxygen and nitrate on sulfate reduction activity of microbial biomass from the surface layer of an intertidal flat (Janssand in German Wadden Sea). Microbial biomass was enriched by continuous culture under three different conditions: exposure to oxygen, two times per day to mimick the tidal cycle; exposure to nitrate, also two times per day, and without tidal cycling. Replicate cultures were performed for each condition. The cultures were supplied with synthetic seawater medium with glucose, acetate and a mixture of amino acids as electron donors. This substrate mixture represented the composition of decaying biomass (in terms of the monomers), the main electron donor and carbon source in situ. The microbial activity and community composition were investigated by combined chemical, metagenomic and transcriptomic analyses.

Experimental Procedures

Sampling site and inoculum for enrichment experiments

Sediment was sampled from the intertidal back-barrier flat Janssand in the German Wadden Sea (53.73515 ’N, 007.69913’E) in June, 2012. From the upper part of the flat, the top 2 cm of sandy sediment was collected with a flat trowel. After transport of the sediment to the laboratory, an equal volume of artificial seawater (Red Sea Salt, 33.4 g/l;

http://www.redseafish.com) was added to the sediment and stirred vigorously. The sediment was allowed to settle briefly, after which the liquid was transferred into (1 l) glass bottles that were closed with rubber stoppers and of which the headspace was exchanged with argon. The liquid was kept at 4°C for 2 days and then used as inoculum.

Continuous culture setup and medium

Three sets of replicate continuous cultures were set up. A glass vessel (DURAN, GLS 80, 500 ml) was filled with (0.4 l) inoculum. Each glass vessel was fitted with tubes for inflowing medium, outflowing medium, inflowing gas and outflowing gas. The culture was mixed with a magnetic stirrer (2 Mag Magnetic Motion Mixdrive 1 EXO); speed was between 200 to 400 rpm. After inoculation, oxygen was removed from the culture headspace (0.4 l) by flushing with Argon (10 ml/min) for two days with a mass flow controller (Alicat Scientific). During the first month of cultivation, the cultures were operated in repeated fed batch mode. Fresh medium was supplied at a rate of 0.17 l/day., then the excess of spent medium was removed by dosing argon into the culture. After medium removal, to maintain anoxic conditions, argon (10 ml/min) was supplied to the culture for an additional 1 h without medium removal. After one month and onward, the culture was operated as a chemostat with continuous removal of spent medium via an overflow. The medium supply rate remained the same (0.17 l/day), resulting in a dilution rate of 0.36-0.4 day^{-1}. During the first 54 days , the medium consisted of Red Sea Salt artificial seawater (33.4 g l^{-1}) which contains 28 mM sulfate, supplemented with 20 C-mM organic carbon. The organic carbon mixture consisted of 1.1 mM D-glucose, 1.7 mM

acetic acid and a mixture of amino acids (mM, L-glutamic acid 0.38, L-aspartic acid 0.65, L-alanine 0.85, L-serine 0.46, L-tyrosine 0.099, L-histidine 0.035, L-methionine 0.088. In addition, 0.2 mM Na-phosphate, 17 iM $FeSO_4$, 0.2 ml l^{-1} trace element solution mg l^{-1}, $ZnCl_2$ 69, $MnCl_2.4\ H_2O$ 100, H_3BO_3 60, $CoCl_2.6\ H_2O$ 120, $CuCl_2.2\ H_2O$ 10, $NiCl_2.6\ H_2O$ 25, $Na_2MoO4.2\ H_2O$ 25, $AlCl_3.6\ H_2O$ 25 in 0.1% HCl and 0.2 ml l^{-1} Se/W solution mg l^{-1}, $Na_2SeO_3.H_2O$ 6, $Na_2WO_4.2\ H_2O$ 8 in 0.04% NaOH were added. The medium also contained 20 mM of 4-(2-hydroxyethyl)piperazine-1- ethanesulfonic acid (HEPES) to buffer the pH of the culture. The pH of the medium was adjusted to pH 7.5. Medium was supplied via a sterile glass back growth buffer in order to prevent contamination of the inflowing medium. After day 54 and onwards, back growth of bacteria into the medium supply tubing was prevented completely by separating the inflowing medium into two parts that were supplied simultaneously to each culture. One part contained the HEPES buffer and the other part all other medium components, at pH 4. The culture pH was measured off line and with pH meter (Mettler Toledo, Five Easy ^{tm}) and was in the range of 7.5 to 8 .The supply rate of both parts was modified to maintain the dilution rate at 0.36-0.4 day-1. The OD600 of all cultures was monitored off line spectrophotometrically (Thermo Scientifi Genesys 10S UV-Vis).

After 21 days the headspace of two of the cultures was oxygenated twice daily by supplying air (1 l/min) for 5 min. The air was removed after 30 min by supplying argon (1 l/min) for 5 min. This procedure was repeated every 12 h for the remainder of the experiment. In parallel, nitrate was supplied twice daily to two other cultures by supplying a nitrate solution (1.4 ml/min, 20 mM $NaNO_3$ dissolved in Red Sea Salt) for 7

minutes every 12 hours. The remaining cultures did not receive any additional electron acceptors except for sulfate which was present in the Red Sea salt.

During oxygenation, the oxygen concentration in the culture liquid was measured with Optical Oxygen Meter – FireSting O_2 . At the same time, hydrogen sulfide concentrations in the culture were measured off line according to (Cline, 1969)., while sulfur was measured according to (Kamyshny and Ferdelman, 2010).. In addition, after 327 days the nitrate in the medium was replaced with ^{15}N-nitrate by direct injection of 10 ml of 20mM ^{15}N-nitrate and the production of ^{15}N-nitrogen gas was off-line by mass spectrometry (GAM 400, InProcess Instruments, Bremen, Germany) using 0.5 ml headspace samples. . Nitrate in the culture liquid was determined as in. (Hanke *et al.*, 2014).

DNA and RNA extraction, metagenomic sequencing and in silico procedures

Between days 311 and 327 DNA was extracted from a 10 ml sample of all six cultures according to (Zhou *et al.*, 1996) following incubation in extraction buffer with lysozyme (2.5 mg ml^{-1}) and RNAse (0.1 mg ml^{-1}). For metagenome shotgun sequencing, 1.5 µg of the extracted DNA per sample were mechanically fragmentend using Nebulizers (Roche) with 32 psi applied for 3 min, in 500 µl nebulization buffer (Roche). The fragmented DNA was purified using MinElute PCR purification columns (Qiagen) and eluted in 50 µl low TE (Life Technologies). The entire eluate was used for the preparation of barcoded PGM sequencing libraries with the Ion Xpress^{TM} Plus gDNA Fragment Library Preparation kit (manual Pub. No 4471989, Rev. M, May 2013; Life Technologies). Library insert sizes were between 350 and 400 bp. Libraries were sequenced with the

Personal Genome Sequencer (PGM) on 318 Chips (pooled with other samples), using the chemistry for 400 bp libraries. Base calling was performed with the Torrent Suite v3.6 (DNA 3-6) or v4.0.2 (DNA 1&2) software, with default settings. Reads of the sequenced DNA samples were assembled with the Newbler assembler (v. 2.8) with default settings for genomic DNA assembly for nonpaired reads.

During the same period, RNA was extracted from a 2 ml sample of all six cultures (for those cultures with periodic oxygen and nitrate supply, RNA was extracted both before and after the exposure to oxygen and nitrate. Between sampling and extraction, the samples were stored at -20°C in RNA stabilization solution (RNA*later*® Invitrogen). , Extraction was performed by adding 1 ml of TRI Reagent® Solution (Ambion), bead beating in 2 ml vials filled with 300 µl of glass beads, diameter 0.1 mm, for 45 sec at 6.5 m/s, incubation at room temperature (RT) for 5 min and centrifugation at 4500 x g at 4°C. The supernatant was transferred to new vials, 200 µl chloroform were added, followed by vigorous shaking for 15-30 sec, incubation at RT for 10 min, and centrifugation at 12000 x g for 15 min at 4°C. The upper aqueous phase was transferred to new vials and RNA was precipitated on ice for 20 min after adding 500 µl of isopropanol. After centrifugation at 20000 x g for 25 min at 4°C, the RNA pellets were washed three times with 75% ice cold ethanol, air dried for 15 min, and re-suspended in sterile TE buffer (pH 8.0).

Assembled DNA samples were binned based on multivariate statistics of tetranucleotide frequencies with MetaWatt 2.1 (Strous *et al.*, 2012). For all samples combined, 18 bins were selected for further analysis, with each bin representing a provisional genome of a distinct population. Genome completeness was evaluated by detection of a set of 139

conserved single copy genes (Campbell *et al.*, 2013) with hidden Markov models (HMMER 3.1) and by detection of transfer RNA genes (Laslett and Canback, 2004). Population abundances over all samples was estimated by mapping the filtered sequencing reads to the contigs making up each associated bin. Genes present in each bin were annotated with prokka 1.9 (Seemann, 2014). Transcriptional activities for each gene of each bin were determined by mapping cDNA reads to the annotated contigs. Reported activities were calculated by dividing number of mapped reads/gene length by the total number of reads mapped to coding sequences of the bin/total length of all coding sequencing of the bin. This way, the average transcriptional activity equals 1.0. 16S sequences were detected with hidden Markov models (www.github.com/Victorian-Bioinformatics-Consortium/barrnap) and, independently, reconstructed with Emirge (Miller *et al.*, 2011). 16S rDNA sequences were linked to bins as previously described (Kraft *et al.*, 2014). Approximate maximum likelihood phylogenetic trees were created with FastTree2 (Price *et al.*, 2010) after alignment with MAFFT (Katoh *et al.*, 2002).

Sequence submission

Data submission to 16S rRNA sequence databases and short read archives is in progress.

Results and discussion

Three sets of replicate cultures (6 total) were inoculated with biomass extracted from a marine tidal flat. All cultures were continuously supplied with artificial seawater medium supplemented with a mixture of glucose, seven different aminoacids and acetate. This

mixture mimicked the composition of decaying biomass (in terms of the monomers), the main electron donor and carbon source in situ. After two days, sulfide was detected in all cultures and increased gradually. From day 21 onward, air was supplied to two replicates during 30 minutes twice daily and nitrate was supplied to two replicates during 7 minutes twice daily. The final two replicates did not receive any additional electron acceptors except for sulfate, which was present in the marine medium. The six cultures were maintained for 350 days and the sulfide concentration was monitored as a proxy for sulfate reducing activity. Sulfide concentrations were different between the treatments (Fig. 1). Transient air supply yielded the lowest sulfide concentrations (2.3±0.3mM), followed by the cultures that received nitrate (4.2±0.6mM) and the cultures without additions (6.3± 0.7mM).After 311-327 days, the concentrations of oxygen, nitrate and sulfide were monitored during multiple exposures to oxygen or nitrate. Typical responses to transient air or nitrate are shown in Fig. 2. The oxygen concentration was stable around 1.3% air saturation (3.1 μM) and during air exposure the sulfide concentrations decreased by (0.7±0.4) mM and transient production of elemental sulfur was observed (up to 0.2 mM). In the cultures supplied with nitrate, the nitrate concentration decreased from 0.5 mM to 0.0 mM during 100 min. Using ^{15}N-labeled nitrate we found no production of N_2, which indicated that ammonia may have been the end product of nitrate reduction, but this could not be confirmed experimentally because of the high background ammonia concentration that resulted from ammonification of the supplied amino acids. During nitrate consumption the sulfide concentration decreased slightly. and production of elemental sulfur (up to 0.2 mM) was observed.

After 311-327 days, DNA was extracted from all six cultures and sequenced with an Ion Torrent Personal Genome Machine. The reads were assembled and the assembled contigs were binned to obtain provisional whole genome sequences for the most abundant populations present in each of the cultures. In total, 18 bins were obtained that defined the genetic repertoire of 18 different populations (Table 1). Figure 3 shows each of these bins as a distinct "blob" on percent-GC-versus-sequencing-coverage plots and Figure 4 shows that each bin was characterized by a distinct phylogenetic profile obtained by blasting (blastp) all open reading frames encoded on the contigs that made up each bin against a database that contained a representative of every genus with a publicly available complete or draft whole genome sequence. In parallel, 16S sequences were obtained and could in most cases be linked unambiguously to a bin (Fig 5-7).

The abundances of every population in the six microbial communities sequenced were estimated by mapping sequencing reads to the assembled contigs (Fig 8). The figure shows both differences and similarities between the communities naturally selected by different treatments. A population related to *Desulfotignum* (Fig 5, bin D) was present in all cultures and was the dominant deltaproteobacterial population in the sulfate-only cultures and in the cultures with transient air supply. A population related to *Desulfovibrio* was also selected mainly in the sulfate-only cultures. A population related to *Desulfuromusa* was the dominant deltaproteobacterial population in cultures with transient nitrate. An alphaproteobacterial population (Fig 5, bin A) and two gammaproteobacterial populations (Fig 5, bins B and C) were detected as minor populations in the cultures with transient nitrate and especially in those with transient oxygen. Five populations of Firmicutes (Fig 6, bins G-K) and three populations

representing Spirochaetales (Fig 7, bins L-N) were detected in all cultures with no apparent selective effect of conditions. The relative abundances of these populations varied immensely, also between replicates. Together, they frequently made up around 50% of the total community (Fig 8). One population represented the phylum Hyd24-12. This population was only selected in the sulfate-only cultures. The remaining three minor populations were affiliated with Anaerolinea (bin P) and Bacteroidetes (bins Q and R,). The latter were apparently selected only in cultures with transient oxygen or nitrate.

Some bins were assembled much better than other bins; table 1 shows N50 contig lengths between 1,1 (bin C) and 222 kb (bin Hyd2412). This could be caused by differences in sequencing coverage combined with varying degrees of microdiversity for different populations. Populations that are relatively clonal yield excellent assemblies whereas more microdiverse populations do not yield long contigs regardless of sequencing coverage.

The assembled contigs constituting each bin were annotated and the activity of each gene of each of the bins was determined by next generation sequencing of cDNA obtained from extracted RNA (Supplementary information). For cultures with transient air or nitrate supply, two transcriptomes were analyzed, one before and one after air or nitrate supply, yielding 10 transcriptomes in total for the six cultures. Annotation already showed that the five populations affiliated with Firmicutes (bins G-K) and the three populations affiliated with Sphaeochaeta (bins L-N), as well as those affiliated with candidate phylum Hyd24-12 and Anaerolinea (bin P) were strictly fermentative. Apart from the presence of multiple hydrogen evolving hydrogenases and their associated electron transfer apparatus, these bins lacked a respiratory chain. Further, the

Sphaeochaete populations only encoded a vacuolar type ATP synthase and where thus completely dependent on substrate level phosphorylation steps during fermentation. Apart from hydrogen, it was difficult to determine the end products of fermentation by each of these populations, but at least production of acetate was supported by active transcription of acyl phosphatases and no evidence was found for production of formate (e.g. presence and activity of genes encoding pyruvate formate lyase). Gene activities indicated that the glucose and aminoacids supplied as the carbon source and electron donor with the medium were the main substrates, as shown by highly active sugar and amino acid importers. Some of these fermentative populations appeared to be slightly more specialized then others. For example, bin G exhibited high activty of aminoacid importers and aminoacid metabolism (e.g. glutamate dehydrogenase, parts of the citric acid cycle) and bin K exhibited high activity of sugar importers and glycolysis. All fermentative populations showed high activity of thioredoxins, peroxiredoxins, rubredoxins, and related enzymes which confer protection against oxidative stress during transient exposure to nitrate or air. This protection might have been effective, because transient exposure to air or nitrate did not provoke a strong transcriptomic response for these populations. Thus, although the selected populations were phylogenetically only distantly related to well studied bacteria in pure culture (Fig 6,7), their lifestyle appears to be quite similar

Transient exposure to air selected for populations of Alpha- and Gammaproteobacteria (bins A-C). Annotation showed that at least the populations corresponding to bins A and B were capable of respiration. Complete respiratory chains including complexes I-IV were present and actively transcribed and the same was true for the citric acid cycle.

However, little evidence was present for the capability of denitrification. Compared to the fermentative populations, the respiratory populations showed low transcriptional activity of substrate importers, indicating that they were not actively competing for the same substrates. It is more likely that these populations used mainly fermentation products as electron donors. In addition, the Rhodobacterales population also actively transcribed genes involved in sulfide and sulfur oxidation – at least the genes soxAXYZ were detected and active. Neither the Rhodobacterales (bin A) nor the Alteromonadales (bin B) appeared to use hydrogen as the substrate, as shown by a lack of transcriptional activity of hydrogenases. Both bins exhibited metabolism of polyhydroxybutyrate (PHB) and polyphosphate. The anaerobic accumulation of PHB fueled by polyphosphate hydrolysis, coupled to the aerobic oxidation of PHB and regeneration of polyphosphate would be a feasible strategy for these populations that is well known from bioplastic production (Johnson *et al.*, 2009) and biological phosphorus removal (Wu *et al.*, 2010) Indeed, for bin A, transcriptomic analysis showed a down regulation of polyphosphate kinase and poly-beta-hydroxybutyrate polymerase during the period of air supply. The fermentative populations may have provided the acetate in addition to the acetate provided from the medium to the respiratory Alpha- and Gammaproteobacteria during the anaerobic periods. The data in bin C was incomplete and the transcriptomes had very low coverage for this bin, making metabolic inferences difficult.

Sulfate reduction was performed by two populations, one related to *Desulfotignum balticum* (Fig 5, bin D) and the other affiliated with *Desulfovibrio* (bin F). Of these, the latter was less abundant in all cases, but especially in cultures with transient air exposure. Genes encoding sulfate adenyl transferase, adenyl sulfate reductase and dissimilatory

sulfite reductase were actively transcribed by both populations. The population related to *D. balticum* appeared to use hydrogen as electron donor and actively transcribed the acetyl CoA pathway for carbon dioxide fixation indicating that this population grew at least partially autotrophically. Autotrophic growth via the acetyl CoA pathway was previously described for *D. balticum* (Kuever *et al.*, 2001).

Bin D also constitutively expressed a bd-type terminal oxidase which enabled aerobic respiration and protection of oxygen sensitive enzymes. The *Desulfovibrio* population (bin F) also appeared to consume hydrogen and also expressed a bd-type terminal oxidase constitutively. However, it lacked the capacity to fix carbon dioxide and transcribed genes for formate:hydrogen lyase and formate oxidation..

The remaining Deltaproteobacterial population, related to *Desulfuromusa bakii*, was not capable of sulfate reduction. This population was only selected in cultures with transient nitrate supply and showed a strong global transcriptional response to nitrate availability. In response to nitrate, it transcribed genes for citric acid cycle enzymes, complex I, formate dehydrogenase, periplasmic nitrate reductase and pentaheme nitrite reductase. This population apparently performed nitrate ammonification with substrates such as amino acids and acetate and formate. *Desulfuromusa bakii* and related bacteria are known as sulfur reducing, and often facultative fermentative bacteria (Liesack and Finster, 1994) and it is likely that the population selected here also performed fermentation of amino acids and/or dicarboxylates in the absence of nitrate.

For the populations affiliated with bacteroidetes (bins Q and R) meaningful inferences were impossible because the genomic data was incomplete (Table 1) and only very few

transcripts were detected. Because they were mainly present in cultures with transient oxygen or nitrate supply, a respiratory lifestyle is most likely.

Overall, two main conclusions can be drawn from this study. Firstly, transient exposure to oxygen had only a minor selective effect on the microbial community level. Apparently, detoxification of oxygen by fermentative populations (with peroxiredoxins and functionally related enzymes) and sulfate reducing populations (with bd type terminal oxidases) was trivial, at least for the short exposure times applied in this study. Because the inoculum was obtained from oxic sediments, the observed populations may have been well prepared for this scenario. Only the fermentative population affiliated with the candidate phylum Hyd24-12 appeared to have no strategy to cope with nitrate or oxygen exposure. Nevertheless, considerable differences in sulfide concentration in the culture liquid were observed between the three different treatments (Fig. 1). These differences may be explained by competition for electron donor by aerobic or nitrate-reducing microorganisms and the utilization of sulfide as electron donor. In the culture with exposure to nitrate, the observed difference in net sulfide production corresponds to 10-43% of nitrate used depending on whether sulfide is oxidized to elemental sulfur or sulfate. . The observed production of elemental sulfur is an indication for sulfide oxidation activity, and produced sulfur may be rapidly reduced again after nitrate was depleted. Transient nitrate supply exerted a strong positive selective pressure on the population related to *Desulfuromusa baki*i which apparently performed dissimilatory nitrate ammonifcation. The conditions that provoked ammonification (nitrate supply, high carbon to nitrogen ratio and low growth rates) were consistent with what was previously reported (Kraft *et al.*, 2014).

The second conclusion is that independent of conditions, fermentative/sulfate reducing consortia were selected that apparently converted a large part of the high quality carbon substrates (glucose, amino acids) into hydrogen and carbon dioxide, leading to the refixation of carbon dioxide by the dominant sulfate reducing population. This is a surprising and counter-intuitive finding that merits future investigation.

[Figure legends]

Figure 1. Sulfide production with time in (a) replicate cultures with transient oxygen supply, (b) replicate cultures with transient nitrate supply and (c) replicate cultures only supplied with sulfate as electron acceptor.

Figure 2. Oxygen, nitrate and sulfide concentrations during transient exposure to oxygen (a, b) or nitrate (c, d).

Figure 3. Assignment of “blobs” on the GC versus coverage plot of assembled contigs after binning. Cultures with transient air supply (a,b), transient nitrate supply (c,d) and cultures only supplied with sulfate as electron acceptor (e,f).Capital letters A-R refer to bin identifiers. Parenthesized letter mean that a bin was detected but was assembled better in a different sample.

Figure 4. Contig length distribution and phylogenetic profile (family level) of each of the 18 bins obtained. Median e values for each family are indicated.

Figure 5. Phylogenetic position of assembled proteobacterial 16S rRNA sequences and assignment of these sequences to the bins.

Figure 6. Approximate maximum likelihood phylogenetic analysis of assembled 16S rRNA sequences affiliated with Firmicutes and assignment of these sequences to the bins. For each sequence recovered (shown in blue), source contigs and average coverage (over all samples) are indicated. “EMIRGE” indicates independent recovery of a sequence with iterative read mapping.

Figure 7. Approximate maximum likelihood phylogenetic analysis of assembled 16S rRNA sequences affiliated with Sphaerochaeta and assignment of these sequences to the bins. For each sequence recovered (shown in blue), source contigs and average coverage (over all samples) are indicated. “EMIRGE” indicates independent recovery of a sequence with iterative read mapping.

Figure 8. Estimated abundances of the major populations in cultures with transient air supply (a,b), with transient nitrate supply (c,d) and cultures only supplied with sulfate as electron acceptor (e,f), obtained by mapping sequencing reads to the assembled contigs of each bin.

Table 1. Properties of the 14 bins obtained by tetranucleotide binning of metagenomes obtained from the six cultures.

Bin	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**
Affiliation	Rhodobacterales	Alteromonadales	Chromatiales	Desulfobacterales	Desulfuromonadales	Desulfovibrionales	Firmicutes	Clostridiales	Clostridiales
Size (kb)	3.85	3.08	1.55	4.77	4.10	4.11	4.27	4.45	4.59
Number of contigs (#)	3272	1897	1650	594	237	885	2310	351	562
N50 contig length (kb)	1.5	2.5	1.1	126	58.9	14.6	2.8	27.6	107
GC content (%)	60.1	50.6	47.6	51.9	50.2	53.9	40.7	37.6	39.9
Number of CSCGs (#)	131	115	71	132	131	138	121	112	119
Number of tRNAs (#)	41	34	16	43	50	60	62	31	51
Metawatt bin	Z4L8	Z2L5	Z6M16	Z5M2	Z3M2	Z6M4	Z5L7	Z1L1**	Z6L2
Bin	**J**	**K**	**L**	**M**	**N**	**Hyd2412**	**P**	**Q**	**R**
Affiliation	Clostridiales	Clostridiales	Spirochaetales	Spirochaetales	Spirochaetales	Novel phylum	Anaerolinea	Bacteroidetes	Bacteroidetes
Size (kb)	6.69	3.86	3.36	3.57	3.78	2.92	2.51	1.68	2.30
Number of contigs (#)	3459	248	40	317	691	96	1147	1661	2657
N50 contig length (kb)	3.3	118	144	160	10.1	222	6.0	1.2	0.99
GC content (%)	34.5	45.4	53.5	36.8	35.7	56.6	52.8	43.5	42.1
Number of CSCGs (#)	182	126	129	88	93	98	128	69	100
Number of tRNAs (#)	41	39	47	47	36	39	36	11	11
	Z5L8	Z3M0	Z5L0	Z4M0	Z4L2	Z5L1	Z6H13	Z1M13	Z4L13

*) Number of Conserved Single Copy Genes detected (out of a set of 139). Numbers higher than 139 indicate the presence of DNA originating from more than a single population in the bin. Numbers lower than 139 indicate the provisional genome sequence associated with the bin may be incomplete.
**) Bin Z1L1 was trimmed to the cloud at sequencing coverage 30x on GC versus coverage plot.

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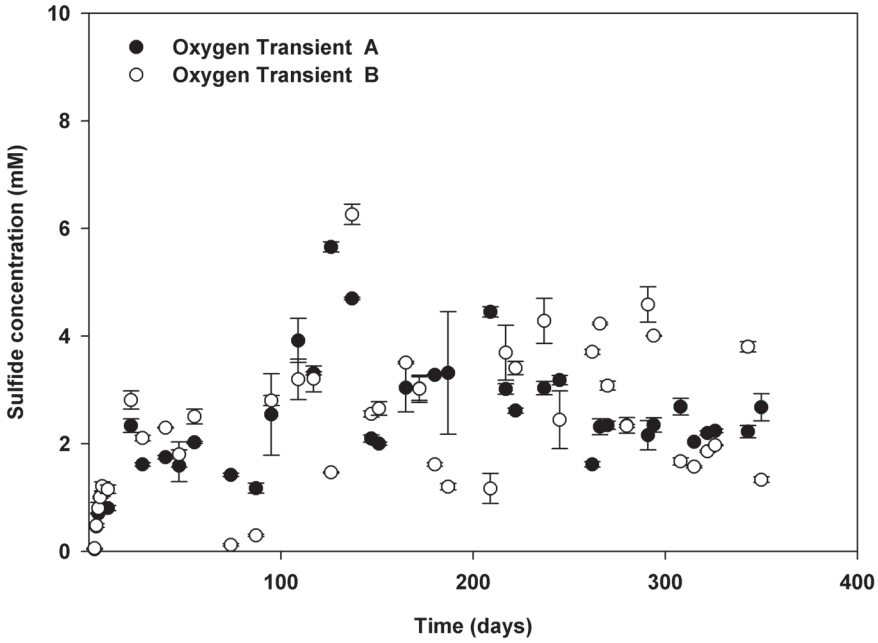
a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Oxygen Transient B
Sulfide concentration (mM)
Time (days)
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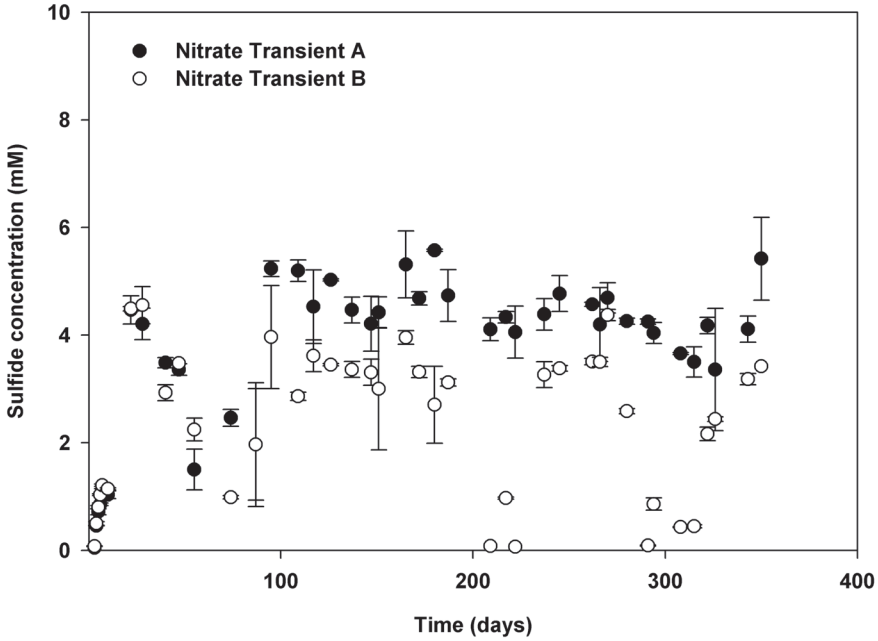
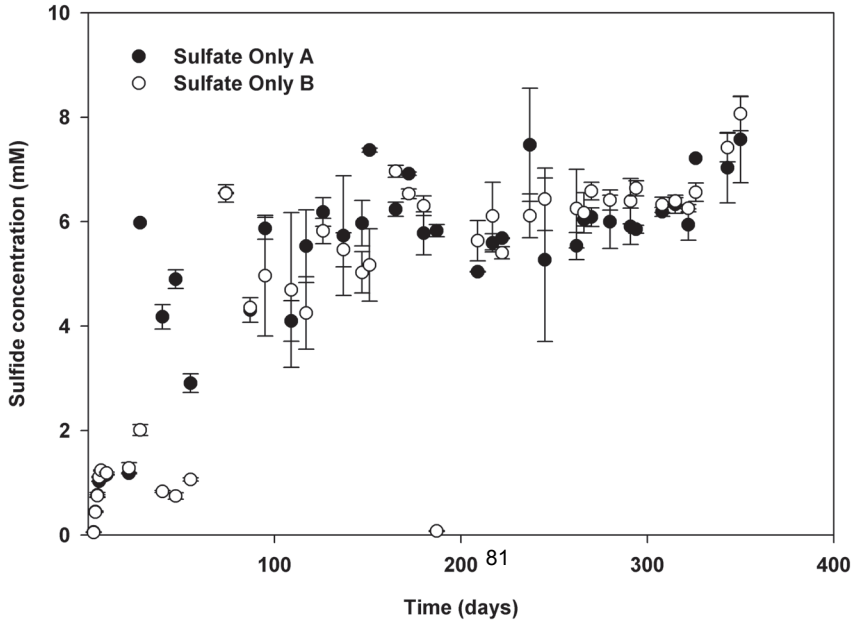
2
4
6
8
10
100
200
300
400

b


Nitrate Transient A
Nitrate Transient B
Sulfide concentration (mM)
Time (days)
0
2
4
6
8
10
100
200
300
400

c


Sulfate Only A
Sulfate Only B
Sulfide concentration (mM)
Time (days)
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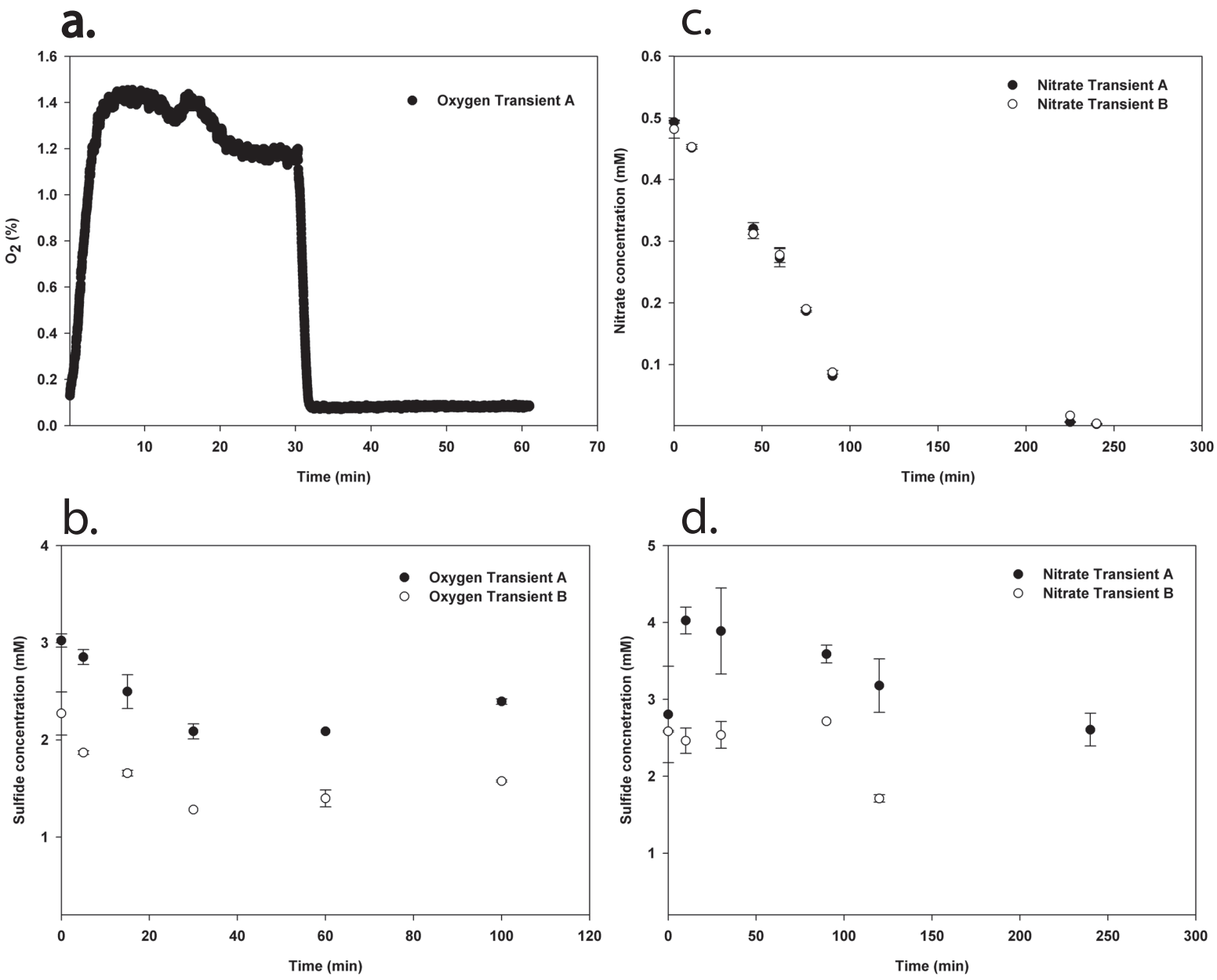
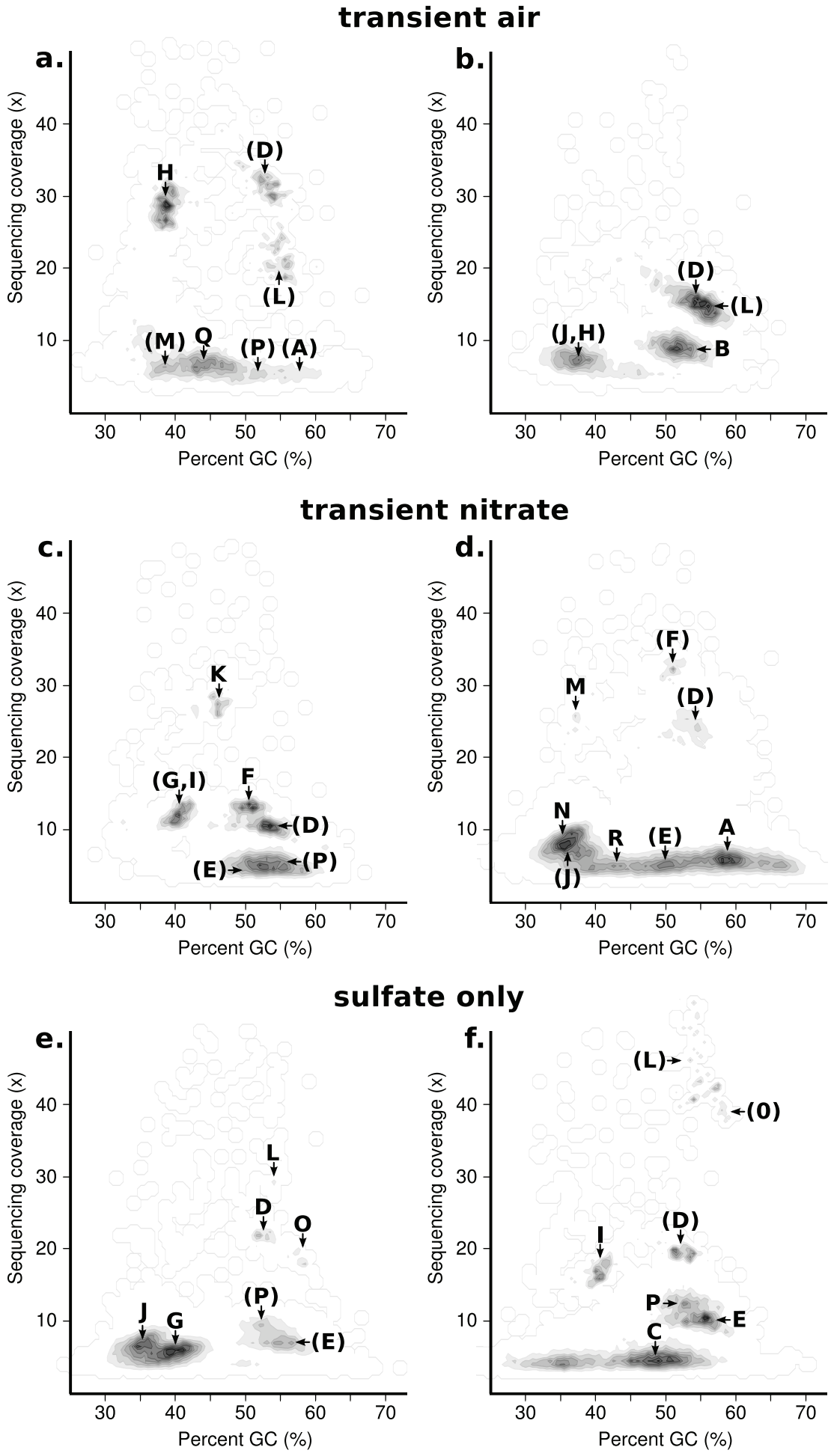
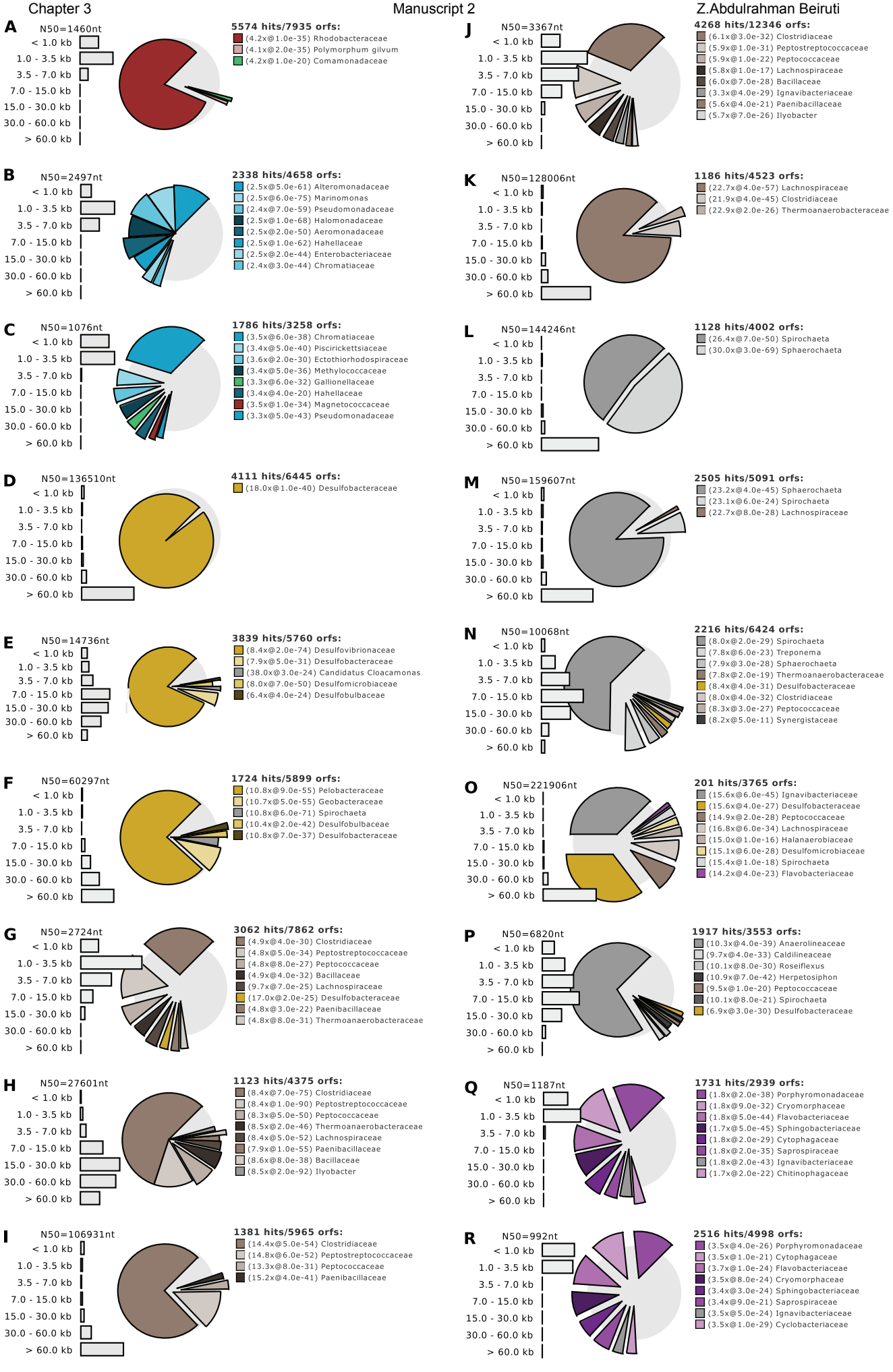
2
4
6
8
10
100
200
300
400


a.
Oxygen Transient A
O_2 (%)
Time (min)
c.
Nitrate Transient A
Nitrate Transient B
Nitrate concentration (mM)
Time (min)
b.
Oxygen Transient A
Oxygen Transient B
Sulfide concentration (mM)
Time (min)
d.
Nitrate Transient A
Nitrate Transient B
Sulfide concnetration (mM)
Time (min)


transient air
a.
b.
transient nitrate
c.
d.
sulfate only
e.
f.
Sequencing coverage (x)
Percent GC (%)
H
(D)
(L)
(M)
O
(P)
(A)
(J,H)
B
K
(G,I)
F
(E)
(F)
M
N
R
(J)
I
C
(0)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I
N50=106931nt
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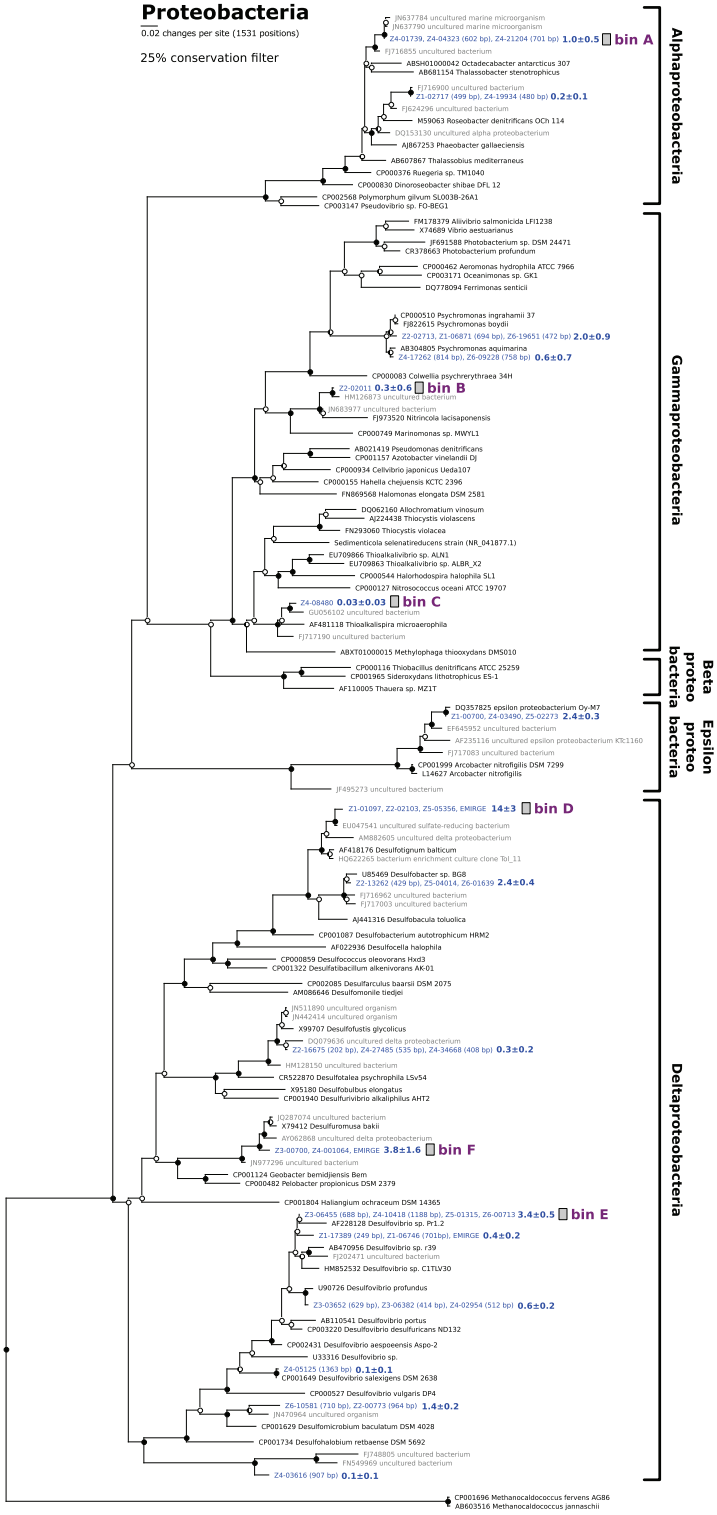
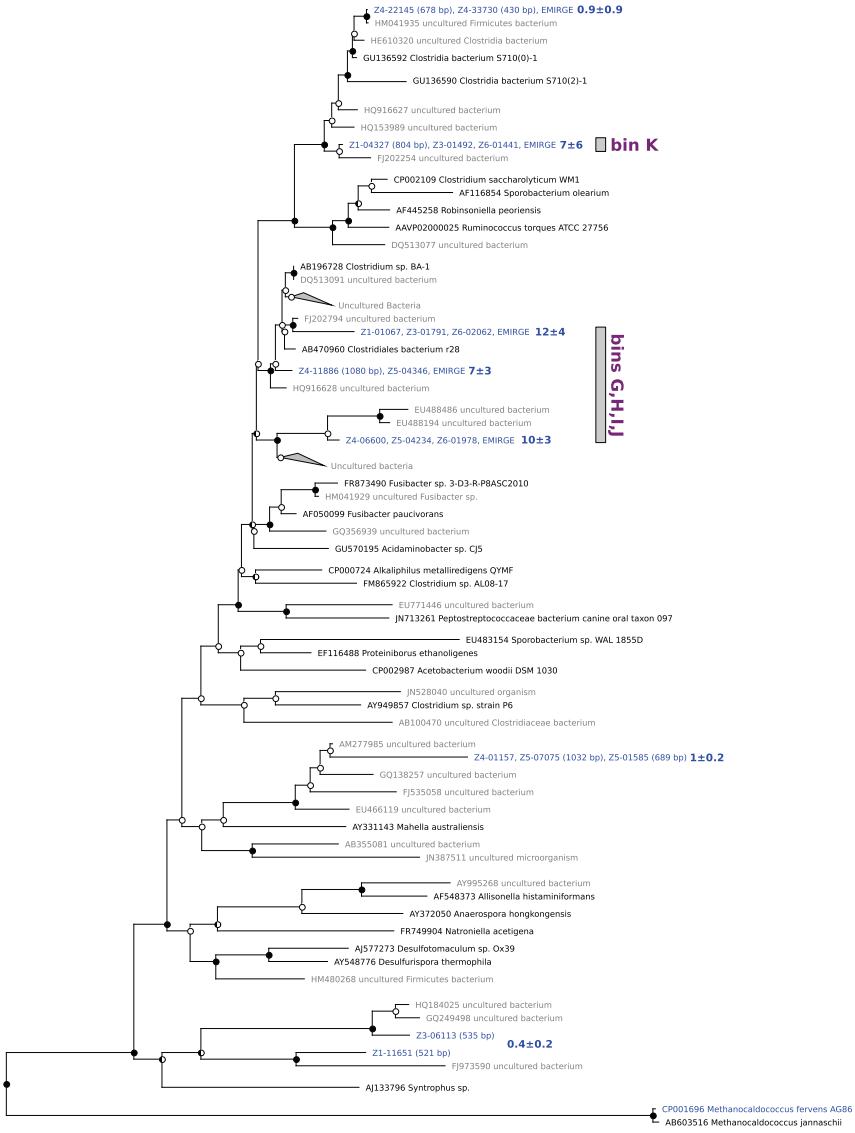
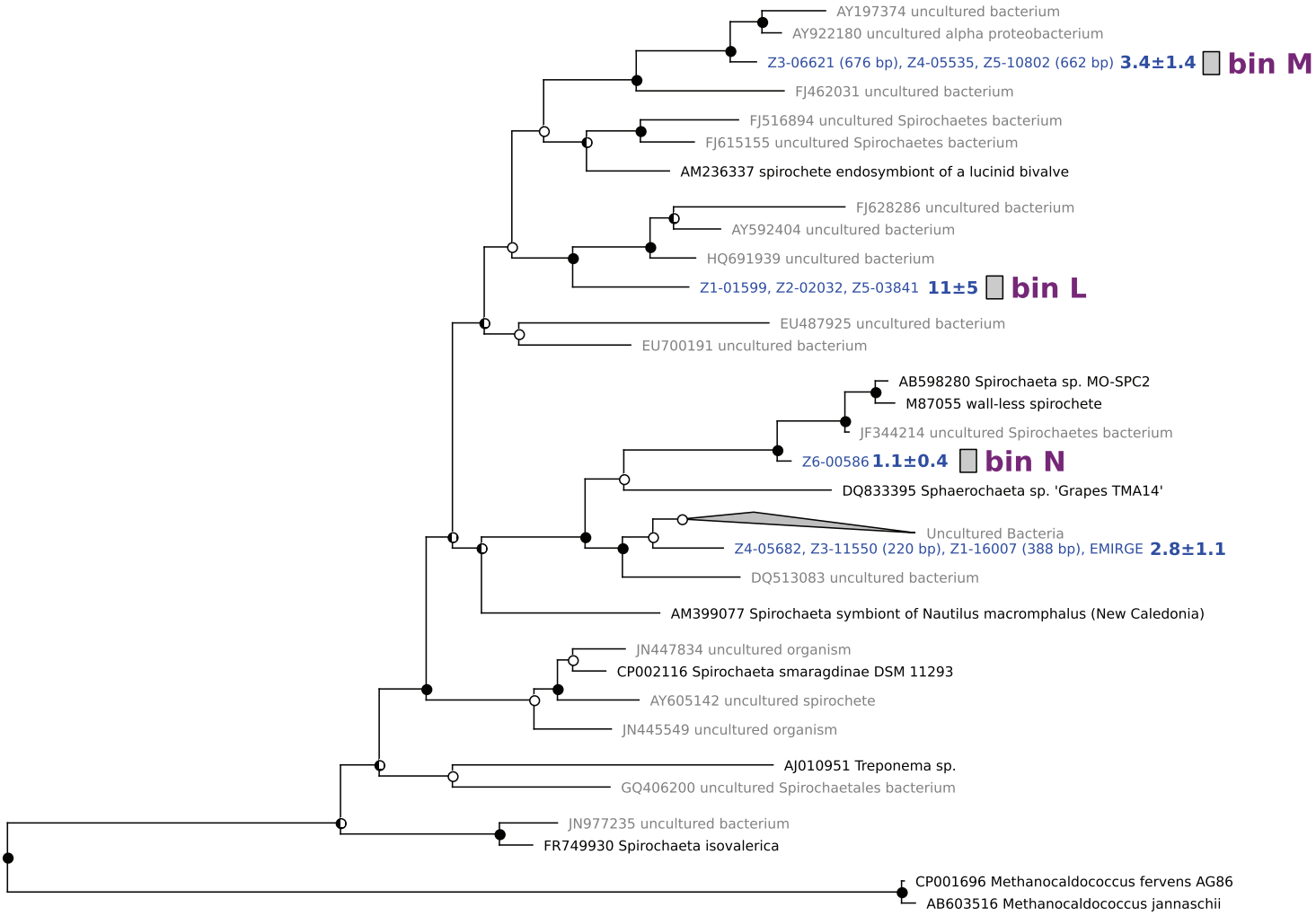

Proteobacteria
0.02 changes per site (1531 positions)
25% conservation filter
JN637784 uncultured marine microorganism
JN637790 uncultured marine microorganism
Z4-01739, Z4-04323 (602 bp), Z4-21204 (701 bp) 1.0±0.5 bin A
FJ716855 uncultured bacterium
ABSH01000042 Octadecabacter antarcticus 307
AB681154 Thalassobacter stenotrophicus
FJ716900 uncultured bacterium
Z1-02717 (499 bp), Z4-19934 (480 bp) 0.2±0.1
FJ624296 uncultured bacterium
M59063 Roseobacter denitrificans OCh 114
DQ153130 uncultured alpha proteobacterium
AJ867253 Phaeobacter gallaeciensis
AB607867 Thalassobius mediterraneus
CP000376 Ruegeria sp. TM1040
CP000830 Dinoroseobacter shibae DFL 12
CP002568 Polymorphum gilvum SL003B-26A1
CP003147 Pseudovibrio sp. FO-BEG1
Alphaproteobacteria
FM178379 Aliivibrio salmonicida LFI1238
X74689 Vibrio aestuarianus
JF691588 Photobacterium sp. DSM 24471
CR378663 Photobacterium profundum
CP000462 Aeromonas hydrophila ATCC 7966
CP003171 Oceanimonas sp. GK1
DQ778094 Ferrimonas senticii
CP000510 Psychromonas ingrahamii 37
FJ822615 Psychromonas boydii
Z2-02713, Z1-06871 (694 bp), Z6-19651 (472 bp) 2.0±0.9
AB304805 Psychromonas aquimarina
Z4-17262 (814 bp), Z6-09228 (758 bp) 0.6±0.7
CP000083 Colwellia psychrerythraea 34H
Z2-02011 0.3±0.6 bin B
HM126873 uncultured bacterium
JN683977 uncultured bacterium
FJ973520 Nitrincola lacisaponensis
CP000749 Marinomonas sp. MWYL1
AB021419 Pseudomonas denitrificans
CP001157 Azotobacter vinelandii DJ
CP000934 Cellvibrio japonicus Ueda107
CP000155 Hahella chejuensis KCTC 2396
FN869568 Halomonas elongata DSM 2581
DQ062160 Allochromatium vinosum
AJ224438 Thiocystis violascens
FN293060 Thiocystis violacea
Sedimenticola selenatireducens strain (NR_041877.1)
EU709866 Thioalkalivibrio sp. ALN1
EU709863 Thioalkalivibrio sp. ALBR_X2
CP000544 Halorhodospira halophila SL1
CP000127 Nitrosococcus oceani ATCC 19707
Z4-08480 0.03±0.03 bin C
GU056102 uncultured bacterium
AF481118 Thioalkalispira microaerophila
FJ717190 uncultured bacterium
ABXT01000015 Methylophaga thiooxydans DMS010
Gammaproteobacteria
CP000116 Thiobacillus denitrificans ATCC 25259
CP001965 Sideroxydans lithotrophicus ES-1
AF110005 Thauera sp. MZ1T
Beta proteo bacteria
DQ357825 epsilon proteobacterium Oy-M7
Z1-00700, Z4-03490, Z5-02273 2.4±0.3
EF645952 uncultured bacterium
AF235116 uncultured epsilon proteobacterium KTc1160
FJ717083 uncultured bacterium
CP001999 Arcobacter nitrofigilis DSM 7299
L14627 Arcobacter nitrofigilis
Epsilon proteo bacteria
JF495273 uncultured bacterium
Z1-01097, Z2-02103, Z5-05356, EMIRGE 14±3 bin D
EU047541 uncultured sulfate-reducing bacterium
AM882605 uncultured delta proteobacterium
AF418176 Desulfotignum balticum
HQ622265 bacterium enrichment culture clone Tol_11
U85469 Desulfobacter sp. BG8
Z2-13262 (429 bp), Z5-04014, Z6-01639 2.4±0.4
FJ716962 uncultured bacterium
FJ717003 uncultured bacterium
AJ441316 Desulfobacula toluolica
CP001087 Desulfobacterium autotrophicum HRM2
AF022936 Desulfocella halophila
CP000859 Desulfococcus oleovorans Hxd3
CP001322 Desulfatibacillum alkenivorans AK-01
CP002085 Desulfarculus baarsii DSM 2075
AM086646 Desulfomonile tiedjei
JN511890 uncultured organism
JN442414 uncultured organism
X99707 Desulfofustis glycolicus
DQ079636 uncultured delta proteobacterium
Z2-16675 (202 bp), Z4-27485 (535 bp), Z4-34668 (408 bp) 0.3±0.2
HM128150 uncultured bacterium
CR522870 Desulfotalea psychrophila LSv54
X95180 Desulfobulbus elongatus
CP001940 Desulfurivibrio alkaliphilus AHT2
JQ287074 uncultured bacterium
X79412 Desulfuromusa bakii
AY062868 uncultured delta proteobacterium
Z3-00700, Z4-001064, EMIRGE 3.8±1.6 bin F
JN977296 uncultured bacterium
CP001124 Geobacter bemidjiensis Bem
CP000482 Pelobacter propionicus DSM 2379
CP001804 Haliangium ochraceum DSM 14365
Z3-06455 (688 bp), Z4-10418 (1188 bp), Z5-01315, Z6-00713 3.4±0.5 bin E
AF228128 Desulfovibrio sp. Pr1.2
Z1-17389 (249 bp), Z1-06746 (701bp), EMIRGE 0.4±0.2
AB470956 Desulfovibrio sp. J39
FJ202471 uncultured bacterium
HM852532 Desulfovibrio sp. C1TLV30
U90726 Desulfovibrio profundus
Z3-03652 (629 bp), Z3-06382 (414 bp), Z4-02954 (512 bp) 0.6±0.2
AB110541 Desulfovibrio portus
CP001320 Desulfovibrio desulfuricans ND132
CP002431 Desulfovibrio aespoeensis Aspo-2
U33316 Desulfovibrio sp.
Z4-05125 (1363 bp) 0.1±0.1
CP001649 Desulfovibrio salexigens DSM 2638
CP000527 Desulfovibrio vulgaris DP4
Z6-10581 (710 bp), Z2-00773 (964 bp) 1.4±0.2
JN470964 uncultured organism
CP001629 Desulfomicrobium baculatum DSM 4028
CP001734 Desulfohalobium retbaense DSM 5692
FJ748805 uncultured bacterium
FN549969 uncultured bacterium
Z4-03616 (907 bp) 0.1±0.1
Deltaproteobacteria
CP001696 Methanocaldococcus fervens AG86
AB603516 Methanocaldococcus jannaschii

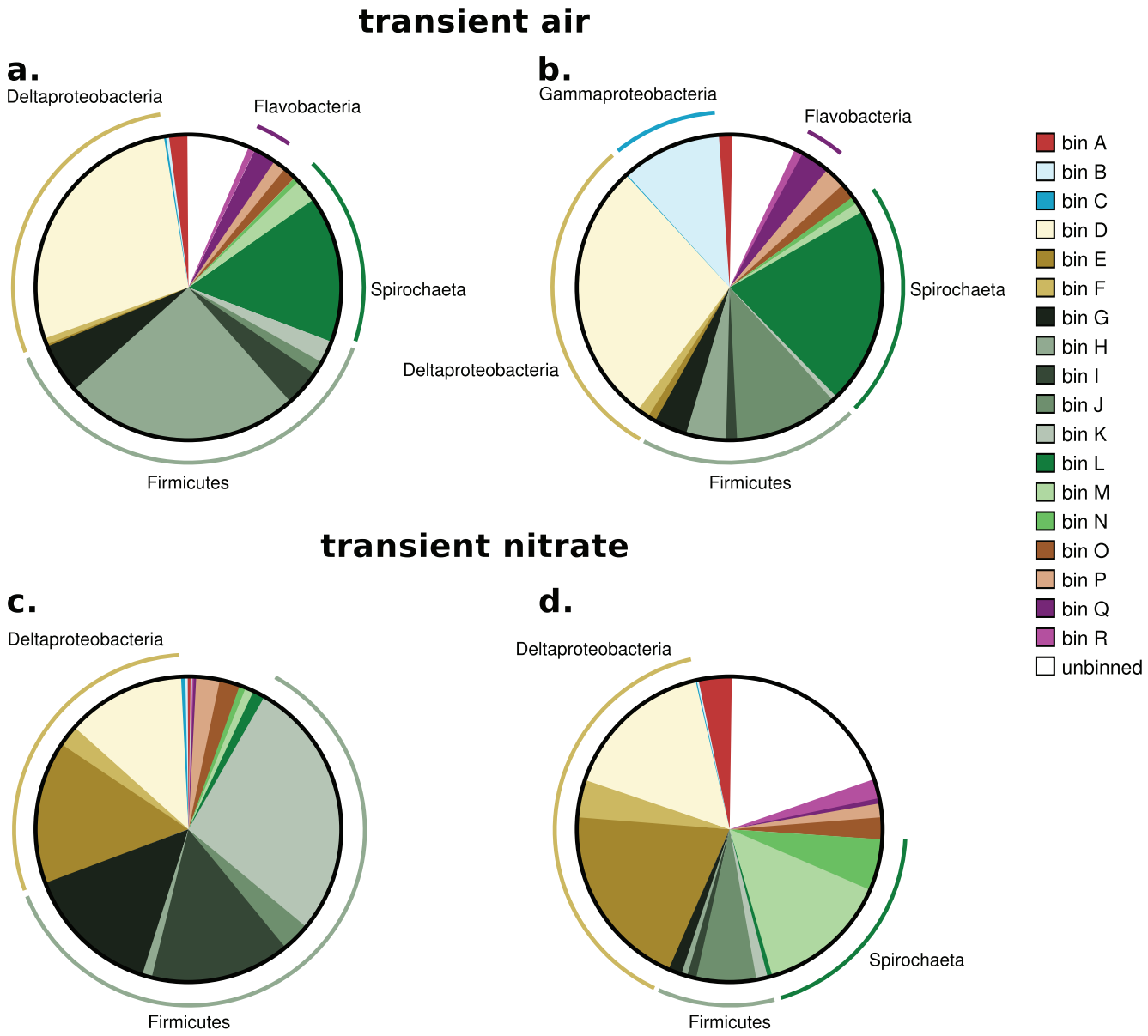
Firmicutes

0.02 changes per site (1283 positions)



0.02 changes per site (1534 positions)




transient air
a.
Deltaproteobacteria
Flavobacteria
Spirochaeta
Firmicutes
b.
Gammaproteobacteria
Flavobacteria
Spirochaeta
Deltaproteobacteria
Firmicutes
bin A
bin B
bin C
bin D
bin E
bin F
bin G
bin H
bin I
bin J
bin K
bin L
bin M
bin N
bin O
bin P
bin Q
bin R
unbinned
transient nitrate
c.
Deltaproteobacteria
Firmicutes
d.
Deltaproteobacteria
Spirochaeta
Firmicutes
sulfate only
e.
Deltaproteobacteria
Novel phylum
Firmicutes
Spirochaeta
f.
Deltaproteobacteria
Novel phylum
Firmicutes
Spirochaeta

The supplementary data of chapter 3 (Manuscript 2) is provided with thesis as USB stick.

Chapter 4

Manuscript 3

Chapter 4

Manuscript 3

"*Thammenomicrobia*": Insights into a novel bacterial phylum with widespread occurrence in anaerobic, organic-rich ecosystems

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My Contribution: Running the chemostat experiment, chemical analysis of sulfide, DNA extraction, writing cultivation method in the material and method part in addition to providing the sulfide figure.

In preparation

"Thammenomicrobia": Insights into a novel bacterial phylum with widespread occurrence in anaerobic, organic-rich ecosystems

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Running title: *Thammenomicrobia*, a novel widespread bacterial phylum

Keywords: continuous culture / sulfate reduction / fermentation / microbial community assembly / metagenomics / transcriptomics

Abstract

Continuous cultures combined with 'omics approaches have proven to be a powerful tool in resolving the identity, the metabolic capabilities and the physiology of many uncultured or unappreciated microbial lineages. Here we present detailed insights into a novel bacterial species termed "*Candidatus* Thammenomicrobium ektimisum", which dwells in anaerobic, sulfidic, organic-rich sediments and appears to be a non-motile, obligate fermenter. The organism was enriched in a continuous culture inoculated with sediment of the Janssand tidal flat located in the German Bight and was characterized based on metagenomics and substance concentrations in the culture. The metagenome of "*Candidatus* Thammenomicrobium ektimisum" provides a first glimpse into the metabolic capabilities of the novel phylum "*Thammenomicrobia*" (formerly candidate division Hyd24-12). Phylogeny based on a concatenated alignment of bacterial single copy genes suggests that the "*Thammenomicrobia*" are a basal phylum in the superphylum FCB (*Fibrobacteres*, *Chlorobi*, *Bacteroidetes*). 16S rRNA sequences affiliated to this phylum occur in methane- and/or organic-rich, sulfidic marine ecosystems worldwide, but also in anaerobic wastewater systems and long-term enrichment cultures favoring the anaerobic oxidation of methane (AOM). These results suggest an adaptation of the organism to fermentation of microbial exudates and metabolic byproducts in sulfide-rich environments and is a first step in resolving the role of heterotrophy at methane seep ecosystems and AOM enrichment cultures.

Introduction

Next generation sequencing and metagenomics have greatly improved our understanding of global microbial biodiversity in recent years (Gilbert and Dupont, 2011). The reconstruction of microbial genomes from environmental samples yielded unforeseeable insights into what was described as the „microbial dark matter" (Rinke *et al.*, 2013). Based on these genomes it becomes now possible to infer metabolic capabilities and indicate ecological roles of organisms and clades that so far can not be cultured(Iverson *et al.*, 2012). One prominent example of such an enigmatic clade is a candidate division that has been termed Hyd24-12 according to SILVA-based taxonomy (Quast *et al.*, 2013). This phylum-level clade contains sequences that were retrieved from marine ecosystems, such as cold seeps(Knittel *et al.*, 2003), mud volcanoes (Niemann *et al.*, 2006), methane hydrates(Mills *et al.*, 2005), organic-rich, anoxic shelf sediments (Elsabé M *et al.*, 2012) , anoxic hypersaline microbial mats (Schneider *et al.*, 2013) and associated to sponges (Simister *et al.*, 2011) . However, despite its worldwide occurrence in methane-rich and anoxic environments the clade Hyd24-12 so far lacks a cultured representative and its metabolic capabilities and ecological niche remain elusive. Organisms belonging to Hyd24-12 were also found in numerous long-term enrichment cultures under strict anaerobic conditions, where the primary electron donor was methane and the primary electron acceptor was sulfate. Although, these conditions favor the enrichment of organisms directly involved in the anaerobic oxidation of methane (AOM), such as anaerobic methane-oxidizing archaea (ANME) and sulfate-reducing bacteria (SRB) the enrichments also contained organisms that appear to have a heterotrophic

lifestyle (Wagner, Ruff et al., in preparation). These organisms may metabolize exudates of the autotrophic organisms or byproducts that are produced during AOM. The biogeochemistry and ecology of methane-fuelled ecosystems and the physiology of the organisms mediating the aerobic and anaerobic methanotrophy is subjected to intense research, which has yielded valuable insights into the relevance of these autotrophic organisms concerning the global carbon cycle and marine biodiversity. However, little is known about the heterotrophic organisms that also thrive at these widespread ecosystems. In addition, organisms belonging to candidate division Hyd24-12 were found in organic-rich anaerobic wastewater treatment systems and anoxic, sulfate-reducing enrichments of tidal marine sediments. Contrastingly, they were not detected in a broad set of environmental samples from oxic/suboxic ecosystems, such as coastal sands, deep-sea surface sediments and hydrothermal vents (Ruff, 2013). Despite these indications suggesting an anaerobic lifestyle, the assessment of the metabolic capabilities and possible ecological niche of Hyd24-12 remained a yet unaccomplished challenge. Here, we present first detailed insights concerning the metabolic capabilities, the physiology and the phylogenetic placement of an organisms belonging to this enigmatic clade using metagenomics of a continuous culture. Based on the data we propose that the widespread candidate division Hyd24-12 is a novel phylum of global occurrence, we indicate its placement it in the ever growing tree of life and suggest a name for the species as well as the phylum.

Materials and Methods

Sampling site and inoculum for enrichment experiments

Sediment was sampled from the intertidal back-barrier flat Janssand in the German Wadden Sea (53.73515 ’N, 007.69913’E) in June, 2012. From the upper part of the flat, the top 2 cm of sandy sediment was collected with a flat trowel. After transport of the sediment to the laboratory, an equal volume of artificial seawater (Red Sea Salt, 33.4 g/l; http://www.redseafish.com) was added to the sediment and stirred vigorously. The sediment was allowed to settle briefly, after which the liquid was transferred into (1 l) glass bottles that were closed with rubber stoppers and of which the headspace was exchanged with argon. The liquid was kept at 4°C for 2 days and then used as inoculum.

Continuous culture setup and medium

The continuous culture consisted of a glass vessel (DURAN, GLS 80, 500 ml) and was filled with (0.4 l) inoculum. The vessel was fitted with tubes for inflowing medium, outflowing medium, inflowing gas and outflowing gas. The culture was mixed with a magnetic stirrer (2 Mag Magnetic Motion Mixdrive 1 EXO) at 200 to 400 rpm. After inoculation, oxygen was removed from the culture headspace (0.4 l) by flushing with Argon (10 ml/min) for two days with a mass flow controller (Alicat Scientific). During the first month of cultivation, the culture was operated in repeated fed batch mode. Fresh medium was supplied continuously for 2 days at (0.17 /day). Next 0.34 l of spent medium

were replaced with Argon with a mass flow controller (Alicat Scientific). After medium removal, to maintain anoxic conditions, Argon (10 ml/min) was supplied to the culture for an additional 1 h without medium removal. After one month and onward, the culture was operated as a chemostat with continuous removal of spent medium via an overflow. The medium supply rate remained the same (0.17 l/day), resulting in a dilution rate of 0.36-0.4day^{-1}. During the first 54 days, the medium consisted of Red Sea Salt artificial seawater (33.4 g l^{-1})) which contains 28 mM sulfate, supplemented with 20 C-mM organic carbon. The organic carbon mixture consisted of 1.1 mM D-glucose, 1.7 mM acetic acid and a mixture of amino acids (in mM L-glutamic acid 0.38, L-aspartic acid 0.65, L-alanine 0.85, L-serine 0.46, L-tyrosine 0.099, L-histidine 0.035, L-methionine 0.088). In addition, 0.2 mM Na-phosphate, 17 μM $FeSO_4$, 0.2 ml l^{-1} trace element solution (in mg l^{-1}, $ZnCl_2$ 69, $MnCl_2$×4 H_2O 100, H_3BO_3 60, $CoCl_2$×6 H_2O 120, $CuCl_2$×2 H_2O 10, $NiCl_2$×6 H_2O 25, Na_2MoO4×2 H_2O 25, $AlCl_3$×6 H_2O 25) in 0.1% HCl and 0.2 ml l^{-1} Se/W solution (in mg l^{-1}, Na_2SeO_3×H_2O 6, Na_2WO_4×2 H_2O 8) in 0.04% NaOH were added . The medium also contained 20 mM of 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) to buffer the pH of the culture. The pH of the medium was adjusted to pH 7.5. Medium was supplied via a sterile glass back growth buffer in order to prevent contamination of the inflowing medium. After day 54 and onwards, back growth of bacteria into the medium supply tubing was prevented completely by separating the inflowing medium into two parts that were supplied simultaneously to each culture. One part contained the HEPES buffer and the other part all other medium components at pH 4. The culture pH was measured off line and with pH meter (Mettler Toledo, Five Easy ^{tm}) and was in the range of 7.5 to 7.8 . The supply rate of both parts

was modified to maintain the dilution rate at 0.36-0.4 day^{-1}. The OD600 of all cultures was monitored off line spectrophotometrically (Thermo Scientific Genesys 10S UV-Vis). Hydrogen sulfide concentration in liquid was monitored colorimetrically in culture samples as previously described. (Cline, 1969).

Metagenomics

On day 312, DNA was extracted from a 10 ml sample of sample taken from the culture according to (Zhou *et al.*, 1996). Following incubation in extraction buffer with lysozyme (2.5 mg ml^{-1}), RNAse (0.1 mg ml^{-1}). For metagenome shotgun sequencing, 1.5 µg of the extracted DNA were mechanically fragmented using Nebulizers (Roche) with 32 psi applied for 3 min, in 500 µl nebulization buffer (Roche). The fragmented DNA was purified using MinElute PCR purification columns (Qiagen) and eluted in 50 µl low TE (Life Technologies). The entire eluate was used for the preparation of barcoded PGM sequencing libraries with the Ion Xpress™ Plus gDNA Fragment Library Preparation kit (Life Technologies). Library insert sizes were between 350 and 400 basepairs (bp). The Library were sequenced with the Personal Genome Sequencer (PGM) on a 318 Chip (pooled with other samples), using the chemistry for 400 bp libraries. Base calling was performed with the Torrent Suite v3.6 (DNA 5-6) software, with default settings. Reads of the sequenced DNA samples were assembled with the Newbler assembler (v. 2.8) with default settings for genomic DNA assembly for nonpaired reads.

Generated cDNA libraries were sequenced with the Personal Genome Sequencer (PGM) on 318 Chips (pooled with other samples), using the chemistry for 200 bp libraries. Base calling was performed with the Torrent Suite v3.6 software, with default settings. Contigs were binned based on multivariate statistics of tetranucleotide frequencies with MetaWatt v2.1 . Genome completeness was evaluated by detection of a set of 139 conserved single copy genes with hidden Markov models (HMMER 3.1)(Campbell *et al.*, 2013) and by detection of transfer RNA genes (Laslett and Canback, 2004). Genes present in bin X were annotated with prokka 1.9 (Seemann, 2014).

Phylogenetic tree reconstruction

Phylogenetic affiliation was analyzed using the software package ARB based on the non-redundant SILVA small subunit reference database release 115 (July 2013). 16S rRNA gene sequences were screened for chimeras using the software Mallard, they were aligned using SINA and the alignment was manually optimized according to the secondary structure. The 16S rRNA phylogenetic tree was calculated using a maximum-likelihood algorithm (PHYML), a positional variability filter excluding highly variable regions and 100 bootstraps. For tree calculation, only nearly full-length sequences (>1300 bases) were considered.

Phylogenomic tree reconstruction

Phylogenomic affiliation of the novel clade was calculated based on 37 bacterial single copy genes found in bin X. Only three bacterial single copy genes were not detected according to the list of marker genes provided by Phylosift v1.0.1. (Darling *et al.*, 2014). The extraction of single copy marker genes from the contigs and the subsequent concatenated alignment was performed using PhyloSift v1.0.1 and the alignment was masked with ZORRO . The phylogenomic tree was calculated using the settings for slow and accurate maximum likelihood and minimal evolution implemented in FastTree v2.1.7 . Subsequently, the tree was optimized with ARB and the leaves were grouped according to their phylogenetic affiliation on phylum level..

Sequence submission

Data submission to 16S rRNA sequence databases and short read archives is in progress.

Results and Discussion

Physiology of the continuous culture

A 0.4 l continuous culture was inoculated with 400 ml extracted cells from the upper 2 cm of a tidal flat sediment. The continuous culture was initially operated in repeated fed batch mode for one month. After that it was operated as a chemostat for an additional 12 months. At all times, it was fed with a marine mineral medium at a dilution rate of 0.4 day^{-1}. The medium contained glucose, aminoacids and acetate as the electron donors and excess sulfate as the only electron acceptor. During the first 150 days, the concentration of sulfide in the culture gradually increased until it stabilized at 6 mmol/l (Figure 1). This indicated that continuous culture selected for a microbial community that performed sulfate reduction. Sulfate reducing bacteria usually oxidize fatty acids, lactate, alcohols or hydrogen as carbon and energy sources (Rabus *et al.*, 2013). Therefore, it was expected that the selected community consisted of fermentative and sulfate-reducing populations. The fermenting bacteria would convert the supplied glucose and at least part of the supplied amino acids into fermentation products which could then be used by the sulfate reducers.

Phylogeny of bin X

To identify the fermentative and sulfate-reducing populations and determine their metabolic potential, total DNA was extracted from the culture on day 312 (Figure 1),. The DNA was sequenced on an ion torrent personal genome machine, yielding 2,203,064 reads with an average read length of 271 nucleotides. These reads were assembled into

23,303 contigs with an N50 length of 3,809 basepairs at an average sequence coverage of 9.9×. Figure 2 shows the distribution of the assembled contigs on a percent GC versus sequencing coverage plot. Five distinct "clouds" of contigs were observed that likely originated from a specific population. Phylogenetic classification of the open reading frames (ORFs) predicted for each of the contigs of these clouds showed that the contigs at (53.5 % GC, 28× coverage) were affiliated with *Spirochaetales*, the contigs at (51.9% GC, 22× coverage) were affiliated with *Desulfobacterales*. Interestingly, at (56.6 % GC, 17× coverage) a cloud was observed that was characterized by an inconsistent phylogenetic profile (cloud X in Fig. 2). Less than 10% of the ORFs could be classified and those that could be classified were affiliated with distantly related bacterial lineages. This indicated that these contigs might originate from a population that was completely unrelated to any microorganism in the database used for classification. Alternatively, these contigs might originate from different unrelated populations. However, because the contigs in this cloud were very long (N50 contig length 220 kb) this possibility was unlikely.

To acquire additional clues on the phylogenetic origin of these contigs, 16S rRNA sequences were detected in the assembled contigs and, independently, reconstructed from the sequencing reads by iterative read mapping with Emirge (Miller *et al.*, 2011). With both procedures an almost identical 16S rRNA gene sequence was found that was affiliated with a candidate division so far known as "Hyd24-12" in the SILVA taxonomy . Other sequences in clade Hyd24-12, were only detected in anoxic environments and seem to form at least four subclades (Figure 3). The phylogeny of other 16S rRNA genes recovered was consistent with the phylogenetic profiles of the other clouds in Figure 2

and did not provide any support for the possibility that the contigs in cloud X were of mixed phylogenetic origin. The assembled 16S rRNA gene affiliated with Hyd24-12 was present on contig00791 (5419 bp) with a GC content of 52.3% and a sequencing coverage of 36×, well outside the range of cloud X or any of the other clouds on Figure 2. However, no conclusion could be drawn from this because 16S rRNA genes have atypical nucleotide compositions and usually a higher coverage, because often a genome contains multiple 16S rRNA genes. Thus, it was most likely that the contigs of cloud X constituted a provisional whole genome sequence of an uncultivated representative of candidate phylum Hyd24-12.

To identify all the relevant contigs independent of sequencing coverage, all contigs were binned based on multivariate statistics of tetranucleotide frequencies. All contigs present in cloud X were binned to a single bin of 96 contigs (longest contig 538 kb), independent of the confidence threshold of the binner. Out of 139 conserved single copy genes present in most bacteria, 98 genes were detected in the bin and only 3 duplicates were detected. A set of 39 transfer RNA genes were detected. Mapping of reads to contigs showed that these contigs were highly interconnected, with many reads mapping to multiple contigs inside this bin and only very few reads mapping to contigs inside and outside this bin. Together these data provide strong evidence that indeed this bin represents a provisional genome sequence of a single population.

To investigate whether this population might represent a candidate division we extracted the aminoacid sequences of 37 bacterial single copy genes found on contigs in bin X. This provided additional evidence that most parts of the genome were covered by the metagenomic analysis, since the list of marker genes provided by PhyloSift v1.0.1.

(Darling *et al.*, 2014) contains 40 bacterial single copy genes. Phylogenetic analysis was performed based on a concatenated alignment of the amino acid sequences of these genes together with over 4000 gene sequences provided by PhyloSift representing the major bacterial biodiversity. The analysis resulted in a tree strongly supported by bootstrap analysis. The placement of Hyd24-12 suggested that this clade is a novel bacterial phylum that seems to be a basal lineage in the superphylum FCB (*Fibrobacteres*, *Chlorobi*, *Bacteroidetes*) (Figure 4). This observation is supported by a phylogentic tree based on the DNA dependent RNA polymerase encoded by the genes *rpoABC* which were located on contig00003 (495,502 bp) (not shown). The nearest sister phylum of Hyd24-12 is *Cloacimonetes* and the overall topology of FCB is very similar to a previously proposed marker gene based phylogeny (Rinke *et al.*, 2013). It has to be noted that phylogeny based on 16S rRNA gene identity also suggested that Hyd24-12 is a phylum level clade, however its affiliation differed from the one in the marker gene based tree as its closest relatives were candidate division BRC1 and *Lentisphaeria* (not shown).

Metabolic capabilities found in bin X

Annotation of the genes encoded on the contigs of bin X suggested that cells of the corresponding population had a typical gram negative cell envelope with a complete peptidoglycan biosynthesis pathway and an active outer membrane transport system (tonB/ExbBD). Glycolysis and the pentose phosphate pathway were complete. The presence of most essential genes involved in lipid biosynthesis, amino acid metabolism and nucleotide metabolism indicated that these bacteria were not dependent on others for the generation of the core building blocks of their cells. However, they were unable to

synthesize quinols and heme and lacked a respiratory chain. The ATP synthase was of the vacuolar type, indicating that they were unable to synthesize ATP by oxidative phosphorylation. The citric acid cycle was incomplete and flagellar biosynthesis was absent. Apparently, these cells were non-motile and obligate fermenters. It's nearest relative *Candidatus* Cloacamonas acidaminivorans (Pelletier *et al.*, 2008) was found in an anaerobic, organic-rich wastewater treatment and seems to derive all its energy from the conversion of amino acids , suggesting a similar lifestyle.

Conclusion

Based on the provided data we suggest a provisional novel species "*Candidatus* Thammenomicrobium ektimisum" and a novel phylum "Thammenomicrobia". "*Candidatus* Thammenomicrobium ektimisum" (*thammeno* (gr.) – buried, *ektimisa* (gr.) – appreciated) is an anaerobic, apparently obligate facultative organism, which seems to preferably occur in methane- and/or organic-rich, sulfidic sediments, such as methane cold seeps. *Thammenomicrobia* are distributed globally in these seep ecosystems and may thus represent important players in geochemical cycles that were so far overlooked..

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[Figure legends]

Figure 1. Sulfide production with time in cultures only supplied with sulfate as electron acceptor.

Figure 2. (left) Assignment of "clouds" on the GC versus coverage plot of assembled contigs after binning. (right) Contig length distribution and phylogenetic profile (family level) of each of the 18 bins obtained. Median e values for each family are indicated.

Figure 3. Phylogenetic affiliation of 16S rRNA gene sequences within the "*Thammenomicrobia*". 16S rRNA genes retrieved from the continuous culture metagenome and from two anaerobic methane-oxidizing (AOM) enrichment cultures are shown in bold. The topology and estimated sequence divergence suggests that at least four order-level clades exist within the new phylum. The origin of the sequences in each subclade may indicate slightly different physiologies as they were predominantly found in certain ecosystems. The scale bar shows 10% estimated sequence divergence.

Figure 4. Phylogenomic affiliation of "*Thammenomicrobia*" based on a maximum likelihood tree calculated using a concatenated alignment of 37 bacterial single copy genes. The tree indicates that the novel phylum is part of the superphylum FCB (*Fibrobacteres*, *Chlorobi*, *Bacteroidetes*). * Tree continues towards *Proteobacteria*, *Firmicutes* and *Actinobacteria*. ** Tree continues towards Outgroup, The scalebar indicates 10% estimated sequence divergence.

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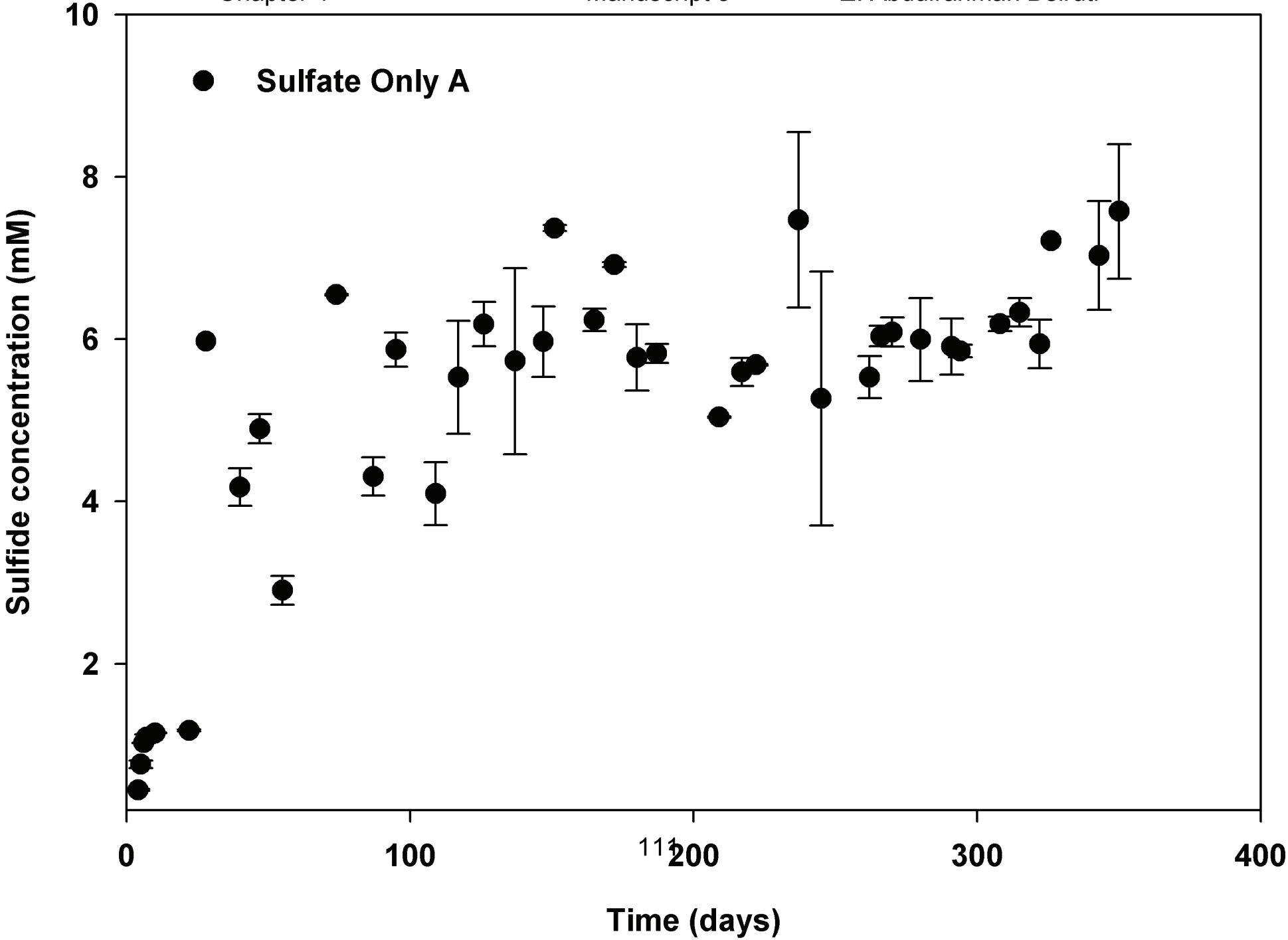
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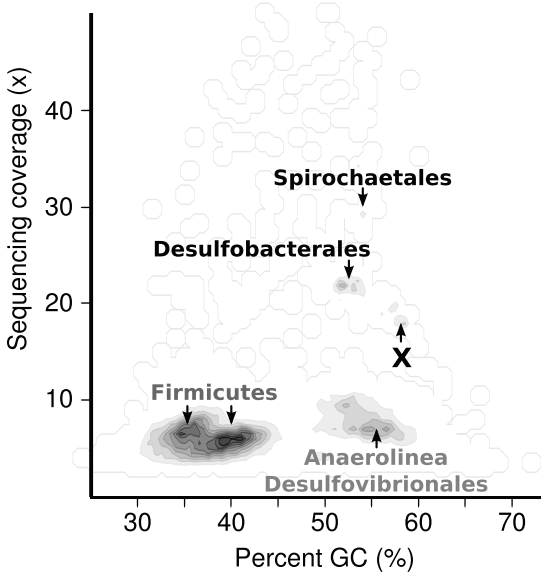
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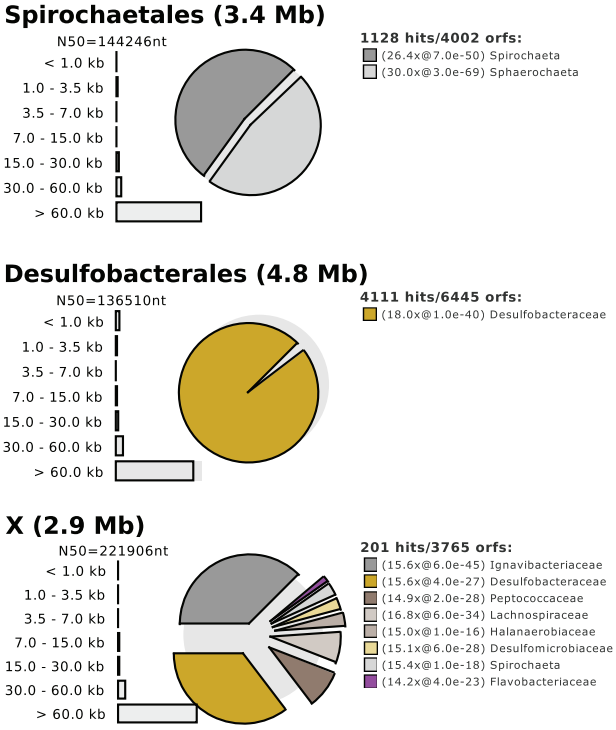
Percent GC (%)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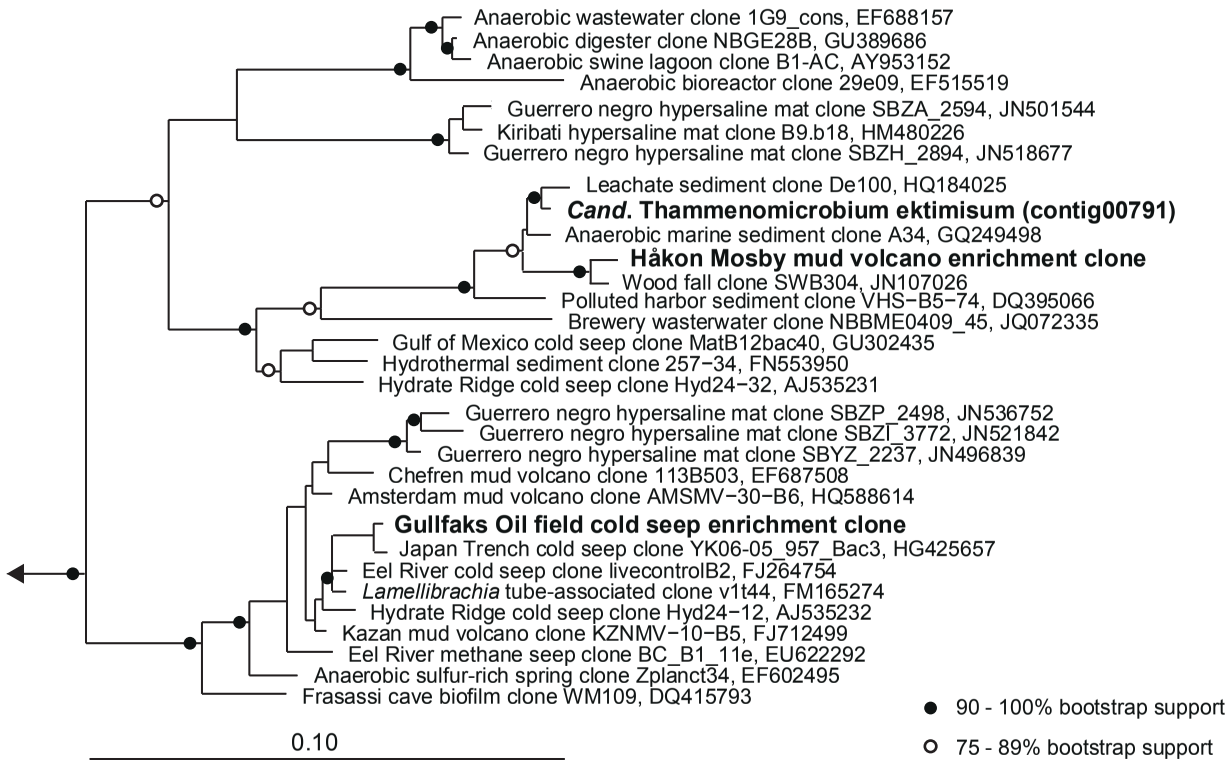
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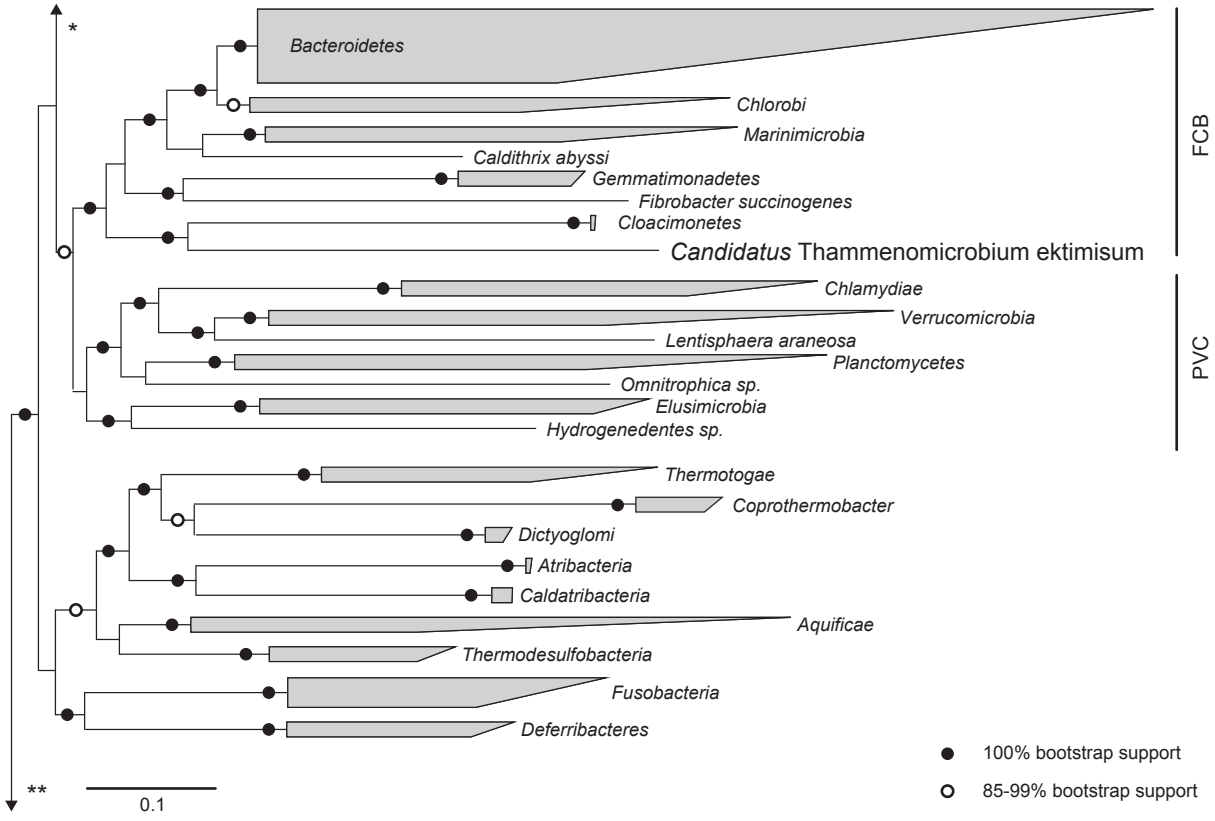
75 - 89% bootstrap support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Chapter 5

General Discussion and Outlook

Chapter 5

General Discussion and outlook

This thesis describes the enrichment of consortia consisting of sulfate reducing and fermentative bacteria with continuous cultivation. The cultures were inoculated with bacteria, mechanically extracted, from the top 2 cm of the intertidal sand flats Janssand in the German Wadden Sea. This habitat is characterized by tidal pumping: During high tide, oxic seawater percolates through the sediment and stimulates aerobic metabolism. During low tide, the pore water does not flow, leading to the rapid depletion of oxygen and so enabling anaerobic metabolism. Locally and temporarily oxygen may still be provided during low tide by water movement facilitated by the benthic fauna. Thus, this is a highly dynamic habitat, not a typical habitat that would support large numbers of sulfate reducing or fermentative bacteria, yet they have been detected in these top sediments (Mußmann *et al.*, 2005a; Billerbeck *et al.*, 2006; Gittel *et al.*, 2008; Jansen *et al.*, 2009)

The inocula for the two sets of experiments were collected in March 2011, around the spring bloom and June 2012, at the onset of summer. The two sets of experiments were performed in slightly different ways. The continuous cultures started in March (Chapter 2) were mixed vigorously by re-circulating gas from the culture headspace, leading to a large potential exchange of volatile compounds (e.g. hydrogen) between the culture liquid and gas, potentially leading to the loss of these compounds from the cultures. Indeed, this loss was observed for hydrogen sulfide, resulting in a reduced sulfide concentration inside the culture (~3.4 mM). The continuous cultures started in June (Chapters 3 and 4) were mixed less vigorously by magnetic stirring with a much

reduced potential for the loss of volatile compounds to the gas. The resulting sulfide concentrations were higher in this case (up to 7 mM). Other differences between the two sets of experiments were the presence of HEPES to the medium, use of an initial repeated- fed-batch phase, and a different period of time between sediment sampling and inoculation (6 or 2 days). Previous studies form (Gittel *et al.*, 2008) showed a difference in the sulfate concentration of the surface layers with the different seasons. Also the abundance of microbial communities in Janssand like *Roseobacte*r showed also variance during the difference seasons (Lenk *et al.*, 2012). So, together with the experimental differences different sampling times may have contributed to the differences in outcome between experiments.

Continuous cultivation was used to engineer a meaningful and defined habitat in the laboratory that enabled a stable selective pressure (Herbert *et al.*, 1956). The experimental setup selected for suspended cells, whereas in situ the cells were known to be present as biofilms on sand grains. One can argue that with this difference the environmental conditions in the culture can never be “meaningful”. However, the experimental setup was at least a step forward compared to the batch cultures that are still commonly used for the enrichment of bacteria: First, all cultures were supplied with a seawater medium that contained a mixture of 9 different carbon compounds as the carbon and energy source. This mixture consisted of glucose, acetate and seven different amino acids, in a ratio that mimicked the composition of decaying biomass, the main carbon and energy source in situ, in terms of its monomers. This enabled the co-enrichment of fermentative and sulfate reducing bacteria, two ecological guilds that also interact in natural habitats. Second, the carbon mixture was the controlling or limiting factor in the

continuous culture. Sulfate was present in excess. This led to very low substrate concentrations in the culture, much lower than can be achieved with batch cultures and enabled the selection of K-strategists (oligotrophs) and against R-strategists, also known as "microbial weeds". In continuous culture, the growth rate of the cultivated bacteria is under experimental control. In the experiments of this thesis, the dilution rate was ~0.4 day^{-1}, equivalent to a doubling time of 42 h, much longer than typically found for pure cultures and also longer than the average doubling time estimated for the sampling site (~10 h).

All experiments were performed in duplicate and the metagenomic analysis of the two replicates showed that in all cases the dominant populations performing sulfate reduction were identical between replicates. This indicated that the environmental conditions exerted a strong selective force within that ecological niche. A population of *Desulfovibrio* dominated the March set of experiments and a population related to *Desulfotignum balticum* the June experiments. However, the fermentative populations enriched in replicate cultures were more variable. Multiple fermentative populations competed or at least co-existed without any apparent selective force of the conditions applied. The abundant fermentative populations consisted of different Firmicutes, Spirochaetales and a representive of the candidate division Hyd24-12. *Desulfovibrio* populations were previously found *in situ* (in low numbers) at the sampling site (Mußmann *et al.*, 2005a). According to (Gittel *et al.*, 2008) the most abundant SRB *in situ* were related to Desulfobulbaceae and Desulfobacteriace. Firmicutes were only detected in the deeper layers of the sampled sediment, and not in the surface sediments (Gittel, 2007; Gittel *et al.*, 2008). The microbial communities enriched in the continuous

cultures do not represent the abundant populations of the sampling site. The selection of Firmicutes might be caused be the supply of monomeric carbon compounds. In situ, Flavobacteria are the most dominant potentially fermentative populations and they are known to take up higher molecular weight substrates. It is conceivable that the ecological success of Firmicutes in the cultures led to the selection of sulfate reducing populations different from the ones that are predominant in situ. For example, it might be possible that the indigenous Flavobacteria produce lactate and formate instead of hydrogen (see below) as fermentation products, as was recently shown for *Formosa agarophila* (Mann *et al.*, 2013).

In the March experiments, the relative abundances of fermentative and sulfate reducing bacteria were approximately 9:1. In the June experiments this ratio was approximately 3:1. It is possible that in the March experiments, hydrogen was produced as a fermentation product and was partially lost to the gas, leading to a lower availability of electron donors for the sulfate reducers and explaining their low relative abundance in these experiments. Although transcriptomic analyses were not performed in this case, at least the fermentative populations were capable of hydrogen production and the enriched *Desulfovibrio* population was capable of hydrogen oxidation, as shown by the metagenomics analyses. For the June experiments, hydrogen production and consumption was supported by transcriptomics analyses. It was difficult to infer from the metagenomes and transcriptomes which other fermentation products were exchanged between the fermentative and sulfate reducing populations. At least production of acetate was supported by the active transcription of acyl phosphatases and no evidence was

found for production of formate (e.g. presence and activity of genes encoding pyruvate formate lyase).

The transcriptionally highly active acetyl-CoA pathway for autotrophic carbon dioxide fixation detected for the dominant sulfate reducer of the June experiments was certainly one of the most surprising observations of this thesis. This result suggested that this population, affiliated with *D. balticum*, assimilated carbon dioxide as a significant carbon source. It also suggested that hydrogen must have been a major, if not the main fermentation product. Fermentation of most of the carbon provided in the form of glucose and amino acids into hydrogen and carbon dioxide would lead to a highly uneven ratio in abundance between fermentative and sulfate reducing populations, as shown in chapter two with a stoichiometric model of the metabolism of the complete consortium. However, the higher abundance of sulfate reducers observed in chapter 3 actually suggests that, apart from carbon dioxide, the sulfate reducers also assimilated organic molecules, e.g. acetate, succinate and lactate.

Even though sulfate reducing bacteria were found in the upper 2 cm of tidal flat sediments (Gittel *et al.*, 2008), they are generally known as strictly anaerobes (Muyzer and Stams, 2008; Rabus *et al.*, 2013). The tolerance and the survival of sulfate reducing bacteria in the presence of oxygen is thus an ecologically and biochemically relevant topic (Rabus *et al.*, 2013). Oxygen can directly suppress sulfate reduction by deactivation of sensitive enzyme systems and/or by enabling growth of aerobic bacteria that outcompete sulfate reducers for substrates (Rabus *et al.*, 2013). Oxygen can suppress activity or only the growth of sulfate reducing bacteria. It was previously found that at a concentration of 15 μM of oxygen, the activity of four different strains was negatively

affected (Marschall *et al.*, 1993) and the growth of *Desulfovibrio vulgaris* was completely arrested at an oxygen concentration of 1 μM (Johnson *et al.*, 1997) . In chapter 3 of this thesis it was shown that transient exposure to oxygen (3 μM for 30 min, in the presence of ~ 2 mM sulfide) did not affect gene transcription of the sulfate reducing populations related to *Desulfotignum* and *Desulfovibrio* that were enriched under these transiently oxic conditions. Furthermore, the relative abundance of sulfate reducers in the transiently oxic cultures was slightly higher than in the control indicating that also their growth was uncompromised by oxidative stress. The lower sulfide concentrations most likely resulted from re-oxidation of the sulfide to elemental sulfur and/or sulfate by small populations of co-enriched Proteobacteria. The transient nature of the exposure (only 30 min) might have limited its impact, compared to what was reported in previous studies.

Lower sulfide concentrations were, to a lesser extent, also observed in cultures transiently exposed to nitrate, However, in those cultures sulfide might have been re-oxidized to sulfur by a population related to *Desulfuromusa bakii*, which, based on transcriptomics analyses, also performed ammonification of the supplied nitrate to ammonia. *Desulfuromusa bakii* and related bacteria are known as sulfur-reducing and facultatively fermentative bacteria (Liesack and Finster, 1994) . This population was incapable of sulfate reduction, enriched in both replicates, and, based on its strong transcriptional response to nitrate, it is likely that it performed fermentation of amino acids and/or dicarboxylates in the absence of nitrate. Rabus et al, (2013) reviewed the capability to perform ammonification reported for some strains of sulfate reducing bacteria (Keith and Herbert, 1983; McCready *et al.*, 1983; Mitchell *et al.*, 1986; Seitz and Cypionka, 1986), such as *Desulfobulbus propionicus* (Widdel and Pfennig, 1982) and

Desulfobacterium catecholicum (Szewzyk and Pfennig, 1987). Apparently, the selective enrichment described in this thesis produced communities with separation of sulfate reduction and ammonification to different populations. As was the case for oxygen, no negative effect of the presence of nitrate (0.1-0.5 mM for 2 h) was observed for the sulfate reducing populations, as gene transcription was unaffected and relative population abundances were similar to the control experiments. Previously 0.46 mM of nitrate was shown to reduce the growth rate of *Desulfovibrio desulfuricans* by 90% (Dalsgaard and Bak, 1994). The lack of an apparent effect reported in Chapter 3 might be explained by the transient nature of the exposure and the fact that populations of sulfate reducing bacteria were selected that were able to cope with these conditions. On the other hand, the same sulfate reducing bacteria were selected without the transient nitrate exposures.

Chapter 4 describes the enrichment and characterization of "*Candidatus* Thammenomicrobium ektimisum", a representative of the candidate division known as Hyd24-12. Phylogenomic analysis showed that this clade of so far uncultivated bacteria was a sister clade to Chlorobia/Flavobacteria/Bacteroidetes and distantly related to Cloacimonetes. It was only enriched in the complete absence of oxygen and nitrate indicating a strictly anaerobic lifestyle, even though it was enriched from a periodically oxic habitat (Billerbeck *et al.*, 2006; Gao *et al.*, 2009). Annotation of its provisional whole genome sequence showed that this organism was obligatory fermentative. It is interesting to note that the current exploration of "microbial dark matter" yields so many new obligatory fermentative species. This phylum-level Hyd24-12 clade appears to have a global distribution with sequences retrieved from many anoxic marine ecosystems such as cold seeps (Knittel *et al.*, 2003), mud volcanoes (Niemann *et al.*, 2006), methane

hydrates (Mills *et al.*, 2005), organic-rich shelf sediments (Elsabé M *et al.*, 2012), anoxic hyper saline microbial mats (Harris *et al.*, 2012; Schneider *et al.*, 2013) and associated to sponges (Simister *et al.*, 2011).

Overall, the results of this thesis show that continuous cultivation in combination with metagenomics and transcriptomics enable the meaningful study of consortia of sulfate reducing and fermentative bacteria, without their isolation in pure culture. Continuous cultivation was essential to create defined and stable environmental conditions defining a known selective force. This was the first study of its kind and the conditions so far did not yield communities close to those found in the natural habitat that serves as the inoculum. In future studies the carbon monomers could be replaced with polymers, such as polysaccharides and proteins. Most likely, this will select for different fermenting populations which may in turn lead to the selection of different sulfate reducers. The most striking observation so far was the detection of carbon dioxide assimilation by sulfate reducers despite the fact that the consortium as a whole grew heterotrophically. In follow up studies, autotrophic growth of sulfate reducing bacteria could assessed more directly in natural samples, for example with nanosims or stable isotope probing. The results also showed that presence of oxygen or nitrate may be less of a problem to "strict" anaerobes in mixed cultures or natural samples than was previously inferred in pure culture studies (Rabus *et al.*, 2013). The recently discovered occurrence of so called "cryptic sulfur cycling" supports this conclusion (Alam *et al.*, 2012). Finally, the enrichment of strict anaerobes like "*Candidatus* Thammenomicrobium ektimisum" from an at least transiently oxic habitat shows the power of the selective force that can be enforced during laboratory enrichment and shows that, although "everything is every

where" is most probably an overstatement, dispersion of microbes in nature is a prevailing phenomenon, and many things are in many places.

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Erklärung

Hiermit erkläre ich, dass ich die vorgelegte Dissertation selbständig und ohne unzulässige fremde Hilfe angefertigt und verfasst habe, dass alle Hilfsmittel und sonstigen Hilfen angegeben und dass alle Stellen, die ich wörtlich oder dem Sinne nach aus anderen Veröffentlichungen entnommen habe, kenntlich gemacht worden sind;

Zainab Abdulrahman Beiruti

Bremen, 22-Juli-2014

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