Role of chemolithoautotrophic microorganisms involved in nitrogen and sulfur cycling in coastal marine sediments

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De rol van chemolithoautotrofe micro-organismen die deelnemen in de stikstof- en zwavelcyclus in mariene kustsedimenten

(met een samenvatting in het Nederlands)

Proefschrift

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„Hinterher ist man immer schlauer…“

Für meine Familie

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1. Introduction

Marine sediments are dynamic habitats shaped by interactions of biotic and abiotic processes including the redox reactions performed by microorganisms to gain energy for growth (Schrenk et al., 2010). The biogeochemical cycling of carbon-, nitrogen- and sulfur compounds in marine sediments is tightly coupled in space and time. Due to electron donor and acceptor availability, an overlap of the activity of metabolically diverse microorganisms involved in carbon-, nitrogen- and sulfur cycling occurs.

Aerobic mineralization processes of organic matter reaching the sediment surface are mainly mediated by the metabolism of phototrophic and chemoorganoheterotrophic microorganisms. These processes result in the consumption of oxygen (O_2) within the first few millimeters/centimeters of marine sediments. Underneath the O_2 penetration depth, anaerobic mineralization processes occur due to the vertical distribution of electron acceptors in the sediment: O_2 → nitrate (NO_3^-) → manganese (IV) oxide (MnO_2) → ferric iron (Fe^{3+}) → sulfate (SO_4^{2-}) → carbon dioxide/bicarbonate (CO_2/HCO_3^-) reflecting the gradual decrease of the redox potential. Most microorganisms involved in anaerobic mineralization processes grow chemoorganoheterotrophically, such as most denitrifying bacteria, manganese- or iron reducing bacteria, most sulfate reducing bacteria, fermenting bacteria, methane oxidizing bacteria and archaea. As a result, reduced inorganic compounds, such as ammonium (NH_4^+), nitrite (NO_2^-), NO_3^-, ferrous iron (Fe^{2+}) and reduced sulfur species, such as sulfide (H_2S), elemental sulfur (S^0), iron sulfide (FeS), thiosulfate ($S_2O_3^{2-}$) and sulfite (SO_3^{2-}), accumulate in the sediment (Jørgensen, 2006; Herbert, 1999), which can subsequently be reoxidized by chemolithoautotrophic microorganisms.

Chemolithoautotrophic microorganisms (bacteria and archaea) gain energy for growth using chemical compounds as energy source (chemo-) in contrast to phototrophic organisms using light. Chemolithoautotrophs are able to use reduced inorganic compounds (litho-) as electron donor and inorganic carbon, CO_2 and/or HCO_3^-, as their only carbon source (-autotroph). The electron donors used by chemolithoautotrophic microorganisms comprise reduced nitrogen and sulfur compounds, as well as Fe^{2+}, H_2 and carbon monoxide (CO). Other microorganisms are able to assimilate organic carbon, while using inorganic electron donors, which is termed chemolithoheterotrophy, whereas those able to use inorganic or organic compounds as carbon source while utilizing inorganic electron donors are called mixotrophs. Many microorganisms use organic electron donors and fix organic carbon in a process known as chemoorganoheterotrophy. Most chemolithoautotrophs are facultative chemolithoautotrophs, i.e. are able to grow chemolithoautotrophically as well as using a different metabolism. As chemolithoautotrophs are not able to synthesize adenosine triphosphate (ATP) from nicotinamide adenine dinucleotide (NADH) oxidation, most obligate chemolitoautotrophs couple the oxidation of their electron donors to the reduction of quinone or cytochromes supplying reducing equivalents for carbon fixation and biosynthesis through reverse electron transport (Kim and Gadd, 2008). Many perform aerobic respiration using O_2, while anaerobic chemolithoautotrophs use NO_3^-, SO_4^{2-}, CO_2 or metal oxides as electron acceptors (Jørgensen, 2006).

Chemolithoautotrophic microorganisms are metabolically versatile and able to use a broad variety of electron donors for inorganic oxidation in coastal marine sediments (Table 1). Aerobic and anaerobic ammonia oxidizing bacteria, sulfide oxidizing and sulfate reducing bacteria are believed to be the main contributors to chemolithoautotrophic carbon fixation in coastal

Table 1: Versatility of chemolithoautotrophic reoxidation reactions/processes performed by microorganisms in coastal marine sediments (modified from Jørgensen, 2006).

Electron donating reaction	**Process**	**Chemolithoautotrophic microorganisms (e.g.)**
$NH_4^+ + {}^3/_2\ O_2 \rightarrow NO_2^- + 2H^+ + H_2O$	ammonia oxidation	ammonia oxidizing bacteria (AOB) (*Nitrosomonas*), ammonia oxidizing archaea (AOA) (*Nitrosopumilus*)
$NO_2^- + ½\ O_2 \rightarrow NO_3^-$	nitrite oxidation	nitrifying bacteria *(Thiobacillus, Nitrobacter)*
$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$	anaerobic ammonia oxidation	anammox bacteria (*Scalindua* spp.)
$H_2S + 2O_2 \rightarrow SO_4^{2-} + 2H^+$ (other possible donors: SO_3^{2-}, $S_2O_3^{2-}$, S^0)	sulfur oxidation	colorless sulfur bacteria (*Thiobacillus, Beggiatoa*)
$H_2S + NO_3^- + H^+ + H_2O \rightarrow SO_4^{2-} + NH_4^+$ (other possible donors: S^0, $S_2O_3^{2-}$)	sulfide oxidation coupled to nitrate reduction to ammonia (DNRA)	denitrifying sulfur bacteria (*Thiobacillus denitrificans*), nitrate reducing sulfur bacteria (*Thiomargarita*), large sulfur oxidizing bacteria (*Beggiatoa*)
$4Fe^{2+} + O_2 \rightarrow 4Fe^{3+} + 2H_2O$ (or Mn^{2+})	iron and manganese oxidation	iron bacteria (*Ferrobacillus*, *Shewanella*)
$2H_2 + O_2 \rightarrow 2H_2O$	hydrogen oxidation	hydrogen oxidizing bacteria (*Hydrogenobacter thermophilus*)
$4H_2 + SO_4^{2-} + 2H^+ \rightarrow 4H_2O + H_2S$	hydrogen oxidation coupled to sulfate reduction	some sulfate reducing bacteria (*Desulfovibrio spp.*)
$H_2 + CO_2 \rightarrow CH_4$	methanogenesis	methanogenic archaea
$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O$	acetogenesis	acetogenic bacteria

marine sediments. In addition, many archaea are chemolithoautotrophs and representatives of the phylum Thaumarchaeota (formerly known as 'marine group I Crenarchaeota') have been determined to oxidize ammonia to gain energy for inorganic carbon fixation (Wuchter et al., 2003 and 2006; Könneke 2005, Brochier-Armanet et al., 2008) suggesting the contribution of archaea to the reoxidation of reduced inorganic compounds and inorganic carbon fixation in coastal marine sediments.

Chemolithoautotrophy in coastal sediments is generally considered to be insignificant in terms of biogeochemistry, due to the low growth yields of these microorganisms and the competition with photo- and heterotrophic processes, which are the main source of labile organic matter in coastal sediments (Jørgensen and Nelson, 2004). However, although chemolithoautotrophic microorganisms might play a minor role in terms of growth, they are able to reoxidize important amounts of reduced inorganic substrates (Cypionka, 2006). The dependency of chemolithoautotrophic microorganisms on the availability of reduced inorganic compounds produced by chemoorganoheterotrophic microorganisms suggests a tight coupling between different microbial metabolic pathways in marine sediments. Unfortunately, direct measurements of chemolithoautotrophic activity are rare. Two rate measurements of dark CO_2 fixation in shallow subtidal sediments of the Baltic Sea suggest that up to 20% of the inorganic carbon produced by mineralization appears to be reoxidized by chemoautotrophic microorganisms (Enoksson and Samuelsson, 1987; Thomsen and Kristensen, 1997). Only recently, chemolithoautotrophic sulfur oxidizing Gammaproteobacteria have been shown to contribute substantially to CO_2 fixation by combined fluorescence *in situ* hybridization and microautoradiography (MAR FISH) analysis in the oxygen-, nitrate- and sulfide transition zone (0−3 cm) of intertidal sandy sediments (Lenk et al., 2011). Inorganic carbon fixation in coastal and ocean margin sediments, characterized by high organic carbon loadings, has been estimated to account for about 48% of the total oceanic chemoautotrophic carbon fixation rate (Middelburg, 2011). Considering the combination of high respiration rates and dominantly anoxic conditions, coastal and ocean margin sediments are presumably a hot spot for chemolithoautotrophy. The activity of chemolithoautotrophic microorganisms in coastal sediments has global implications for the cycling of carbon and the availability of nitrogen and sulfur compounds in marine environments. However, the diversity of chemolithoautotrophic microorganisms involved in nitrogen and sulfur cycling of coastal marine sediments, as well as the temporal and spatial distribution of their abundance and activity, is still poorly understood, especially in subsurface sediment layers. In the upper marine sediments, dark CO_2 fixation is believed to be predominantly driven by oxidation of reduced nitrogen and sulfur compounds.

1.1. Chemolithoautotrophic contribution to nitrogen cycling in marine coastal sediments

Inorganic nitrogen concentrations (NH_4^+, NO_3^-) in marine sediments are determined by the sedimentation and decomposition rate of organic matter, as well as by diffusive transport rates and bioturbation. The cycling of nitrogen in coastal marine sediments consists of a series redox reaction performed by phototrophic, heterotrophic and autotrophic microorganisms and underlies complex regulatory mechanisms, comprising physicochemical and biological factors (Herbert, 1999; Joye and Anderson, 2006). The complexity of the cycle is revealed in Fig. 1.

Nitrogen fixation, e.g. the conversion of molecular nitrogen (N_2) to bioavailable organic ni-

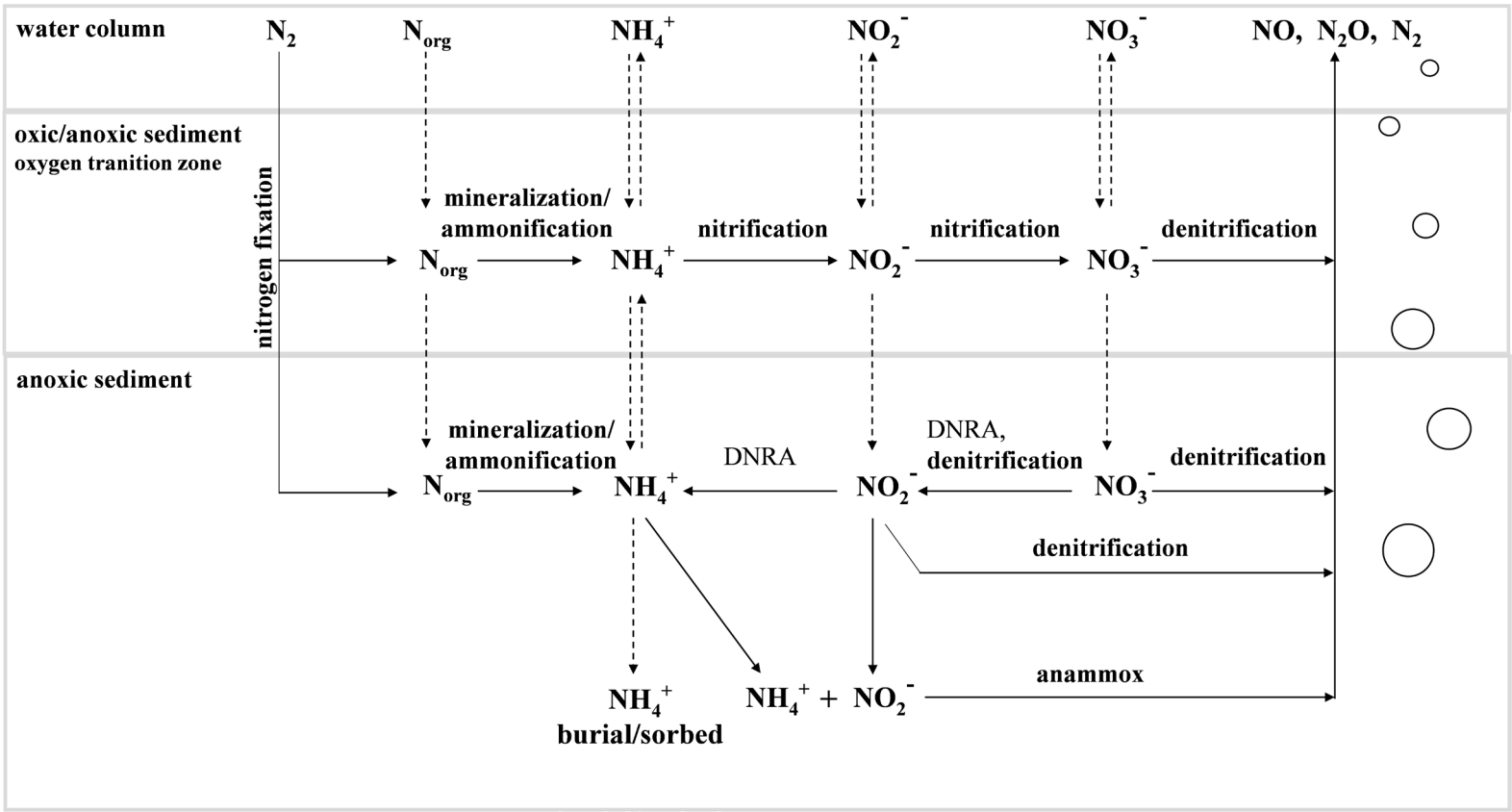

Figure 1: Nitrogen cycling in coastal marine sediments (simplified and modified from Herbert, 1999; Joye and Anderson, 2006). Dashed lines indicate diffusive, advective, or surface exchange between compartments; solid lines represent microbial mediated processes. Bold process indicates chemolitho-autotrophic contribution.

trogen compounds (N_{org}, e.g. proteins), is the initial step of the nitrogen cycling in marine sediments. During the mineralization of organic nitrogenous matter ammonification, the release of NH_4^+ occurs. The NH_4^+ released by ammonification is available for primary production and for ammonia oxidation in the O_2 transition zone of the sediment (Herbert, 1999). The fate of NH_4^+ regenerated in sediments may either be the adsorption to particles, the flux from the sediment to the overlying water, the consumption by biological processes within the sediment or at the sediment–water interface, the oxidation to NO_2^- and then NO_3^- by nitrifying microorganisms, or the transformation to molecular nitrogen (N_2) via the denitrification and anammox process (Joye and Anderson, 2006).

1.1.1. Nitrification

Nitrification is a two-step process, first ammonium is oxidized to NO_2^- by chemolithoautotrophic ammonium oxidizing bacteria (AOB) and archaea (AOA), which is then further oxidized to NO_3^- by nitrite oxidizing bacteria (NOB) (Kim and Gadd, 2008). Aerobic ammonium oxidation is a two-step process, in which ammonium is oxidized to hydroxylamine catalyzed by the enzyme ammonia monooxygenase (AMO). In bacteria, hydroxylamine is further oxidized to NO_2^- by the enzyme hydroxylamine oxidoreductase (HAO), whereas in archaea, the oxidation of hydroxylamine to NO_2^- may be catalyzed via nitroxyl oxidoreductase (NxOR) (Schleper and Nichol, 2010). In coastal marine sediments, AOB belong to the Gammaproteobacteria (*Nitrosococcus oceanus*) and to Betaproteobacteria (*Nitrosomonas* spp. and *Nitrosospira* spp.), based on the phylogenetic analysis of 16S rRNA genes (Herbert, 1999). Ammonia oxidizing archaea (AOA) belong to the distinct phylum of Thaumarchaeota (Brochier-Amanet et al., 2008). Most AOBs are obligate chemolithoautotrophs, whereas Thaumarchaeota can grow chemolithoautotrophically (e.g. "*Candidatus* Nitrosopumilus" maritimus; Könneke et al., 2005) and possibly mixotrophically (e.g. "*Nitrososphaera viennensis*"; Tourna et al., 2011), besides heterotrophic growth cannot be ruled out (Pester, 2011). The communities of AOB and AOA seem to be favored by different physicochemical parameters, such as salinity, ammonia and O_2 concentrations (Mosier and Francis, 2008; Santoro et al., 2008), with AOA preferring lower salinities and lower ammonia concentrations. The oxidation of NH_4^+ plays an important role in generating nitrogen oxides (NO_2^- and NO_3^-) for microorganisms utilizing these to gain energy for carbon fixation, such as anammox and denitrifying bacteria.

1.1.2. Anammox

Bacteria of the phylum Planctomycetes have been discovered to perform anaerobic ammonia oxidation (anammox) reaction, which consist on conversion of NH_4^+ and NO_2^- into N_2 while growing chemolithoautotrophically (Strous et al., 1999, Kuypers et al., 2003). In addition to NO_2^-, anammox bacteria are also able to use Fe^{3+}, manganese oxides and NO_3^- as electron acceptors in their metabolism (Strous et al., 2006). All five described anammox genera belong to the order Brocadiales and are related to the order Planctomycetales (Jetten et al., 2009), but at present only "*Candidatus* Scalindua" has been detected in marine sediments and O_2 minimum zones (OMZs) (Daalsgard et al., 2005; Woebken et al., 2008; Lam et al., 2009). The contribution of the anammox process to the total nitrogen removal in marine sediments has been estimated to be between 0–80% (Dalsgaard, 2005).

1.1.3. Denitrification

After the consumption of dissolved O_2 in the sediment pore water, organic matter decomposition continues via denitrification, anammox or dissimilatory nitrate reduction to ammonium (DNRA) (Zumft, 1997; Seitzinger, 2006). Whereas DNRA results in bioavailable ammonia, denitrification leads to the release of gaseous end products such as nitric oxide (NO), nitrous oxide (N_2O), and dinitrogen gas (N_2) to the atmosphere. Dissimilatory denitrification, the microbial oxidation of organic matter with NO_3^- or NO_2^- as terminal electron acceptor, is considered to be the sequence of nitrate respiration, nitrite respiration combined with NO reduction, and N_2O respiration: $NO_3 \rightarrow NO_2 \rightarrow NO \rightarrow N_2O \rightarrow N_2$. The denitrification process is performed by facultative chemoorganoheterotrophic bacteria, some archaea and foraminifera (Zumft, 1997; Risgaard-Petersen et al., 2006), and occurs as well in chemolithoautotrophic organisms performing the oxidation of an inorganic compound such as H_2S coupled to nitrogen oxide reduction. Recently, sulfide-dependent denitrification, performed by autotrophic members of Alpha-, Beta- Gamma-, and Epsilonproteobacteria (especially *Thiobacillus denitrificans* and *Sulfurimonas denitrificans*), has been suggested to play a major role in the denitrification process in the oxygen- and sulfide- transition zone of coastal marine sediments (Shao, 2010).

The processes of nitrification and denitrification in sediments are the major source of NO_3^- for denitrification in some estuaries and shelf regions. In systems with low NO_3^- concentrations (<10 μM) in the oxygenated bottom water, coupled nitrification/denitrification accounts for 90% of the NO_3^- that is required for denitrification (Risgaard-Petersen, 2003). However, coupled nitrification/denitrification is a mechanism that can be responsible for substantial nitrogen removal from the sediment, which is in turn not available for primary producers. Nitrogen removal by denitrification, including anammox, might control the rate of eutrophication in coastal marine systems that receive large amounts of anthropogenic nitrogen (Herbert, 1999).

1.2. Chemolithoautotrophic sulfur cycling in coastal marine sediments

Below the oxygen penetration depth (OPD) and after the depletion of electron acceptors, such as oxygen, NO_3^-/NO_2^-, and metal oxides, sulfate reduction to H_2S becomes the prevalent microbial redox process in marine sediments (Jørgensen, 2006). Sulfate reduction, the reduction of elemental sulfur (S^0) and sulfur disproportionation can result in H_2S production and upward diffusion. Most H_2S is reoxidized by chemical and microbial mediated sulfur oxidation or buried as iron sulfide and pyrite (FeS, FeS_2).

1.2.1. Sulfate reducing microorganisms

Sulfate reducing microorganisms have a key role in the sulfur cycle, they use SO_4^{2-} as a terminal electron acceptor in the degradation of organic matter, which results in the production of H_2S (Muyzer and Stams, 2008), which subsequently can be oxidized aerobically and anaerobically by sulfur oxidizing bacteria (e.g. *Thiobacillus* or *Beggiatoa* spp.). During dissimilatory sulfate reduction, SO_4^{2-} is activated by the enzyme ATP sulfurylase, catalyzing the formation of adenosine phosphosulfate (APS) from SO_4^{2-} and phosphate (ATP). The SO_4^{2-} in APS is directly reduced to SO_3^{2-} by the cytoplasmic enzyme adenosine-5' phosphosulfate (APS) reductase, which is then further reduced to H_2S by the enzyme sulfite reductase. Sulfate reducing microorganisms are metabolically and genetically divers, some grow chemoorganoheterotrophically by using acetate, propionate, butyrate or lactate as electron acceptor, whereas many chemolithoautotrophic repre-

sentatives use hydrogen as electron donor and SO_4^{2-} as electron acceptor while utilizing CO_2 as carbon source (e.g. *Desulfovibrio* spp.). Sulfate reducing bacteria belong to the class of Deltaproteobacteria, as well as to the phyla of the Firmicutes and Nitrospirae. Within the archaea, sulfate reducing representatives belong to the genus *Archaeglobus* within the phylum of Euryarchaetoa, and to the phylum of Crenarchaeota (Muyzer and Stams, 2008). The activity of sulfate reducing microorganisms is especially important in organic-rich sediments underlying highly productive waters of continental shelves and slopes (Jørgensen, 1982a, b; Jørgensen and Kasten, 2006). It has been estimated that sulfate reduction can account for more than 50% of the organic carbon mineralization in marine sediments (Jørgensen, 1982a, b).

1.2.2. Sulfur oxidizing microorganisms in marine sediments

Sulfur oxidation, i.e. the oxidation of reduced sulfur compounds, such as H_2S, SO_3^{2-}, $S_2O_3^{2-}$, S^0, to SO_4^{2-} can be performed by taxonomical divers chemolithoautotrophic bacteria and some archaea, using mainly O_2 and NO_3^- as electron acceptor, which ultimately links the carbon-, nitrogen- and sulfur cycling in marine sediments. Sulfide oxidation starts with the conversion of H_2S (sulfur, thiosulfate) to sulfite. Some sulfur oxidizing chemolithoautotrophs use the enzyme sulfite oxidase, which transfers electrons directly to cytochrome *c* generating ATP during electron transport and proton motive force formation. Others use the enzyme adenosine-5' phosphosulfate (APS) reductase, which is an essential enzyme of sulfate reducing bacteria, which results in SO_4^{2-} production in sulfur oxidizers (Meyer and Kuever, 2007b).

Chemolithoautotrophic bacteria belonging to the class of Betaproteobacteria (*Thiobacillus*), Gammaproteobacteria (*Thiomicrosprira*), and to the Epsilonproteobacteria (*Acrobacter*, *Sulfurimonas*, *Sulfurovum*), have been shown to contribute to chemolithoautotrophic sulfide oxidation in coastal marine sediments (Jørgensen and Nelson, 2004; Campbell et al., 2006; Lenk et al., 2011). Chemolithoautotrophic sulfur oxidizing bacteria are taxonomically broad, while chemolithoautotrophic sulfur oxidizing archaea seem to be restricted to Sulfolobales (Crenarchaeota) using metal oxides, Thermoplasmatales (Euryarchaeota) using elemental sulfur, and only two genera seem to be able to oxidize H_2S, i.e. *Acidianus sulfidivorans* and *Ferroglobus placidus*, using NO_3^- and Fe^{3+} as electron acceptors (Hafenbradl et al., 1996; Plumb et al., 2007).

Additionally, large sulfur bacteria, Gammaproteobacteria of the order Thiotrichales (*Beggiatoa*, *Thioploca*, *Thiomargarita*), contribute substantially to sulfide oxidation in coastal marine sediments. Under low O_2 conditions, Beggiatoaceae are able to store NO_3^- in an internal vacuole at the sediment surface, before gliding into anoxic and sulfidic layers, where they oxidize H_2S to S^0 with the stored NO_3^-. Here, S^0 is stored in globules and transported back to the surface to complete the oxidation to sulfate. Most members of the marine Beggiatoaceae family are chemolithoautotrophs, some species are able to use an organic carbon source for mixotrophic or chemooarganoheterotrophic growth (Mußmann et al., 2003; Jørgensen and Nelson, 2004).

1.3. Autotrophic CO_2 fixation pathways of microorganisms

Up to date six different CO_2 fixation pathways used by taxonomically diverse bacteria and archaea have been discovered (Fig. 2). In the Calvin-Benson-Bassham (CBB) cycle, CO_2 reacts with ribulose 1,5-bisphosphate to gain two 3-phosphoglycerate, from which the sugar is formed. Apart from eukaryotes such as plants and algae, the CBB cycle is utilized by cyanobacteria, many aerobic or facultative aerobic Proteobacteria belonging to the Alpha-, Beta-, and Gamma- classes

(including AOB), *Sulfobacillus* spp., iron and sulfur oxidizing Firmicutes, some mycobacteria, and bacteria of the phylum Chloroflexi (*Oscillochloris*) (Berg et al., 2010). The CBB cycle prevails under oxic conditions, whereas the more energy efficient and less O_2 sensitive reductive tricarboxylic acid (rTCA) cycle is restricted to microaerobic and anaerobic conditions (Berg et al., 2010). The rTCA cycle reverses the reactions of the oxidative citric acid (Krebs-) cycle and operates in sulfate reducing Delta- and sulfur oxidizing Epsilonproteobacteria, green sulfur bacteria and in microaerophilic bacteria of the phylum *Aquificae* (Hügler et al., 2005; Campbell et al., 2006).

The strictly anaerobic reductive acetyl-coenzyme A (acetyl-CoA) pathway is a noncyclic pathway, in which one CO_2 molecule is reduced to CO and one to a methyl group, from which acetyl-CoA is synthesized subsequently. The acetyl-CoA pathway functions in acetogenic and methanogenic bacteria, as well as in anammox bacteria (Planctomycetes), sulfate reducing Deltaproteobacteria (*Desulfobacterium* sp.), and in Euryarchaeota (Archaeglobales). The 3-hydroxypropionate bicycle is currently restricted to members of the *Chloroflexacae* family. The 3-hydroxypropionate/4-hydroxybutyrate cycle occurs in aerobic Crenarchaeota (*Sulfolobales*) and marine Thaumarchaeota (AOA), whereas the dicarboxylate/4-hydroxybutyrate cycle functions in anaerobic Crenarchaeota of the orders Thermoproteales and Desulfurococcales. The prevalence of CO_2 fixation pathways utilized by chemolithoautotrophic microorganisms in seasonally hypoxic coastal marine sediments is poorly known at present (Berg et al., 2010, and 2011).

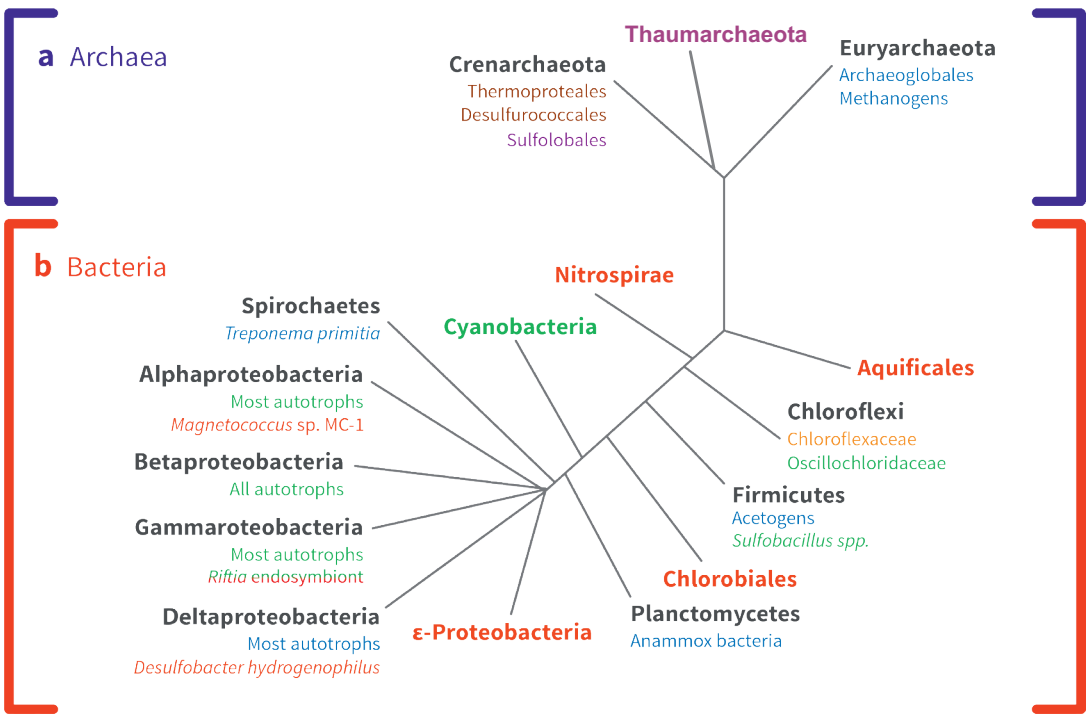

Figure 2: Phylogenetic classification of autotrophic bacteria and archaea; green: Calvin-Benson-Bassham cycle, red: reductive tricarboxylic acid cycle, blue: reductive acetyl-CoA cycle, orange: 3-hydroxypropionate bicycle, purple: 3-hydroxypropionate-/ 4-hydroxybutyrate cycle, brown: dicarboxylate/4-hydroxybutyrate cycle (Figure modified from Hügler and Sievert, 2011).

1.4. Physicochemical parameters affecting microbial chemolithoautotrophy in coastal marine sediments

Various abiotic and biotic factors control the abundance and activity of chemolithoautotrophic microorganisms involved in carbon-, nitrogen-, and sulfur cycling in marine sediments. Generally, O_2 is one of the most important ecological factors influencing chemolithoautotrophy, especially in the oxygen transition zones of coastal marine sediments, where O_2 is depleted within the first millimeters. The O_2 concentration shapes the distribution of different autotrophic carbon fixation pathways, as well as the availability of suitable electron donors and acceptors, metals and coenzymes (Berg, 2011). Whereas nitrification is predominantly an obligate aerobic process, denitrification is a facultative anaerobic process, most denitrifiers prefer to respire O_2 instead of NO_3^- when O_2 concentrations exceed 20 mM (Tiedje, 1982). Anammox bacteria are obligate anaerobes and despite their O_2 tolerance, the activity seems to be limited to anoxic conditions (Dalsgaard et al., 2005; Risgaard-Petersen et al., 2004, and 2005).

Coastal regions, especially estuaries and coastal areas with limited water exchange, often obtain large amounts of anthropogenic nitrogen leading to eutrophication, (Howarth and Marino, 2006). Hypoxia is one of the common features of eutrophication and the phenomenon of seasonal hypoxia has expanded over the last decades in coastal regions. Seasonal hypoxia causes a decrease in the availability of electron acceptors, i.e. O_2 and NO_3^- in the bottom water (Rosenberg and Diaz, 2008), suggesting consequences for chemolithoautotrophic microorganisms involved in carbon-, nitrogen-, and sulfur cycling in marine coastal sediments and thus for the biogeochemistry in marine sediments (Middleburg and Levin, 2009).

Extensive sulfate reduction leads to the accumulation of free H_2S in anoxic sediments, which is diffusing upwards (Jørgensen and Nelson, 2004). Sulfide has been shown to inhibit ammonia oxidation (Joye and Hollibaugh, 1995), as well as enzymes catalyzing the last steps of denitrification (Sørensen, 1980; Porubsky et al., 2009). The effect of H_2S on anammox is relatively unclear, but there is certain evidence, that the activity of anammox bacteria is inhibited in sulfidic waters of oxygen minimum zones (Daalsgaard 2003; Jensen et al., 2008), suggesting that the abundance and activity of anammox bacteria might as well be inhibited by rising H_2S concentrations in coastal marine sediments.

However, it is well known that high concentrations of free H_2S are toxic for benthic animals, such as bioturbating organisms (Vaquer-Sunyer and Duarte, 2008). The key role of bioturbation in nutrient cycling is well established (Meysman et al., 2006) and has been suggested to extend the area of O_2 availability in deeper sediment layers, supporting aerobic microbial processes, such as nitrification, which in turn might fuel anammox and denitrification (Meyer et al., 2005). Recently, bioturbation has been shown to induce changes in the sedimentary microbial community composition, resulting in distinct communities within the burrow (Laverock et al., 2010).

1.5. Methods to study microbial chemolithoautotrophy

The majority of microorganisms that occur in natural environments cannot be cultured or isolated using traditional cultivation techniques, which limits our knowledge of environmental microbial diversity (Rappé et al., 2003). In the last decades, a wide range of cultivation independent approaches have been developed to understand the abundance, diversity and metabolic activity of microbial communities in the environment using a wide range of organic and inorganic

biomarker molecules, such as DNA/RNA and lipids, as well as the incorporation of labeled substrates into biomarker molecules.

1.5.1. Abundance and diversity of chemolithoautotrophic microorganisms using DNA-based methods

The analyses of DNA sequences of microorganisms has expanded our knowledge of the taxonomic and functional diversity of marine microbes to a large extent. Molecular methods revealed a diverse community of microorganisms with diverse metabolic capabilities, new species and novel pathways of organic matter cycling (Delong, 1992; Fuhrman et al., 1992). Advances in molecular microbiology allow for DNA sequence analysis and comparison to sequences that are already available in sequence databases. The development of quantitative PCR (qPCR) for the real time quantification of selected DNA sequences (genes) can be used to estimate the abundance of specific groups of microorganisms. In general, two kinds of genes are normally targeted, the 16S rRNA gene and functional genes.

The 16S ribosomal RNA is part of the 30S small subunit of a prokaryotic ribosome, and the 16S rRNA gene sequence has been used to reconstruct the microbial phylogeny (Woese and Fox, 1977). The 16S rRNA gene sequence is especially useful because as it is present in all Domains of life, i.e. Bacteria, Archaea and Eukarya. Variable regions of the 16S rRNA gene are diagnostic for specific groups of organisms, whereas highly conserved regions can be used to assess the diversity of the microbial community. Sequencing of the highly conserved and species specific 16S rRNA gene has been extensively used to characterize the microbial community in marine surface and subsurface sediments (Madsen, 2008). Nevertheless, the rapid development of new generation sequencing methods allows for high throughput sequencing, and 16S rRNA gene amplicon sequencing has been shown to be a powerful tool to study the diversity of the microbial community as well as the relative abundance of specific groups in a high resolution in the marine environment (Gilbert and Dupont, 2011; Klindworth et al., 2013; Cruaud et al., 2014).

As the 16S rRNA gene gives only limited and indirect information about the metabolism, i.e. the contribution to CO_2 fixation and reoxidation pathways, functional gene analysis, i.e. genes encoding for key-enzymes involved in different CO_2 fixation and reoxidation pathways, is a powerful tool to study the diversity and abundance of chemolithoautotrophic microorganisms. Gene sequences, i.e. the *cbbL* gene coding for ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) and the *aclB* gene coding for the ATP-citrate lyase, functioning in the CBB and rTCA cycle respectively, have been used to assess the diversity of chemolithoautotrophic microorganisms in intertidal mudflat sediments as well as in hydrothermal vent sediments and in sediments with an oxygen-sulfide chemocline (Nigro and King, 2007; Campbell et al., 2003, and 2006). However, the temporal and spatial distribution of the diversity and the abundance of chemolithoautotrophic microorganisms using the CBB or rTCA cycle have rarely been studied in coastal marine sediments. Some important key-enzymes and their corresponding functional genes for inorganic carbon assimilation and reoxidation pathways are summarized in Table 2.

The key-enzyme of aerobic ammonia oxidation is a membrane-bound putative ammonia monooxygenase (AMO), catalyzing the oxidation of ammonium to hydroxylamine in Bacteria (Rotthauwe et al., 1997) and Archaea (Venter et al., 2004; Könneke et al., 2005; Yakimov et al., 2009). The metabolic gene *amoA*, encoding AMO, has been used to analyze the diversity and abundance of AOA and AOB in estuarine sediments (Beman and Francis, 2006; Mosier and

Table 2: Important functional genes of CO_2 fixation and reoxidation pathways. Primer sequences are reported in the given references.

	Pathway	Key enzyme	Functional gene	References
CO_2 fixation	Calvin-Benson-Bassham (CBB) cycle/ reductive pentose phosphate cycle	ribulose1,5-bisphosphate carboxylase/ oxygenase (RuBisCO)	*cbbL*	Nigro and King, 2007
	reductive tricarboxylic acid (rTCA) cycle/ Arnon–Buchanan cycle	ATP-citrate lyase (ACL)	*aclB*	Campbell et al., 2003
reoxidation	Ammonia oxidation	archaeal ammonia monooxygenase (AMO)	*amoA*	Yakimov et al. (2011)
		β-proteobacterial ammonia monooxygenase (AMO)	*amoA*	Rotthauwe et al. (1997); Hornek et al. (2006)
	Anaerobic ammonia oxidation (anammox)	hydrazine synthase (HZS)	*hzsA*	Harhangi et al., 2012
		cytochrome cd1-containing nitrite reductase (NIR)	*nirS*	Lam et al., 2009
	Denitrification	cytochrome *cd*1-containing nitrite reductase (NIR)	*nirS*	Bracker et al., 1998
	Denitrification coupled to sulfur oxidation	adenosine-5' phosphosulfate (APS) reductase	*aprA*	Meyer & Kuever, 2007c

Francis, 2008; Abell et al., 2010) and in sandy coastal sediments (Santoro et al., 2008). However, the spatial and temporal distribution of the diversity and abundance of microbial ammonia oxidizers, as well as of anammox bacteria, denitrifiers, sulfate reducers and sulfur oxidizers, in seasonally hypoxic sediments, is still unknown. The *hzsA* and *nirS* gene of anammox bacteria, encoding for the hydrazine synthase (HZO) and cytochrome *cd*1-containing nitrite reductase (NIR), have been proposed as suitable biomarker to detect anammox bacteria diversity and abundance in the marine environment (Kartal et al., 2011; Harhanghi et al., 2012; Lam et al., 2009) as well as in marine sediments (Li et al., 2011). The cytochrome *cd*1-containing nitrite reductase, coded by the *nirS* gene, operates as well in the dissimilatory denitrification process, catalyzing the reduction of NO_2^- to NO, the first gaseous product in the sequence of denitrification (Zumft, 1997; Braker, 1998). The *nirS* gene has been used to study the diversity and to estimate the abundance of denitrifying microorganisms in estuarine sediments (Smith at al., 2007; Mosier and Francis, 2010). The diversity of sulfate reducing and sulfur oxidizing microorganisms have been studied using the *aprA* gene encoding the disssimilatory adenosine-5' phosphosulfate (APS) reductase, which is a key enzyme in sulfate reduction and sulfur oxidation processes. The APS reductase catalyzes the conversion of APS to AMP and SO_3^{2-} in sulfate reducing microorganisms, and operates in the reverse direction in sulfur oxidizing microorganisms. The highly conserved *aprA* gene has been shown to be a suitable biomarker to profile the community structure of sulfate reducing and sulfur oxidizing microorganisms in marine and estuarine sediments (Meyer and Kuever, 2007c, Muyzer and Stams, 2008).

1.5.2. Archaeal and bacterial membrane lipids as taxonomic markers

Membrane lipids are considered as taxonomic markers that can be used to estimate the abundance, distribution, and physiological status of certain microbial groups (White, 2007). In addition, these lipids can be preserved in sediments upon cell depth and thus being used as biomarkers of the presence of certain organisms both in present and past ecosystems. On the contrary, other biomolecules like DNA have a more rapid turnover and they cannot be used for this purpose. In recent years, intact polar lipids (IPLs) are increasingly applied for tracing 'living' bacteria and archaea in the environment (Lipp et al., 2008; Rossel et al., 2008; Lipp and Hinrichs, 2009). IPLs with polar head groups are present in living cells but upon cell lysis the polar head groups are lost, releasing the core lipids (CLs) that may be preserved in the fossil record. Since IPLs degrade relatively quickly after cell death (Harvey et al., 1986), it is possible to associate the presence of IPLs in the environment with the occurrence of their living producers (Zink et al., 2003; Biddle et al., 2006; Huguet et al., 2010; Schubotz et al., 2009). Specifically, IPLs with phospho- head groups have been shown to degrade rapidly after cell death, while IPLs with glycosidic polar head groups might be preserved in the sediment record (White et al., 1979; Harvey et al., 1986; Bauersachs et al., 2010; Lengger et al., 2012).

Membrane lipids from bacteria and eukaryotes are composed of fatty acid chains that are linked to the glycerol moiety through ester bonds and organized in a bilayer structure. On the other hand, archaeal membrane lipids are characterized by ether bonds, isoprene-based alkyl moieties, and an opposite stereochemistry of the glycerol phosphate backbone, i.e. *sn*-glycerol-1-phosphate (G1P). Archaeal membrane lipids are typically a variation of two main structures, *sn*-2,3-diphytanylglycerol diether (archaeol) with phytanyl (C_{20}) chains in a bilayer structure, and *sn*-2,3-dibiphytanyl diglycerol tetraether (glycerol dibiphytanyl glycerol tetraether, GDGT), in which the two glycerol moieties are connected by two C_{40} isoprenoid chains, allowing the

Table 3: Membrane lipid composition of the main groups of archaea involved in chemolithoautotrophy and sulfur metabolism in marine sediments (modified from Villanueva et al., 2014). Species specific GDGT occurrence and distributions of pure cultures are described in detail in Schouten et al. (2013) and references therein.

Phylum	Order	Metabolism	Membrane lipid
Thaumarchaeota (AOA)	Cenarchaeales	ammonia oxidizer autotroph	GDGT-0 to 4, crenarchaeol
	Nitrosopumilales	ammonia oxidizer autotroph/mixotroph	GDGT-0 to 4, crenarchaeol
	Nitrososphérales	ammonia oxidizer autotroph	GDGT-0 to 4, crenarchaeol
Crenarchaeota	Desulfurococcales	sulfur dependent	GDGT-0 to 4,
	Sulfolobales	sulfur dependent	GDGT-0 to 4, GDGT-5 to 8
	Thermoproteales	sulfur dependent	GDGT-0 to 4, GDGT-5 to 8
Euryarchaeota	Thermoplasmatales	sulfur dependent	GDGT-0 to 4, GDGT-5 to 8
	Thermococcales	sulfur dependent	archaeol, GDGT-0
	Archaeglobales	sulfur dependent	GDGT-0
	ANME-cluster	methane oxidation chemoorganoautotroph	GDGT-0 to 4

formation of monolayer membranes (Koga and Morii, 2007). GDGTs have been found to be ubiquitous in the marine water column and sediments (Schouten et al., 2000). Isoprenoid GDG-containing 0–8 cyclopentane moieties (Fig. 3) are commonly found in different archaeal groups, such as Thaumarchaeota (Wuchter et al., 2006), extremophiles (Huber et al., 1998; Lattuati et al., 1998; Ellen et al., 2009), methanogens (Koga et al., 1998; Gattinger et al., 2002; Zhang et al., 2011), and anaerobic methane-oxidizing archaea, ANME (Pancost et al., 2001). However, the isoprenoid GDGT crenarchaeol (Sinninghe Damsté et al., 2002a), containing four cyclopentane moieties and a cyclohexane moiety, is considered to be characteristic of Thaumarchaeota (Schouten et al., 2000; Sinninghe Damsté et al., 2002a; Zhang et al., 2006; Pester et al., 2011; Pitcher et al., 2011a). The culture-based GDGT compositions of the main archaeal groups, which are potentially involved in chemolithoautotrophy and sulfur metabolism in marine sediments, are summarized in Table 3.

The distribution of thaumarchaeotal GDGTs in the marine environment has been shown to be affected by temperature, i.e. with increasing temperature there is an increase in the relative abundance of cyclopentane-containing GDGTs (Schouten et al., 2002; Wuchter et al., 2004, and 2005). Based on this relationship, the TEX_{86} paleotemperature proxy was developed and calibrated using sea surface temperature and it has been widely applied for more than a decade

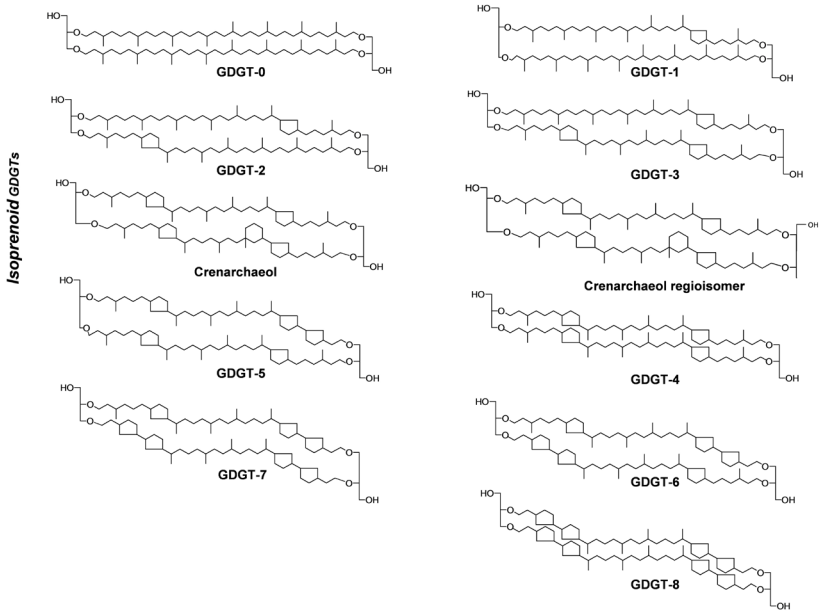

Figure 3: Structures of isoprenoid GDGTs (0–8) and crenarchaeol (mod. from Schouten et al., 2013).

(Schouten et al., 2002). However, evidences increasingly demonstrate that temperature is not the only variable affecting the GDGT distribution (Schouten et al., 2013, Pearson et al., 2013). For example, TEX_{86} responds to temperature in a different way in enrichments (Schouten et al., 2007a) and cultures (Pitcher et al., 2011a) than in suspended particulate matter (SPM) or when fossilized in surface sediments (Kim et al., 2010). It is also known that TEX_{86} does not reflect surface temperatures but rather subsurface temperatures (Huguet et al., 2007; Lopes dos Santos et al., 2010; Kim et al., 2010), and that archaeal production in the deeper water column may also contribute to the signal of GDGTs in sediments (Pearson et al., 2001; Shah et al., 2008). In addition, the TEX_{86} signal may also be affected by input from other archaea, which could potentially synthesize GDGTs used in TEX_{86}. For example, it has been suggested that *in situ* produced lipids of benthic and methanogenic archaea might contribute to the GDGT pool in marine sediments, which might be relevant for the reliability of the TEX_{86} proxy (Weijers et al., 2006a; Blaga et al., 2009; Sinninghe Damsté et al., 2012a). In addition, some studies have suggested that benthic archaea might be able to recycle core lipid GDGTs in marine sediments (Takano et al., 2010; Liu et al., 2011).

Recent advances in high performance liquid chromatography (HPLC)- mass spectrometry (MS) methods have allowed the analysis of core GDGTs as well as IPL GDGTs. IPL GDGTs have been analyzed by separating them from the core lipids using silica gel chromatography and hexane/EtOAc mixtures as eluent (Oba et al., 2006; Pitcher et al., 2009) followed by acid hydrolysis and high pressure liquid chromatography coupled via an atmospheric pressure chemical ionization interface to a mass spectrometer (HPLC−APCI−MS) (Hopmans et al., 2000; Sturt et al., 2004; Schouten et al., 2007a). With this method, IPL derived core GDGTs can be quantified using internal standards, but it does not allow to specify the kind of head group that was attached to the GDGT. In contrast, the development of HPLC combined with electrospray chemical ionization mass spectrometry (HPLC−ESI−MS), allows to analyze GDGT IPLs, with

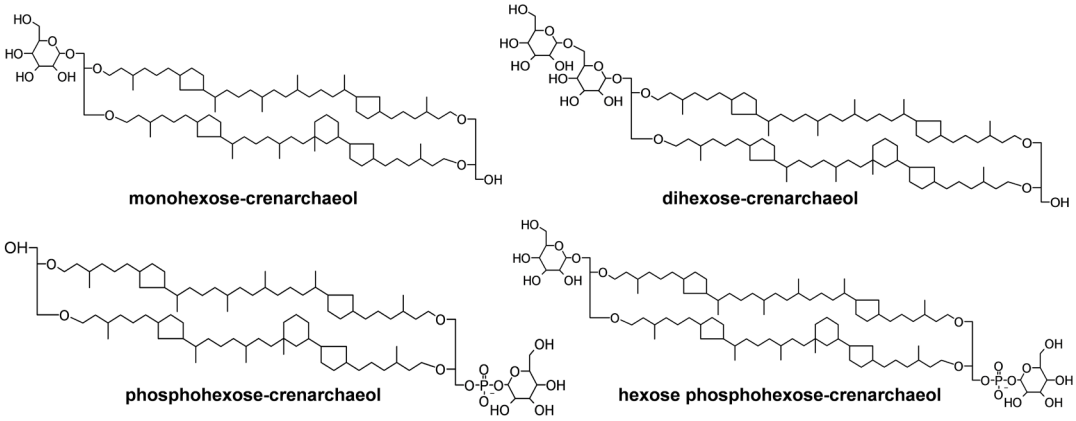

Figure 4: Main head groups of crenarchaeol detected in cultures of "*Ca.* Nitrosopumilus maritimus", "*Ca.* Nitrososphaera gargensis" and "*Ca.* Nitrosoarchaeum limnia", as well as in marine sediments.

the polar head group still attached to the core (Rütters et al., 2002; Zink et al., 2003; Sturt et al., 2004). Core and intact polar GDGTs have been analyzed by HPLC-ESI-MS^2 in selected reaction monitoring (SRM) (Schouten et al., 2008a), in AOA sediment enrichment cultures, including "*Candidatus* Nitrosoarchaeum limnia" (Pitcher et al., 2011a), as well as in marine sediments (Lengger et al., 2012). Polar headgroups of creanarchaeol have been seen to be mainly comprised by monohexose (MH), dihexose (DH), phosphohexose (PH) and hexose-phosphohexose (HPH) (Fig. 4) (Pitcher et al., 2011a). Results suggest that HPH-crenarchaeol is the most suitable lipid biomarker to trace living Thaumarchaeota of marine group I.1a due to the labile nature of the phosphate bond (Harvey et al., 1986; Schouten, et al., 2010) in comparison with glycosidic crenarchaeol-IPL-GDGTs. Additionally, it has been shown that the HPH-crenarchaeol concentration corresponds well to DNA-based thaumarchaeal abundance (Pitcher, 2011a and b; Schouten et al., 2012a; Lengger et al., 2012).

Bacterial membrane lipids are composed by fatty acids linked by ester bonds to the glycerol-3-phosphate backbone. The phospholipid-linked fatty acids (PLFAs) have been extensively used to characterize bacteria and eukaryotic microorganisms in different environmental settings including estuarine sediments (Guckert et al., 1985). PLFAs have complex structures with different carbon lengths, unsaturations, methylations and isomers (Vestal and White, 1989) and in some cases, PLFA signatures are specific enough to pinpoint shifts in the bacterial and eukaryotic community composition (Boschker and Middleburg, 2002) (Table 4), but it does not offer a high taxonomic resolution compared to DNA/RNA-based methods. PLFA concentrations can be analyzed by gas chromatography (GC)-flame ionization detection (FID) and MS according to a method previously described (Boschker et al., 1998).

Other membrane lipids, present in microbial cells can be used as biomarkers of the presence of their producer. For the case of chemolithoautotrophic microorganisms of importance in marine sediment, the specific lipids contained in anammox bacteria are a clear example. Anammox bacteria contain a membrane bound compartment in the cytoplasm, the anammoxosome, in

Table 4: PLFA signatures of the main groups of chemoautotrophic bacteria in marine sediments. The relative contributions of the main fatty acids to the total PLFA composition and complete PLFA signatures are described in the listed references (mod. from Vasquez-Cardenas, 2016). For details on the nomenclature of PLFAs see supplementary information.

Metabolism	PLFA signature	Reference
Ammonium oxidizers	16:1ω7c 16:0	Blumer et al., 1969
Nitrite oxidizers	16:1ω7c 16:0 18:1ω7c	Lipski et al., 2001 Blumer et al.,1969
Sulfate reducers (Deltaproteobacteria)	14:0 15:0 i15:0 16:1ω7c 16:0 i17:1ω7c 17:1ω6c 18:1ω7c	Taylor and Parks, 1983 Edlund et al., 1985 Suzuki et al., 2007 Pagani et al., 2011
Mat forming sulfur oxidizers (Gammaproteobacteria)	14:0 16:1ω7c 16:1ω7t 16:0 18:1ω7c	Zhang et al., 2005 Li et al., 2007
Sulfur oxidizers (Epsilonproteobacteria)	14:0 16:1ω7c 16:0 18:1ω7c	Inagaki et al., 2003 Takai et al., 2006 Donachie et al., 2005
Iron and Sulfur oxidizers	16:1ω7c 16:0 cy17:0 cy19:0	Knief et al., 2003

which the anammox reaction takes place (van Niftrik et al., 2008). The anammoxosome consists of ladderane lipids with three or five linearly arranged cyclobutane rings (Sinninghe Damsté et al., 2002b). Ladderane lipids can be either ester- or ether bound to glycerol and major polar headgroups are phosphatidylcholine (PC), phosphoetanolamine (PE) and phosphoglycerol (PG) (Boumann et al., 2006; Rattray et al., 2008). Whereas ladderane core lipids have been shown to be suitable biomarkers for anammox bacteria abundance in the fossil sediment record, the C_{20}-[3]-monoether ladderane lipid attached to a PC head group has been shown to be a suitable

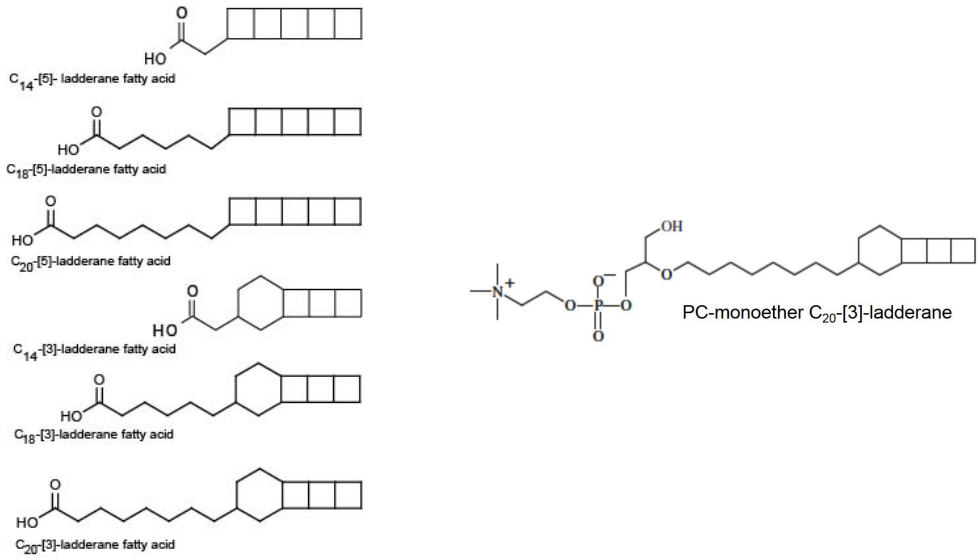

Figure 5: Chemical structures of ladderane core lipids and the intact PC-monoether ladderane lipid.

biomarker for living anammox bacteria in continental shelf sediments (Jaeschke et al., 2010). Ladderane core lipids can be analyzed as fatty acid methyl esters (FAMEs) by HPLC-APCI-MS^2 in selective reaction monitoring (SRM) mode as previously described (Hopmans et al., 2006) and modified to include short chain fatty acids (Rush et al., 2012). The PC-monoether ladderane lipid can be analyzed by HPLC-MS^2 (Jaeschke et al., 2009), and quantified using an external PC-monoether ladderane standard. Main structures of ladderane core lipids and the intact PC-monoether ladderane lipid are summarized in Fig. 5.

1.5.3. Determining the activity of chemolithoautotrophic microorganisms

The determination of active (chemolithoautotrophic) microorganisms in marine sediments has been a challenge and has been addressed using different methods by targeting the metabolic process or the activity of specific groups or specific microorganisms.

In sediments, microsensor profiling, i.e. concentration measurement of respiratory gases O_2 and H_2S has been used to identify active microbial processes, such as photosynthesis, respiration and sulfur oxidation (Nielsen et al., 1990; Schramm et al., 1996). The activity of microbes participating in nitrogen cycling processes, such as nitrification, denitrification and anammox, have been estimated by isotope tracer experiments with ^{15}N- and ^{14}N-labeled NO_3^-, NO_2^- and NH_4^+ by the discrimination of isotope pairing in marine sediments (Nielsen, 1992; Rysgaard et al., 1993). Microsensor profiling, as well as isotope tracer experiments, are adequate methods to measure overall process rates, but do not provide any taxonomic information about metabolically active microorganisms.

In contrast, the expression of functional genes, i.e. the quantification of mRNA by reverse transcription (Holmes et al., 2004) and subsequent qPCR, has been proposed to be a good marker to determine the activity of specific microorganisms in the marine environment. The positive gene expression of functional genes may serve as evidence for active *in situ* processes and provide insight into the diversity of active microorganisms (Nogales et al., 2002; Wellington

et al., 2003).

For the case of chemolithoautotrophic microorganisms, the transcript abundance of the archaeal *amoA* gene has been quantified to estimate the activity of Thaumarchaeota in oceanic waters, showing that marine Thaumarchaeota are actively involved in ammonia oxidation in the water column of the Pacific Ocean, as well as in the Peruvian OMZ and in the Mediterranean Sea (Lam et al., 2009; Church et al., 2010; Yakimov et al., 2011). Additionally, gene expression of the bacterial and archaeal *amoA* gene has been analyzed in soil, as well as in estuarine sediments as a measure of the transcriptional activity, indicating the protein production potential (Nicol et al., 2008; Abell et al., 2011). In estuarine sediments, the expression of bacterial *amoA* genes was reduced at low dissolved O_2 (DO) concentrations, whereas the archaeal *amoA* gene expression was not affected, suggesting that AOB and AOA show different responses to changes in sediment DO, which may be a significant factor in niche partitioning of different ammonia oxidizer groups. The gene expression of other functional genes has also been determined as an indicator for the potential activity of specific groups. For example, the *nirS* transcript abundance of denitrifying bacteria has been quantified in estuarine sediments (Nogales et al., 2002; Smith et al., 2007; Bulow et al., 2008), showing the decrease of potentially active denitrifying bacteria from the head to the mouth of the estuary. The *nirS* transcript abundance of anammox bacteria has been used to estimate the potential activity of anammox bacteria, showing the contribution of anammox bacteria to nitrogen removal the Peruvian oxygen minimum zone (Lam et al., 2009).

Stable carbon isotopes have been also used to track the activity of microorganisms in marine sediments. In contrast to radioactive isotopes (^{14}C), stable carbon isotopes (^{12}C, ^{13}C) do not decay in nature and are less harmful. In nature, stable carbon and nitrogen isotopes occur in different abundances. Whereas the isotope with the low mass (e.g. ^{12}C) is highly abundant (98%), the isotope with the higher mass (e.g. ^{13}C) represents less than 2% (Fry, 2006). The difference in the abundance gives the possibility to track the cycling of elements by the metabolic incorporation of ^{13}C into specific molecules. The labeled substrate is added to an environmental sample (e.g. intact sediment core) and biomarkers are purified and analyzed following the consumption of the substrate. Stable isotope probing (SIP) has been applied to track the incorporation of ^{13}C substrates into membrane lipids, such as archaeal GDGTs (Wuchter et al., 2003), IPL derived GDGTs (Pitcher et al., 2011b) and bacterial PLFAs (Boschker et al., 2002). The ^{13}C incorporation into the biphytanes of GDGTs and into PLFAs can be measured by chromatography combustion isotope ratio mass spectrometry (GC-c-IRMS) (Boschker et al., 1998; Wuchter et al., 2003; Pitcher et al., 2011b). GDGT SIP, i.e. the incorporation of ^{13}C labeled bicarbonate into the biphytanyl chains of crenarchaeol revealed the capability of Thaumarchaeota to grow autotrophically (Wuchter et al., 2003), which was later confirmed by the isolation and physiological characterization of *Candidatus* Nitrosopumilus maritimus (Könneke et al., 2005). SIP has been used to label membrane lipids, as well as to track the incorporation of ^{13}C into nucleic acids, such as DNA (DNA-SIP) and RNA (RNA-SIP) (Radajewski et al., 2000, and 2003). PLFA-SIP is the most sensitive method, whereas the DNA-SIP is the less sensitive approach. In contrast to RNA and PLFA, the replication of DNA depends on cell division in presence of the labeled substrate, which makes it difficult to accomplish sufficient incorporation for separation of labeled DNA (Neufeld et al., 2006).

A method that does not imply extraction of labeled compounds, but rather visualizes active microbial cells that have been targeted with a sequence specific labeled probe under the micro-

scope, is fluorescence *in situ* hybridization (FISH). The combination of FISH with catalyzed reporter deposition (CARD-FISH) has been used to estimate the abundance of active microorganisms in the marine environment, targeting DNA and RNA molecules as well as rare molecules, such as mRNA and genes, with signal amplification using horseradish peroxidase (HRP) (Llobet-Brossa et al., 2002; Pernthaler et. al., 2002; Ishii et al., 2004). For example, the abundance of Crenarchaeota has been estimated by CARD-FISH in water samples of the Atlantic Ocean (Wuchter et al., 2006). CARD-FISH has been also combined with other approaches to determine metabolic activity in chemolithoautotrophic microorganisms. For example, catalyzed reporter deposition (CARD)-FISH combined with microautoradiography (MAR) demonstrated that novel groups of Gammaproteobacteria attached to sediment particles have been shown to incorporate ^{14}C-labeled CO_2 by MAR-FISH concluding that these groups performed a chemolithoautotrophic metabolism based on sulfur oxidation, and also suggesting that they play a more important role for H_2S removal and primary production in marine sediments than previously thought (Lenk et al., 2011).

Another novel technique, providing information about elemental, isotopic and molecular characteristics of single cells is nano-scale secondary ion mass spectrometry (NanoSIMS). NanoSIMS can be combined with SIP as well as with CARD-FISH methods. A combination of CARD-FISH and NanoSIMS has been used to track the fate of carbon and nitrogen in microbial aggregates (Behrens et al., 2008). However, the NanoSIMS method is cost-intense and time consuming regarding the sample preparation and fine tuning of the instrument.

1.6. Scope of this thesis

The research described in this thesis is focused on the spatial and temporal distribution of chemolithoautotrophic microorganisms in coastal marine sediments. These microbes have not been studied extensively and there is a growing need to determine their role in the sedimentary carbon-, nitrogen-, and sulfur cycle, as well as to identify microbial key-players that are involved, especially in the oxygen transition zone of seasonally hypoxic sediments.

To this end, the diversity, abundance and activity of chemolithoautotrophic microorganisms, such as AOA, AOB, anammox and denitrifying bacteria, as well as sulfate reducing and sulfur oxidizing microorganisms have been determined. Additionally, the diversity and activity of chemolithoautotrophic microorganisms involved in the CBB and rTCA cycle as well as the metabolism of Thaumarchaeota and the abundance of Archaea have been addressed. In the following studies, we focused on coastal marine sediments, exposed to summer hypoxia, i.e. (1) the seasonal hypoxic basin of the Oystergrounds in the central North Sea, (2) different sediment types (sand, clay, and mud) of the Iceland Shelf, and (3) seasonally hypoxic and sulfidic sediments of saline Lake Grevelingen (The Netherlands). The work presented here is divided in the following chapters:

Chapter 2 shows the seasonal and depth distribution of the abundance and potential activity of archaeal and bacterial ammonia oxidizers (AOA and AOB) and anammox bacteria in coastal marine sediments of the southern North Sea. Specific intact polar lipids as well as the gene abundance and expression of the 16S rRNA gene, the ammonia monooxygenase subunit A (*amoA*) gene of AOA and AOB, and the hydrazine synthase (*hzsA*) of anammox bacteria were quantified in contrasting seasons (i.e. February, March and August). AOA, AOB and anammox bacteria were detected and transcriptionally active down to 12 cm sediment depth in all seasons.

AOA seem to play a more dominant role in aerobic ammonia oxidation compared to AOB, as their abundance was higher in all seasons. Anammox bacteria were abundant and active even in bioturbated and oxygenated sediment layers, especially in August, suggesting that their growth and activity are favored by higher temperatures and lower O_2 concentrations during summer stratification. The metabolic activity of the studied microbial groups is spatially coupled leading to the assumption that the rapid consumption of O_2 allows the coexistence of aerobic and aerobic ammonia oxidizers in the sediments of the Oyster Grounds in the southern North Sea.

In **Chapter 3**, we aimed to determine the metabolism of sedimentary Thaumarchaeota and bacteria by studying the incorporation of ^{13}C-label from bicarbonate, pyruvate, glucose and amino acids into the thaumarchaeal IPL-GDGTs and bacterial/eukaryotal PLFAs during 4−6 days of incubation in intact marine sediment cores from three different sites on the Iceland shelf. The abundance of thaumarchaeal IPL-GDGTs, in particular HPH-crenarchaeol, were detected at all stations and the concentrations remained constant or decreased slightly during incubation, suggesting that living Thaumarchaeota were present in sediments at all stations. In organic-rich sediments a large uptake of ^{13}C label from organic substrates in bacterial/eukaryotic IPL-derived fatty acids was found, implying that heterotrophic bacteria and potentially non-photosynthetic eukaryotes incorporated label into their biomass, whereas the incorporation of labeled bicarbonate into the PLFAs of chemolithoautotrophic bacteria and archaea seem to be of minor relevance or under the detection limit of the method. In sandy sediments, some enrichment of ^{13}C (1−4%) in thaumarchaeotal IPL-GDGTs was detected after the incubation with bicarbonate and pyruvate, but overall no substantial incorporation of ^{13}C into the biphytanyl chains of HPH-crenarchaeol has been detected. We conclude that the low incorporation rates detected suggest a slow growth of sedimentary Thaumarchaeota in contrast to other sedimentary organisms and/or a slow turn-over of thaumarchaeotal IPL-GDGTs in contrast to bacterial/eukaryotic PLFAs.

In **Chapter 4** the abundance and diversity and potential activity of denitrifying and anammox bacteria, as well as of H_2S dependent denitrifying bacteria in seasonally hypoxic and sulfidic sediments of saline Lake Grevelingen (The Netherlands) were studied. The depth distribution of 16S rRNA and *nirS* genes of denitrifying and anammox bacteria, as well as the *aprA* gene of sulfur oxidizing and sulfate reducing bacteria, were estimated down to 5 cm sediment depth at three different locations before (March) and during (August) summer hypoxia. The abundance of denitrifying bacteria was relatively stable, whereas anammox bacteria were more abundant and active during summer hypoxia in sediments with lower H_2S concentrations, suggesting a possible inhibition of anammox bacteria by sulfide. Heterotrophic and autotrophic denitrifiers as well as anammox bacteria and SRB/SOB spatially coexist, but *nirS*-type denitrifiers outnumbered anammox bacteria leading to the conclusion that anammox does not contribute significantly to nitrogen removal in Lake Grevelingen sediments. The transcriptional activity of denitrifying and anammox bacteria as well as of sulfur oxidizing, including sulfide-dependent denitrifiers, and sulfate reducing bacteria was potentially inhibited by the accumulation of H_2S during summer hypoxia. Both denitrifiers and anammox bacteria could be inhibited by the competition for NO_3^- and NO_2^- with cable and Beggiatoa-like bacteria before summer hypoxia. Results suggest that H_2S detoxification mediated by sulfur oxidizers including sulfide-dependent denitrifiers is not sufficient to sustain the activity of denitrifying and anammox bacteria in Lake Grevelingen sediments.

In **Chapter 5** the impact of seasonal hypoxia on the activity and the community structure of

chemolithoautotrophic bacteria was studied in seasonally hypoxic and sulfidic sediments of saline Lake Grevelingen (The Netherlands) before (March) and during summer hypoxia (August). Combined 16S rRNA gene amplicon sequencing and PLFA analysis revealed a major temporal shift in the community structure. Aerobic sulfur oxidizing Gamma- and Epsilonproteobacteria predominated the chemolithoautotrophic community in spring, whereas Deltaproteobacteria were prevalent during summer hypoxia. Chemolithoautotrophic rates, determined by PLFA-SIP, were three times higher in spring than during summer hypoxia, especially in surface sediments. The abundance of *cbbL* (RuBisCO) and *aclB* (ATP-citrate lyase) genes revealed a predominance of microorganisms using the CBB cycle compared to the rTCA cycle independent of the season and location. Gene abundances of both, the *cbbL* and *aclB* genes decreased significantly between the seasons, suggesting that the abundance of chemolithoautotrophic bacteria using the CBB or the rTCA cycle is inhibited by the limited availability of electron acceptors, such as O_2 and NO_3^-, regulated by summer hypoxia.

In **Chapter 6** the archaeal abundance and community composition was studied in seasonally hypoxic and sulfidic surface sediments of Lake Grevelingen (The Netherlands). We combined archaeal 16S rRNA gene amplicon sequencing and the analysis of archaeal GDGT IPLs to determine the biological sources of these archaeal lipids under changing O_2 and H_2S conditions, before (March) and during summer hypoxia (August). In spring, the archaeal community was predominated by aerobic Thaumarchaeota, whereas during summer hypoxia, anaerobic members of the DPANN superphylum prevailed. The abundance of IPL-GDGTs decreased one order of magnitude between March and August, and polar head groups changed from phospho- to glycosidic head groups, suggesting that Thaumarchaeota might be in a stationary growth phase and/or a slow degradation of these GDGTs in the sediment. The switch from a Thaumarchaeota to a DPANN-dominated community during summer hypoxia together with a change in the lipid composition suggest that DPANN archaea may use fossil lipids found in the surface sediment to build up their membranes as they are unable to do so themselves, suggesting that DPANN archaea could be considered as important lipid recyclers in sediment systems and should be taken into account in organic geochemistry interpretations.

2. Seasonality and depth distribution of the abundance and activity of ammonia oxidizing microorganisms in marine coastal sediments (North Sea)

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Microbial processes such as nitrification and anaerobic ammonium oxidation (anammox) are important for nitrogen cycling in marine sediments. Seasonal variations of archaeal and bacterial ammonia oxidizers (AOA and AOB) and anammox bacteria, as well as the environmental factors affecting these groups, are not well studied. We have examined the seasonal and depth distribution of the abundance and potential activity of these microbial groups in coastal marine sediments of the southern North Sea. This was achieved by quantifying specific intact polar lipids (IPLs) as well as the abundance and gene expression of their 16S rRNA gene, the ammonia monooxygenase subunit A (*amoA*) gene of AOA and AOB, and the hydrazine synthase (*hzsA*) gene of anammox bacteria. AOA, AOB and anammox bacteria were detected and transcriptionally active down to 12 cm sediment depth. In all seasons, the abundance of AOA was higher compared to the AOB abundance suggesting that AOA play a more dominant role in aerobic ammonia oxidation in these sediments. Anammox bacteria were abundant and active even in oxygenated and bioturbated parts of the sediment. The abundance of AOA and AOB was relatively stable with depth and over the seasonal cycle, while anammox bacteria abundance and transcriptional activity were highest in August. North Sea sediments thus seem to provide a common, stable, ecological niche for AOA, AOB and anammox bacteria.

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2.1. Introduction

Nitrogen is an essential component for living organisms and thus plays a critical role in controlling primary production (Gruber & Galloway 2008). Microbial aerobic and anaerobic nitrogen cycling processes such as nitrification, denitrification and anaerobic ammonium oxidation are tightly coupled in marine sediments where 50% of marine nitrogen removal occurs (Zhang et al., 2013, and references therein). The discovery of ammonia-oxidizing archaea (AOA) (Könneke et al., 2005; Wuchter et al., 2006) belonging to the phylum Thaumarchaeota (Brochier-Armanet et al., 2008), and bacteria of the Planctomycetes phylum performing the anaerobic ammonia oxidation (anammox) reaction (Strous et al., 1999; Kuypers et al., 2003), has changed our perspective on the marine nitrogen cycle and the involved microbial key-players over the last decade. Ammonia oxidation is the first and rate-limiting step of nitrification in which ammonia is oxidized to nitrite by AOA and ammonia oxidizing bacteria (AOB) (see Prosser and Nicol, 2008 for a review). The anammox process involves the combination of ammonium with nitrite to form dinitrogen gas which is then released from the system (Kuenen et al., 2008).

Several studies focusing on ammonia oxidation in marine sediments have suggested that AOA have a more prominent role in marine nitrification than AOB based on the higher abundance of AOA *amoA* gene copies (Beman & Francis, 2006; Mosier & Francis, 2008; Santoro et al., 2008; Abell et al., 2010). Proposed environmental controls shaping the distribution and activity of AOA in marine and estuarine surface sediments include the presence of sulfide, salinity, dissolved oxygen (DO) concentration, the availability of phosphorous, temperature and primary production (Bernhard et al., 2010; Dang et al., 2010; Erguder et al., 2009; Mosier & Francis, 2008; Sahan & Muyzer, 2008; Sakami et al., 2012; Bale et al., 2013). Beman et al. (2012) studied the seasonal distribution of AOA and AOB *amoA* gene abundance in Catalina Harbor down to 10 cm sediment depth and showed higher abundances of AOA and AOB *amoA* genes during summer compared to winter months as well as coinciding higher $^{15}NH_4^+$ oxidation rates.

For anammox bacteria, studies have shown that temperature, organic carbon content, nitrite concentration, water depth, sediment characteristics and bioturbation play a role in their distribution, abundance and activity in marine and estuarine sediments (Dale et al., 2009; Dalsgaard & Thamdrup, 2002b; Engström et al., 2005; Meyer et al., 2005; Dang et al., 2010; Jaeschke et al., 2009; Li et al., 2010; Zhang et al., 2013; Laverock et al., 2013; Neubacher et al., 2011, 2013). However, most of the studies have focused on the spatial distribution of anammox bacteria abundance and activity only in surface sediments (Dalsgaard & Thamdrup, 2002a; Engström et al., 2005; Thamdrup & Dalsgaard, 2002; Nicholls & Trimmer, 2009; Bale et al., 2014). Limited studies have been focused on the abundance of anammox bacteria with sediment depth or the impact of seasonality on this group (Jaechke et al., 2009; Zhang et al., 2013). Anammox bacteria abundance and activity has been previously detected using anammox specific ladderane biomarker lipids in combination with ^{15}N-labeling experiments in continental shelf and slope sediments of the Irish and Celtic Sea down to eight centimeters depth with spatial differences (Jaeschke et al., 2009). Recently, anammox bacteria abundance was shown to be higher during summer compared to the winter months in surface sediments of three hyper-nutrified estuarine tidal flats of Laizhou Bay (Bohai Sea, China) through the quantification of anammox bacteria 16S rRNA gene (Zhang et al., 2013). A study by Laverock et al. (2013) indicated that the temporal variation in the abundance of N-cycling functional genes is directly influenced by bioturbation activity varying between different bacterial and archaeal N-cycling genes. The relative

abundance of AOB *amoA* genes is significantly affected by bioturbation whereas the relative abundance of AOA *amoA* seems to be controlled by other factors (Laverock et al., 2013). Interestingly, bioturbation and mixing can extend the area of nitrate reduction in sediments leading to the production of nitrite which would fuel the anammox process (Meyer et al., 2005).

The aim of our study was to determine differences in abundance and activity of ammonia oxidizers with sediment depth (down to 12 cm depth) in marine sediments from the southern North Sea, a continental shelf sea. To address this issue, we quantified the abundance of 16S rRNA gene of ammonia oxidizer groups (AOA, AOB, and anammox bacteria), metabolic genes (*amoA* of AOA and AOB, and *hzsA* gene of anammox bacteria), and the intact polar membrane lipids specific for anammox bacteria (i.e. ladderane lipids, Sinninghe Damsté et al., 2002b), and Thaumarchaeota (i.e. crenarchaeol; Sinninghe Damsté et al., 2002a). The potential transcriptional activity of the target genes was analyzed to elucidate the potential activity of the target organisms in the sediment. Our results suggest that both aerobic and anaerobic ammonia oxidizers coexist in the same niche and are actively involved in the nitrogen cycle in oxygenated and bioturbated sediments in the North Sea.

2.2. Material and Methods

2.2.1. Study site and sampling

The Oyster Grounds, an almost circular depression with a maximum depth of 50 m located in the southern North Sea (Weston et al., 2008), is a temporary deposition center for sediment (Raaphorst et al., 1998), which plays an important role in the carbon and nitrogen cycle in the region (Weston et al., 2008). The sedimentation of organic matter in this region leads to organic rich and muddy sediment, which supports high levels of benthic fauna (Van Raaphorst, 1992; Duineveld, 1991).

Sediment cores were collected at a station (4°33.01' E, 54°13.00' N) in the Oyster Grounds during three cruises on board of the R/V Pelagia in February, May and August 2011. Sediment was collected with 10 cm diameter multicores. Bottom water of the overlaying water was collected using a syringe and filtered (through a 0.45 µm 25 mm Acrodisc HT Tuffryn Membrane syringe filter) for nutrient analysis. The cores were sliced into 1 cm slices using a hydraulic slicer or a manual slicer. Samples were collected for lipid and DNA/RNA analysis and kept at –80ºC (DNA/RNA) and –40ºC (lipids) until processing.

Bottom water temperature, water depth and salinity were measured using a SBE911+ conductivity-temperature depth (CTD) system (Seabird Electronics, Inc., WA). The oxygen concentration in the water column was measured using a dissolved oxygen (DO) sensor SBE 43 (Seabird Electronics, Inc., WA) integrated in the CTD system.

2.2.2. Physicochemical parameters

Pore water was extracted from 2.5 mm sediment slices from 0 to 2 cm depth, and from 1 cm sediment slices from 2–12 cm depth by centrifugation (approx. 4000 g, 5 min, through a 0.45 µm 25 mm Acrodisc HT Tuffryn Membrane syringe filter), and the obtained concentrations of the first 2 cm were averaged for the first and second centimeter of the sediment to estimate pore water nutrient concentrations in a 1 cm resolution. Pore water samples were stored in pre-rinsed pony vials and NH_4^+, NO_3^-, NO_2^- and PO_4^{3-} concentrations were analyzed as described by Bale et al. (2013). The total organic carbon (TOC) and total organic nitrogen (TON) content

per gram of freeze dried sediment were determined after acidification of freeze dried sediment (0.5−1 g) with 2N HCl. Residues were analyzed by using a Thermo Finnigan Delta plus isotope ratio monitoring mass spectrometer (irmMS) connected to a Flash 2000 elemental analyzer (Thermo Fisher Scientific, Milan).

2.2.3. Intact polar lipid extraction and analysis

IPLs were extracted following Bale et al. (2013). Hexose-phosphohexose (HPH) crenarchaeol, a specific biomarker lipid for AOA (Schouten et al., 2008b), was analyzed using high performance liquid chromatography mass spectrometry (HPLC/MS) as described by Pitcher et al. (2011a). Due to the lack of a standard the results are reported here as response unit per gram of sediment (r.u. g^{-1}). The anammox bacteria specific intact ladderane phospholipid, C_{20}-[3]-monoether ladderane, attached to a phosphatidylcholine (PC) headgroup (PC-monoether ladderane) was analyzed by HPLC/MS following Jaeschke et al. (2009) and quantified using an external standard consisting of isolated PC-monoether ladderane.

2.2.4. DNA/RNA extraction

DNA and RNA from sediment cores were extracted by using the DNA and RNA PowerSoil® Total Isolation Kit, respectively (Mo Bio Laboratories, Inc., Carlsbad, CA). Nucleic acid concentrations were quantified spectrophotometrically (Nanodrop, Thermo Scientific, Wilmington, DE) and checked by agarose gel electrophoresis for integrity. Extracts were kept frozen at –80°C. The RNA extracts were treated with RNase-free DNase (DNA-free™, Ambion Inc., Austin, TX). RNA quality and concentration were estimated by the Experion RNA StdSens Analysis Kit (Bio-Rad Laboratories, Hercules, CA). DNA contamination was checked by PCR using RNA as a template.

2.2.5. Reverse transcription (RT)-PCR

Reverse transcription (RT) was performed with the Enhanced Avian First Strand synthesis kit (Sigma-Aldrich Co., St Louis, MO) using random nonamers as described previously (Holmes et al., 2004). Two negative controls lacking reverse transcriptase or RNA were included. PCR reactions were performed as described above to confirm the transcription to complementary DNA (cDNA) and the negative controls using the RT reaction as a template.

2.2.6. Quantitative PCR analysis

Quanititative (qPCR) analyses were performed on a Biorad CFX96TM Real-Time System/ C1000 Thermal cycler equipped with CFX ManagerTM Software. Gene copy numbers of the Thaumarchaeota group 1.1a were estimated by using the 16S rRNA gene primers MCGI-391F/ MCGI-554R (Coolen et al., 2007). The AOA *amoA* gene was amplified using the primer combination AmoA-ModF/AmoA-ModR (Yakimov et al., 2011). Abundance of AOB 16S rRNA gene was estimated using primers CTO189F/CTO654R as described by Kowalchuk et al. (1997). Gene copy numbers of the AOB *amoA* gene were estimated using the primer set AOB-amoAF/ AOB-amoAR new (Rotthauwe et al., 1997; Hornek et al., 2006). Abundance of the anammox bacteria 16S rRNA gene was estimated using primers Brod541F/Amx820R as described by Li et al. (2010). Additionally, the anammox bacteria *hzsA* gene was quantified using the primer combination hzsA_1597F/hzsA_1857R as described by Harhangi et al. (2012) (See Table S1 for details). All qPCR reactions were performed in triplicate with standard curves from 10^0 to 10^7 molecules per microliter. Standard curves were generated as described before (Pitcher et al.,

2011b). Gene copies were determined in triplicates on diluted DNA extracts (1:10) and on complementary DNA (cDNA) extracts. The reaction mixture (25 µL) contained 1 U of PicoMaxx high fidelity DNA polymerase (Stratagene, Agilent Technologies, Santa Clara, CA) 2.5 µL of 10x PicoMaxx PCR buffer, 2.5 µL 2.5 mM of each dNTP, 0.5 µL BSA (20 mg mL^{-1}), 0.02 pmol μL^{-1} of primers, 10,000 times diluted SYBR Green® (Life Technologies, Carlsbad, CA) (optimized concentration), 0.5 µL 50 mM of $MgCl_2$ and ultra-pure sterile water. All reactions were performed in iCycler iQTM 96-well plates with optical tape (Bio-Rad, Hercules, CA). Specificity of the reaction was tested with a gradient melting temperature assay. The cycling conditions for the qPCR reaction were the following: 95°C, 4 min; 40–45 × [95°C, 30 s; melting temperature (Tm), 40 s; 72°C, 30 s]; final extension 80°C, 25 s. Specificity for qPCR reaction was tested on agarose gel electrophoresis and with a melting curve analysis (50°C–95°C; with a read every 0.5°C held for 1 s between each read) in order to identify unspecific PCR products such as primer dimers or fragments with unexpected fragment lengths. Melting temperature, PCR efficiencies (E) and correlation coefficients for standard curves are listed in Table S2.

2.2.7. PCR amplification and cloning

Amplifications of the anammox bacteria 16S rRNA gene, AOA and AOB *amoA* genes were performed with the primer pairs specified above (Table S1). PCR reaction mixture was the following (final concentration): Q-solution (PCR additive, Qiagen, Valencia, CA) 1 ×; PCR buffer 1 ×; BSA (200 µg mL^{-1}); dNTPs (20 µM); primers (0.2 pmol μL^{-1}); $MgCl_2$ (1.5 mM); 1.25 U Taq polymerase (Qiagen, Valencia, CA). PCR conditions for these amplifications were the following: 95°C, 5 min; 35 × [95°C, 1 min; Tm, 1 min; 72°C, 1 min]; final extension 72°C, 5 min. PCR products were gel purified (QIAquick gel purification kit, Qiagen, Valencia, CA), cloned in the TOPO-TA cloning® kit (Life Technologies, Carlsbad, CA), and transformed in E. coli TOP10 cells following the manufacturer's recommendations. Recombinant plasmidic DNA was sequenced using M13R (5'-CAG GAA ACA GCT ATG AC-3') primer by Macrogen Inc. (Amsterdam, The Netherlands).

2.2.8. Phylogenetic analysis

Sequences were analyzed for the presence of chimeras using the Bellerophon tool at the GreenGenes website (http://greengenes.lbl.gov/). Sequences were aligned with Mega5 software (Tamura et al., 2011) by using the alignment method ClustalW. Partial sequences of the archaeal *amoA* gene generated in this study were added to the *amoA* gene reference tree provided in Pester et al. (2012) using the ARB Parsimony tool (Ludwig et al., 2004). Phylogenetic trees of AOB *amoA* and the anammox bacterial 16S rRNA genes were computed with the Neighbour-Joining method (Saitou and Nei, 1987) in the Mega5 software. The evolutionary distances were estimated using the Jukes-Cantor method (Jukes and Cantor, 1969) with a bootstrap test of 1,000 replicates. Sequences were deposited in NCBI with the following accession numbers: KJ807530–KJ807556 for partial sequences of the archaeal *amoA* gene, KJ807557–KJ807597 for partial bacterial *amoA* gene sequences and KJ807598–KJ807609 for partial anammox bacterial 16S rRNA gene sequences.

2.2.9. Statistical analysis

Spearman's rank order correlation coefficient (rs) analysis was performed using the SigmaPlotTM (12.0) Exact Graphs and Data Analysis (Systat Inc., San Jose, CA). Additional multivariate anal-

ysis did not show additional correlations of environmental data with gene and expression data therefore results were not included in this study.

2.3. Results

2.3.1. Physicochemical conditions

Oxygen concentrations of the water column corresponding to the sampling time and location of sediment core sampling are shown in Fig. S1. In February, oxygen concentrations decreased throughout the water column from 301.3 to 299 μM (300 μM on average). In May, the oxygen concentration was slightly lower compared to February (282 μM on average) and decreased between 17.8–22.8 m water depth from about 287 to 278 μM. Lowest oxygen concentrations were detected in August compared to February and May (230 μM on average) and decreased between 17.8–24.8 m water depth from 242 to 217.25 μM.

Physicochemical parameters of the bottom water during the cruises of February (winter), May (spring) and August (summer) are shown in Table S3. Bottom water temperatures ranged from 5 to 15.4°C. Salinity values were stable throughout the year ranging from 34.3–34.7 practical salinity units (psu). Ammonia (NH_4^+) concentrations in the bottom water ranged between 1.6 and 3 μM with a maximum concentration in February. Bottom water nitrite (NO_2^-) concentrations varied from 0.6 μM in February to 0.1 μM in May and August. Highest nitrate (NO_3^-) concentrations were detected in February (1 μM) and lowest in August (0.5 μM).

Pore water concentrations of NH_4^+ ranged between 3–53 μM in all three seasons (Fig. 1). The depth trend of ammonia concentrations in the pore water was similar in all seasons with lower concentrations in the upper 4 cm of the sediment (3–34 μM) compared to increasing concentrations of the underlying layers (20–53 μM) down to 12 cm sediment depth. Highest NH_4^+ concentrations were detected in August (40–53 μM) compared to February and May. Nitrite (NO_2^-) concentrations ranged between 0.1–1.2 μM in all seasons (Fig. 1). Depth profiles of pore water nitrite concentrations showed different seasonal trends. In February, nitrite concentrations were relatively low (0.3 μM) in the first 3 cm of the sediment and increased with depth (0.6 μM) whereas in May and August, nitrite concentrations were higher between 1–2 cm sediment depth (0.8 μM) compared to the underlying sediment layers (0.4 and 0.2 μM, respectively). Pore water nitrate (NO_3^-) concentrations strongly varied with sediment depth and season (Fig. 1). In February and May, nitrate concentrations were highly variable with depth and reached maximum values between 3−4 cm (38 μM) and 4−5 cm of the sediment (24 μM), while the concentration strongly decreased in the underlying layers. In August, lower nitrate concentrations were detected compared to the other seasons with slightly elevated values in the first 2 cm of the sediment (7.3 μM) and lower concentrations in the underlying layers (1.3 μM). Phosphate concentrations ranged between 1.4–5.2 μM in all seasons (Fig. 1). Pore water phosphate concentrations were stable in the first 4 cm of the sediment (1.7 μM, averaged) in all three seasons. In February and May, phosphate concentrations increased slightly (3.4 μM, averaged) in the underlying sediment layers whereas the depth profile remained stable in August. TOC and TON percentages of the sediment were relatively stable in all seasons (between 0.15–0.38% and 0.02–0.08% respectively), with highest values in May between 1–2 cm and 4–5 cm sediment depth (Table S4).

2.3.2. Abundance, distribution, activity and diversity of AOA and AOB

The abundance of AOA in the sediments was determined by quantification of the 16S rRNA

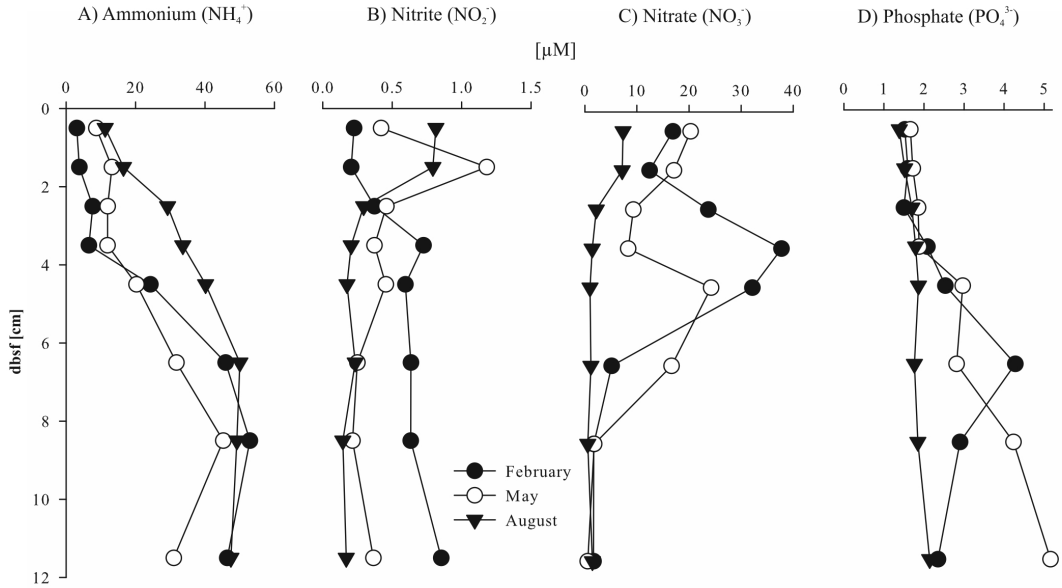

Figure 1: Sediment pore water nutrients: A) NH_4^+; B) NO_2^-; C) NO_3^- and D) PO_4^{3+} [µM] with sediment depth [cm] below sea floor [bsf].

and *amoA* gene copies, as well as by quantification of HPH-crenarchaeol (Figs. 2A–C Table S5). The seasonal variations in depth profiles of AOA 16S rRNA and *amoA* gene abundance were generally similar (Figs. 2A, B). Both AOA gene abundances were higher between 0−5 cm (6×10^6 and 1.4×10^7 gene copies g^{-1}, respectively) compared to 5−12 cm of the sediment (5×10^6 and 8.2×10^6 gene copies g^{-1}, respectively). Gene copy numbers of the AOA *amoA* gene were higher in August in comparison to the values observed in February and May (1.4-fold and 2.7-fold higher, respectively) for the 0–5 cm interval. HPH-crenarchaeol concentration was variable over the first 5 cm of the sediment (between 3×10^6–17×10^6 r.u. g^{-1}), but stable values (around 5.9×10^6 r.u. g^{-1}) were detected in the layers underneath. No clear seasonal differences were detected (Fig. 2C).

The abundance of AOB was determined by quantifying the AOB 16S rRNA and *amoA* gene (Figs. 2D, E). Values were relatively stable with depth with seasonal variations between 0–4 cm of the sediment. Abundance of the AOB 16S rRNA gene showed values similar to the AOA 16S rRNA gene abundance in February and August (on average 5.1×10^6 and 5.8×10^6 gene copies g^{-1}, respectively), while in May AOB 16S rRNA gene copy numbers were lower on average compared to the AOA 16S rRNA gene copy number (4.0×10^6 gene copies g^{-1}, 1.4-fold lower). The AOB *amoA* gene abundance followed the same seasonal trends as that of the AOB 16S rRNA gene, AOA 16S rRNA and *amoA* gene abundances, with higher values in August, but absolute values were one order of magnitude lower than AOA *amoA* gene copy numbers (2.3×10^5–5.2×10^6 gene copies g^{-1}). AOB *amoA* gene abundance was highest in the 0−5 cm depth interval except in May when values between 2−5 cm and in the 5−12 cm depth interval were lower than in the surface sediment.

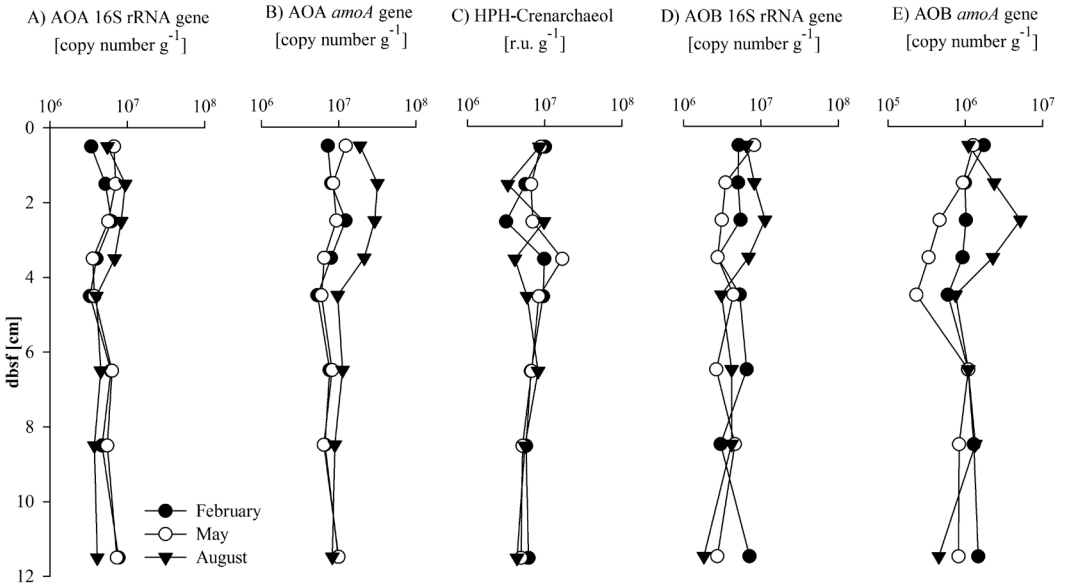

Figure 2: Abundance of A) AOA 16S rRNA gene [copy number g^{-1}]; B) AOA *amoA* gene [copy number g^{-1}]; C) Hexose phosphohexose (HPH)-crenarchaeol [response units g^{-1}], and D) AOB 16S rRNA gene [copy number g^{-1}]; E) AOB *amoA* gene [copy number g^{-1}] with sediment depth [cm] below sea floor [bsf].

The RNA:DNA ratios of the AOA and AOB 16S rRNA and *amoA* genes were calculated as an indicator of the potential transcriptional activity of the targeted microbial group (Figs. 3A–F).In all three seasons, AOA 16S rRNA gene RNA:DNA ratio showed relatively stable values down core ranging between 0.9–5.0 and 1.2–3.4 in May and August but reached slightly higher values (2.2–8.2) in February (Fig. 3A). AOB 16S rRNA gene RNA:DNA ratio was between 0.1–2.1 with a minimum of 0.1 between 4−5 cm of the sediment in February and 0.1–3.7 with a minimum between 2−3 cm sediment depth in May. The AOB 16S rRNA gene RNA:DNA ratio was slightly higher (1.2–4.1) in August (Fig. 3C). Values were relatively stable with depth in August whereas in February and May values were more variable. Compared to the RNA:DNA ratio of the AOA 16S rRNA gene, the AOB 16S rRNA gene RNA:DNA ratio was lower (5-fold in February, 1.5-fold in May and 1.3-fold lower in August) in all seasons (Figs. 3A, C). AOA *amoA* gene transcripts (Fig. 3B) were only detected in February in the first 3 cm and in August throughout the core, and were under the detection limit in May. AOB *amoA* gene RNA:DNA ratio (Fig. 3D) varied between 4×10^{-5}–2×10^{-2} in all seasons with a slight decrease with depth and highest values in August.

The diversity of AOA and AOB was evaluated in two selected depth intervals (0–1 and 9–10 cm sediment depth) of the sediment core recovered in August by targeting the AOA *amoA* gene and the AOB *amoA* gene, respectively (Figs. S2, S3). Amplified AOA *amoA* gene sequences were closely related to AOA *amoA* gene sequences previously isolated from marine and estuarine environments and did not cluster according to depth (Figs. S2A, B). Sequences were mainly affiliated to the Nitrosopumilus subclusters 12 and 16, also known as the stable marine cluster, or to

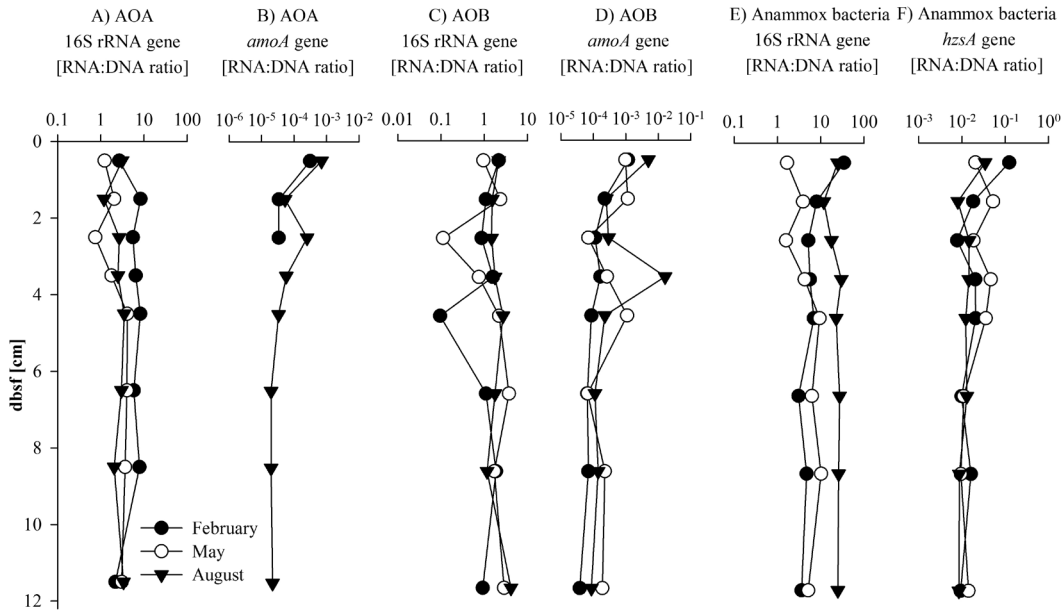

Figure 3: Transcriptional activity given as RNA:DNA ratio of A) AOA 16S rRNA gene; B) AOA *amoA* gene; C) AOB 16S rRNA gene, D) AOB *amoA*; E) Anammox 16S rRNA gene, and F) Anammox bacteria *hzsA* gene with sediment depth [cm] below sea floor [bsf].

the Nitrosopumilus subcluster 4.1, known as the estuarine cluster, according to the phylogenetic classification proposed by Pester et al. (2012). The AOB *amoA* gene sequences were affiliated to AOB *amoA* gene sequences previously isolated from estuarine sediments. Sequences were closely related to the AOB *amoA* gene sequences of *Nitrospira* sp. (accession number X90821) and *Nitrosolobus multiformis* (accession number AF042171). No clustering of AOB *amoA* sequences according to depth was observed (Fig. S3).

2.3.3. Abundance, distribution, activity and diversity of anammox bacteria

The abundance and distribution of anammox bacteria were estimated by quantification of anammox bacteria 16S rRNA and *hzsA* gene copy number, as well as by quantification of the PC-monoether ladderane (Figs. 4A–C). Depth profiles of anammox bacteria 16S rRNA and *hzsA* gene abundances followed similar trends. Gene copy numbers of the *hzsA* gene were one order of magnitude lower (between 3.5×10^5 and 2×10^6 copies g^{-1}) than anammox bacteria 16S rRNA gene copy numbers in all three seasons (Figs. 4 A, B). Values were higher in August compared to those in February and May, especially for the anammox bacteria 16S rRNA gene (3.7-fold higher).

The RNA:DNA ratio for the anammox bacteria 16S rRNA gene varied between 1.6–34.6 (Fig. 3E) with higher values in August compared to February and May (on average 4-fold higher). The *hzsA* gene RNA:DNA ratio (Fig. 3F) depth profiles showed relatively stable values (between 0.008–0.125) throughout the core without significant seasonal differences. The PC-monoether ladderane concentration (Fig. 4C) was variable over the first 5 cm of the sediment (between 0.25–1.25 ng g^{-1}), while in the underlying sediment layers values decreased slightly to more stable

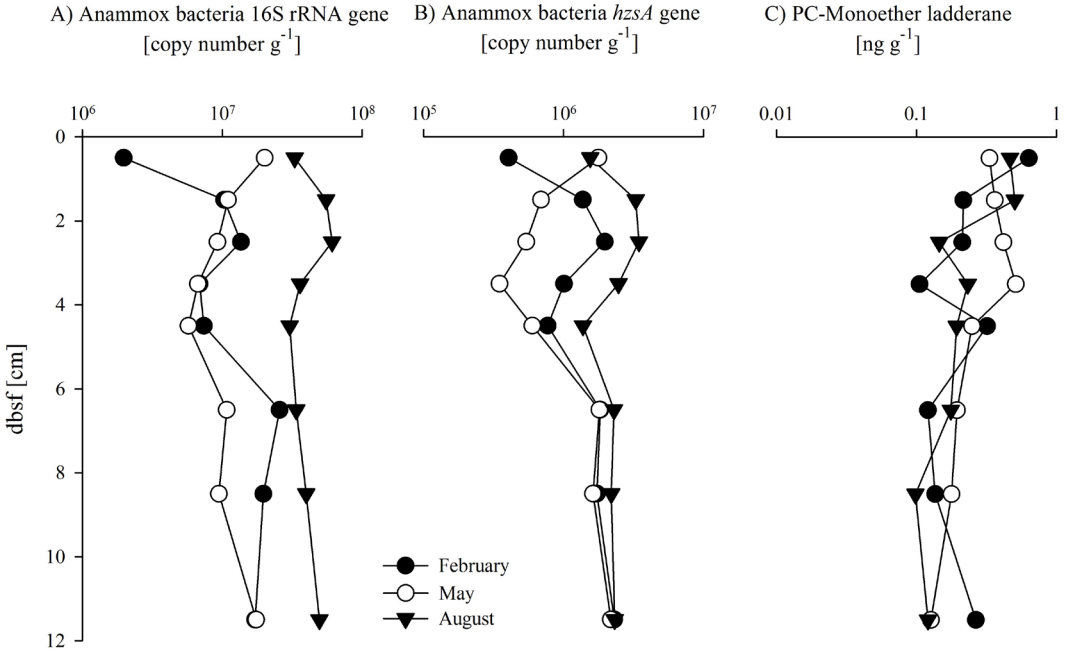

Figure 4: Abundance of A) Anammox bacteria 16S rRNA gene [copy number g^{-1}]; B) Anammox bacteria *hzsA* gene [copy number g^{-1}], and C) PC-monoether ladderane [ng g^{-1}] with sediment depth [cm] below sea floor [bsf].

values (approximately 0.36 ng g^{-1}) down to 12 cm sediment depth. There was no clear seasonal difference observed.

The diversity of anammox bacteria was evaluated in two selected depths (0–1 and 9–10 cm) of the sediment core in August by targeting the 16S rRNA gene of anammox bacteria. The sequences were closely related to "*Candidatus* Scalindua marina" (accession number EF602038) and "*Candidatus* Scalindua brodae" (accession number AY254883) and to sequences previously detected in waters of marine oxygen minimum zones (Woebken et al., 2008). Anammox bacterial 16S rRNA gene sequences from different depths were closely related to each other (Fig. S4).

2.4. Discussion

2.4.1. Aerobic ammonia oxidizers

In this study, we defined two niches in the Oyster Grounds sediment based on the differences in physicochemical parameters and in the abundance of the target organisms, i.e. the upper 4 cm of the sediment, and the underlying sediment. Ammonia concentration in the pore water of the upper part of the sediment (0–4 cm) was consistently lower than in deeper sediment layers (4–12 cm) (Fig. 1), suggesting ammonia diffusion from below formed by mineralization of organic matter by ammonification or dissimilatory nitrate reduction to ammonium (DNRA). In addition, the abundance of AOA and AOB (as well as anammox bacteria, see below) was higher and more affected by seasonality in the upper part than deeper in the sediment, which apparently is a more stable compartment. The higher variability observed in the upper part of the sediment is in agreement with the seasonal mixing of the bottom water layers and sediment deposition

dynamics in this area (Greenwood et al., 2010).

Oxygen availability is expected to play an important role in the dynamics of ammonia oxidizing microorganisms in marine sediments. In the summer months, water stratification leads to a decrease in the dissolved oxygen concentration in the bottom water in the Oyster Grounds area (Greenwood et al., 2010) (see Fig. S1). Indeed, bottom water hypoxia has been shown to cause a decrease in oxygen penetration depth and oxygen consumption in Oyster Grounds sediments by induced short-term hypoxia in intact sediment cores (Neubacher et al., 2011). Lohse et al. (1996) reported an oxygen penetration depth (OPD) of 1.7 cm in February and 0.5 cm in August in sediment at a station close to the Oyster Grounds using oxygen microelectrodes, suggesting permanently anoxic conditions from a depth of approximately >2 cm throughout the year. However, the OPD into the sediment must be interpreted with caution because the Oyster Grounds sediment is known to be an area of intense bioturbation by macro- and meiobenthos even in periods of low oxygen concentration in the bottom water (De Wilde et al., 1984; Duineveld et al., 1991), thus the import of oxygen by macrofaunal burrows into the deeper sediment could be relevant (Kristensen, 2000; Meysman et al., 2006). A study by Laverock et al. (2010) has also shown that bioturbation can induce changes in the microbial community composition, resulting in distinct communities within the burrow. However, in our study we did not observe changes in the diversity of *amoA* gene sequences of AOA or AOB, which might indicate that the changing physicochemical conditions in this system do not select for specific ammonia oxidizing microorganisms. The depth and seasonal variability for AOB was more pronounced than for AOA in the first 4 cm of the sediment, which is consistent with the fact that the relative abundance of AOB is more significantly affected by bioturbation than AOA according to previous studies (Laverock et al., 2013).

AOA and AOB 16S rRNA and *amoA* genes and AOA-IPLs and were detected up to 12 cm depth in Oyster Grounds sediments. The detection of aerobic ammonia oxidizing microorganisms in marine sediment at depths where the oxygen concentration is expected to be undetectable seems counterintuitive with their aerobic metabolism. However, the intense bioturbation reported in the Oyster Grounds sediments, as mentioned above, might provide enough oxygen to support the activity of these microbial groups. In fact, Beman et al. (2012) detected AOB and AOA *amoA* genes up to 10 cm sediment depth as well as readily detectable $^{15}NH_4^+$ oxidation rates up to 9 cm sediment depth with maxima overlapping with a peak in pore water NO_3^- + NO_2^- concentrations between 3–7 cm sediment depth in bioturbated sediments of Catalina Island (CA, USA) . Thus, our results, and those of Beman et al. (2012), suggest that aerobic ammonia oxidizers are indeed living and actively involved in the marine sedimentary nitrogen cycle deeper (i.e. up to at least ca. 10 cm) in coastal marine sediments.

The detection of a potential transcriptional activity of the AOA 16S rRNA gene up to 12 cm depth supports that AOA are indeed active deeper in the marine sediment. The potential AOA 16S rRNA gene transcriptional activity was higher during winter, whereas the abundance was slightly higher during summer months. Previous studies showed higher AOA abundance during winter months in the in the North Sea water column (Wuchter et al., 2006; Herfort et al., 2007; Pitcher et al., 2011c) attributed to the higher availability of ammonia and the lack of competition for ammonia with phytoplankton. However, none of these studies targeted the transcriptional activity of AOA. Recently, Bale et al. (2013) observed that the abundance of the benthic Thaumarchaeota (given by HPH crenarchaeol) in the surface sediment (0–1 cm) at the

Oyster Grounds was on average highest in the summer (August) and lowest in the winter (November), which they attributed to the deposition of algal-bloom-derived organic matter taking place in late spring and summer resulting in the formation of ammonia. However, it should also be noted that Bale et al. (2013) did find that transcriptional activity of the AOA 16S rRNA gene was higher during winter in the surface sediment. The disparity in seasonality between the results of these two studies highlights the difference between the seasonal ammonia dynamics at the surface sediment/water interface (Bale et al., 2013) and with depth in the sediment (this study).

To complement the determination of potential transcriptional activity of AOA based on 16S rRNA gene, we also determined the AOA *amoA* gene RNA:DNA ratio, which was possible for samples from August throughout the core but only in the upper 3 cm depth in February because the quantity of transcripts in the other seasons and depths was under the detection limit of the qPCR assay.

The relationship of *amoA* gene expression and *in situ* ammonia oxidation is still unclear (Lam et al., 2007; Nicol et al., 2008; Abell et al., 2011; Vissers et al., 2013). However, recent studies have observed a good correlation between nitrification rates and abundance of AOA *amoA* gene and transcripts in coastal waters (Urakawa et al., 2014; Smith et al., 2014). Therefore, the detection of AOA *amoA* transcripts in our setting suggests that AOA were active in the summer throughout the sediment in comparison with the winter in which AOA would be only active in the upper part of the sediment (upper 2 cm).

AOB 16S rRNA gene abundance was in the same order of magnitude than AOA 16S rRNA gene abundance, however, AOB *amoA* gene abundance was one order of magnitude lower than AOA *amoA* gene abundance, which can be explained by biases introduced by the primers or by differences in copy number of 16S rRNA and *amoA* genes per cell. In fact, it is assumed that 3.3 ± 1.6 copies of 16S rRNA can exist per cell in AOB (Větrovský et al., 2013), whereas marine Crenarchaeota seem to contain only one copy per genome (Klappenbach et al., 2001; Laverock et al., 2013). For the *amoA* gene it is assumed that AOB genomes contain 2 to 3 copies (Arp et al., 2007) while AOA genomes contain only one copy. In addition, the transcriptional activity of the AOB 16S rRNA gene was lower compared to the one of AOA 16S rRNA gene in all seasons.

On the other hand, the abundance of AOB *amoA* gene transcripts was higher than for AOA and was detected up to 12 cm depth in all seasons which would suggest a higher AOB activity vs AOA. However, a recent study by Urakawa et al. (2014) in water of the Puget Sound Estuary did not observe a correlation between nitrification rates and AOB *amoA* transcripts. In addition, previous studies have detected the presence of AOB *amoA* transcripts in non-active starved cells (Bollmann et al., 2005; Geets et al., 2006; Ward et al., 2011) indicating that the AOB *amoA* transcriptional activity is not a good indicator of AOB nitrification activity due to a longer half-life of AOB *amoA* transcripts than that expected for AOA *amoA* transcripts.

Taking together the AOA and AOB *amoA* gene quantification results, AOA have a more important role in the nitrification process in Oyster Grounds sediments than AOB. In addition, all available evidence points to the presence of some oxygen deeper in the sediments of the Oyster Grounds in all seasons probably due to the intense bioturbation in the area, which favors the activity of both AOB and AOA. In the case of AOA, and due to their higher affinity for oxygen (Martens-Habbena et al., 2009), the niche occupancy of AOA could be potentially larger than for AOB.

Previous studies observed a positive correlation between AOA abundance and low phosphate concentrations and suggested that phosphate plays a role in determining the niches of Thaumarchaeota (Dang et al., 2013; Erguder et al., 2009). In our study, AOA *amoA* gene transcript copy number and RNA:DNA ratio was negatively correlated with phosphate (r_s= –0.745 and –0.739 respectively; both $P \leq 0.005$) suggesting a lower expression of the AOA *amoA* gene with an increase in pore water phosphate concentrations. These results support the assumption that some ecotypes of AOA dominate over AOB in environments with low phosphate availability. Previously, homologues of the pst gene, encoding for the Pst phosphate ABC transporter, which is usually activated in phosphate deficient environments, was found in marine metagenomes of thaumarchaeotal origin (Rusch et al., 2007; Dang et al., 2013) emphasizing the importance of phosphate availability in marine environments. No correlations were found between AOB abundance or transcriptional activity with pore water nitrogen species, but a negative correlation with the sediment depth (r_s= –0.646; $P \leq 0.005$) was observed indicating that sediment depth is a factor determining the AOB abundance and activity in Oyster Grounds sediments which might be explained by the decreasing oxygen availability with depth.

2.4.2. Anaerobic ammonia oxidizers

Anammox bacteria 16S rRNA and *hzsA* gene abundance was detected throughout the analyzed sediment with more pronounced depth and seasonal differences than those observed for AOA and AOB. This was not reflected in the abundance of PC-monoether ladderane lipid.

It is possible that the PC-monoether ladderane is partly derived from fossil material and thus reflects a long-term presence of anammox bacteria averaged throughout the entire year rather than an actively living anammox population. This possibility has been already suggested by Bransdsma et al. (2011) and Bale et al. (2014) who found dissimilarities between the PC-monoether ladderane concentration and gene copy numbers in surface sediments of the Gullmar Fjord and the Southern North Sea, respectively.

We also observed an up to 10-fold difference in the quantification of anammox bacteria 16S rRNA and *hzsA* gene abundance (Figs. 4A, B). This discordance between the anammox bacteria gene markers has been previously observed in environmental samples (Harhangi et al., 2012), and it could be due to primer biases as previously suggested (Bale et al., 2014). Both the anammox 16S rRNA and the *hzsA* gene revealed a higher abundance in August compared to February and May. This may indicate that anammox bacteria are more active and abundant at higher temperatures (15˚C), which is the anammox bacteria temperature optimum in temperate shelf sediments (Dalsgaard and Thamdrup, 2005). Additionally, the oxygen penetration depth is expected to decrease during water stratification between May and October, which would also favor anammox bacterial growth and activity (Dalsgaard and Thamdrup, 2002). Previous studies by Neubacher et al. (2013) observed an increase in anammox activity up to 82% compared to control treatments in mesocosms under sustained hypoxic conditions, which corresponds well with our findings of high anammox activity during summer stratification associated with lower oxygen concentrations in the bottom water (Fig. S1).

The presence of anammox markers in the upper layers of the sediment (0–4 cm) is remarkable as the metabolism of anammox bacteria is expected to be incompatible with the presence of oxygen. However, some studies have reported that anammox bacteria can cope with low oxygen concentrations (Kalvelage et al., 2011; Yan et al., 2012), and that anammox bacteria can

be present and active at high oxygen levels by being dormant or using anaerobic niches (Kuypers et al., 2005; Woebken et al., 2007). It is also possible that a high activity of AOA and AOB and heterotrophic bacteria inhabiting these marine sediments would rapidly consume the oxygen available and allow the activity of anammox bacteria even in presence of oxygen. Some studies have also concluded that the potential for anammox bacteria activity is indeed not affected by bioturbation or physical mixing (Rooks et al., 2012). On the contrary, bioturbation and mixing can extend the area of nitrate reduction and thus the availability of nitrite, which fuels the anammox process (Meyer et al., 2005). Our results thus suggest that anammox bacteria can tolerate the presence of oxygen and co-exist with aerobic ammonia oxidizers and potentially have an important role in the nitrogen cycle in marine sediments. In fact, the potential transcriptional activity of anammox bacteria 16S rRNA and *hzsA* gene was observed throughout the sediment in all seasons. The transcriptional activity of anammox bacteria 16S rRNA gene was higher in the summer as observed for the anammox bacteria abundance. A recent study by Bale et al. (2014) observed a good correlation between the rate of anammox and anammox bacteria 16S rRNA gene transcript abundance in North Sea sediments, which suggests that the transcriptional activity of anammox bacteria 16S rRNA gene is a good proxy of anammox bacteria activity, and confirm a higher anammox activity in the summer for the sediments analyzed in our study. Nevertheless, quantification results of anammox bacteria 16S rRNA gene transcripts have to be interpret with caution because there is evidence that the ribosome content does not decrease during the period of starvation (Schmid et al., 2005; Li et al., 2011). However, the increase of anammox bacteria 16S rRNA gene transcriptional activity during summer was not observed for the *hzsA* gene, which remained stable across sediment depth and also for the different seasons, and gene abundances were generally an order of magnitude lower. The low and constitutive transcriptional activity of the *hzsA* gene indicates that the *hzsA* gene transcript abundance does not reflect variations of anammox bacteria activity in environmental sediment samples, thus the *hzsA* gene transcript abundance does not seem to be an adequate biomarker for the estimation of the activity of anammox bacteria. Further experiments, especially with pure cultures and under controlled physiological conditions, should be performed to clarify the regulation of the expression of the *hzsA* gene.

No evidence of a niche separation of aerobic and anaerobic ammonia oxidizers was observed in the oxygen transition zone of the marine sediments. Surprisingly, aerobic and anaerobic ammonia oxidizers are present and active throughout the year in oxygenated sediment layers as well as in anoxic layers. These results seem to be counterintuitive with regards to the individual oxygen requirements of the targeted microbial groups. We hypothesize that the presence and activity of aerobic ammonia oxidizers (AOA, AOB) is supported by the oxygen supply in deeper anoxic layers due to bioturbation. Likewise, the presence and activity of anammox bacteria in these sediments is then possible by the rapid consumption of oxygen by aerobic ammonia oxidizers and/or other groups, as well as the availability of nitrite provided either by ammonia oxidizers or by an active nitrate reduction present in bioturbated sediments (Meyer et al., 2005).

2.5. Conclusion

Our study has unraveled the coexistence and metabolic activity of AOA, AOB, and anammox bacteria in bioturbated marine sediments of the North Sea, leading to the conclusion that the metabolism of these microbial groups is spatially coupled based on the rapid consumption of

oxygen that allows the coexistence of aerobic and anaerobic ammonia oxidizers. AOA outnumbered AOB throughout the year which may be caused by the higher oxygen affinity of AOA compared to AOB. Anammox bacterial abundance and activity were higher during summer, indicating that their growth and activity are favored by higher temperatures and lower oxygen available in the sediments due to summer stratifying conditions in the water column. During the summer, anammox bacteria are probably not in competition with AOA and AOB for ammonia as the concentrations were relatively high in the sediment pore water most likely as a result of ammonification processes and the activity of denitrifiers and DNRA. Further studies are required to get a complete picture of the nature of these interactions in the oxygen transition zone of coastal marine sediments.

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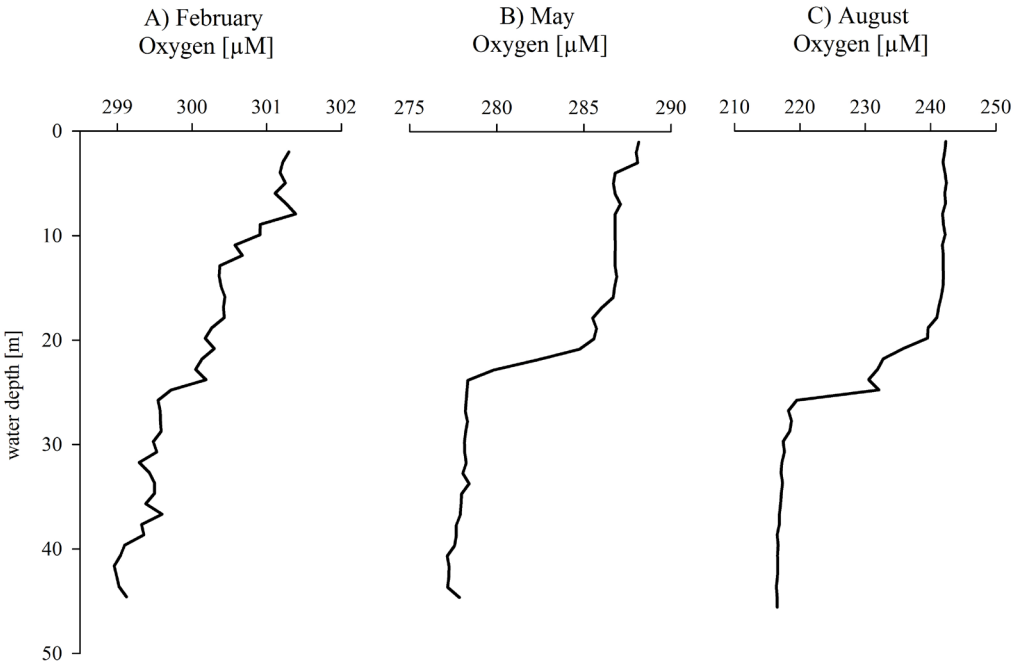

Figure S1: Oxygen profiles [µM] of the water column in February, May and August.

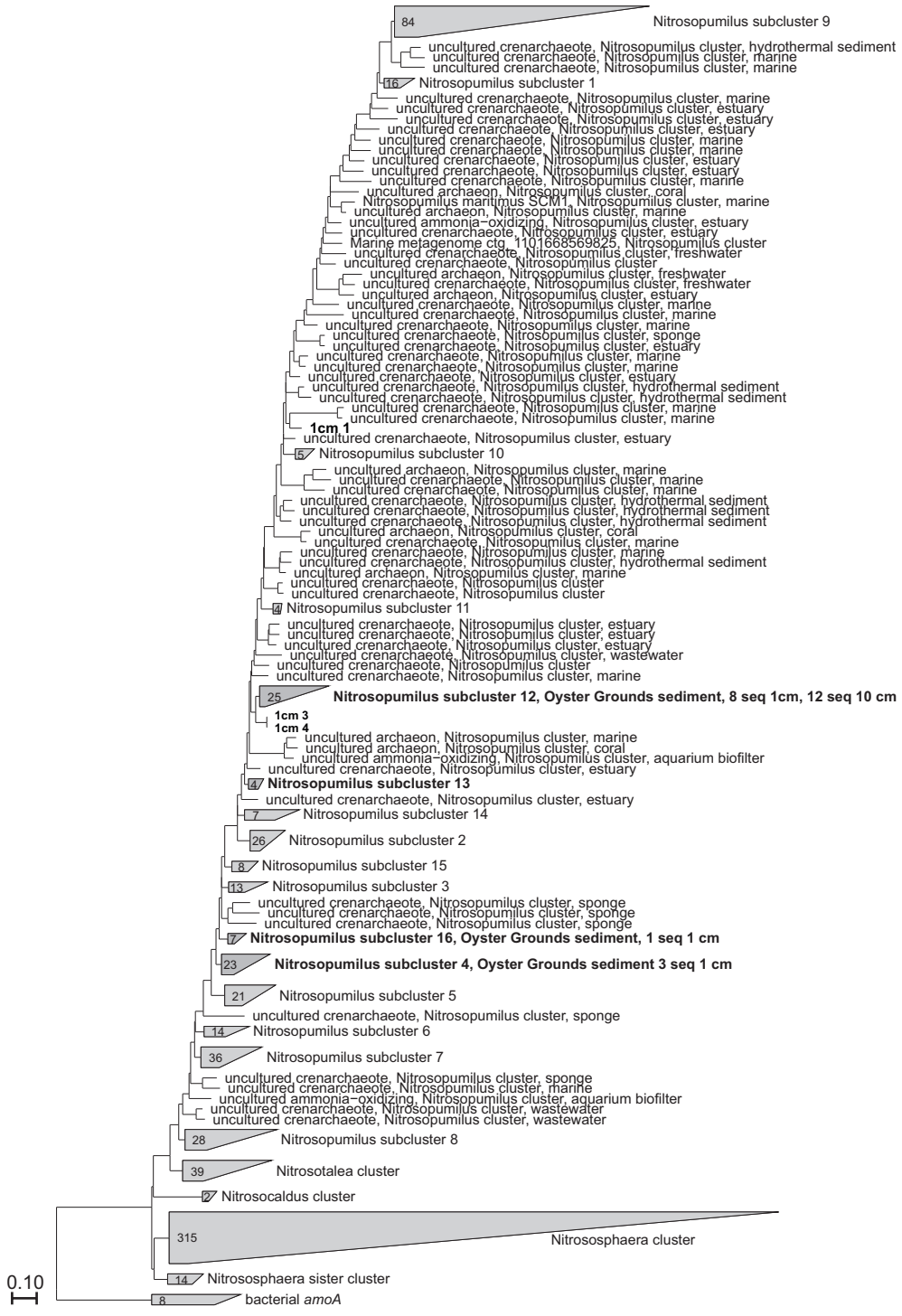

Figure S2A: Phylogenetic tree of AOA *amoA* gene sequences retrieved in this study and closest relatives (Bold: sequences retrieved at 1cm sediment depth and sequences retrieved at 10 cm sediment depth; August 2011 and clusters containing sequences retrieved in this study, find details in Fig. S2B); the scale bar indicates 10% sequence divergence.

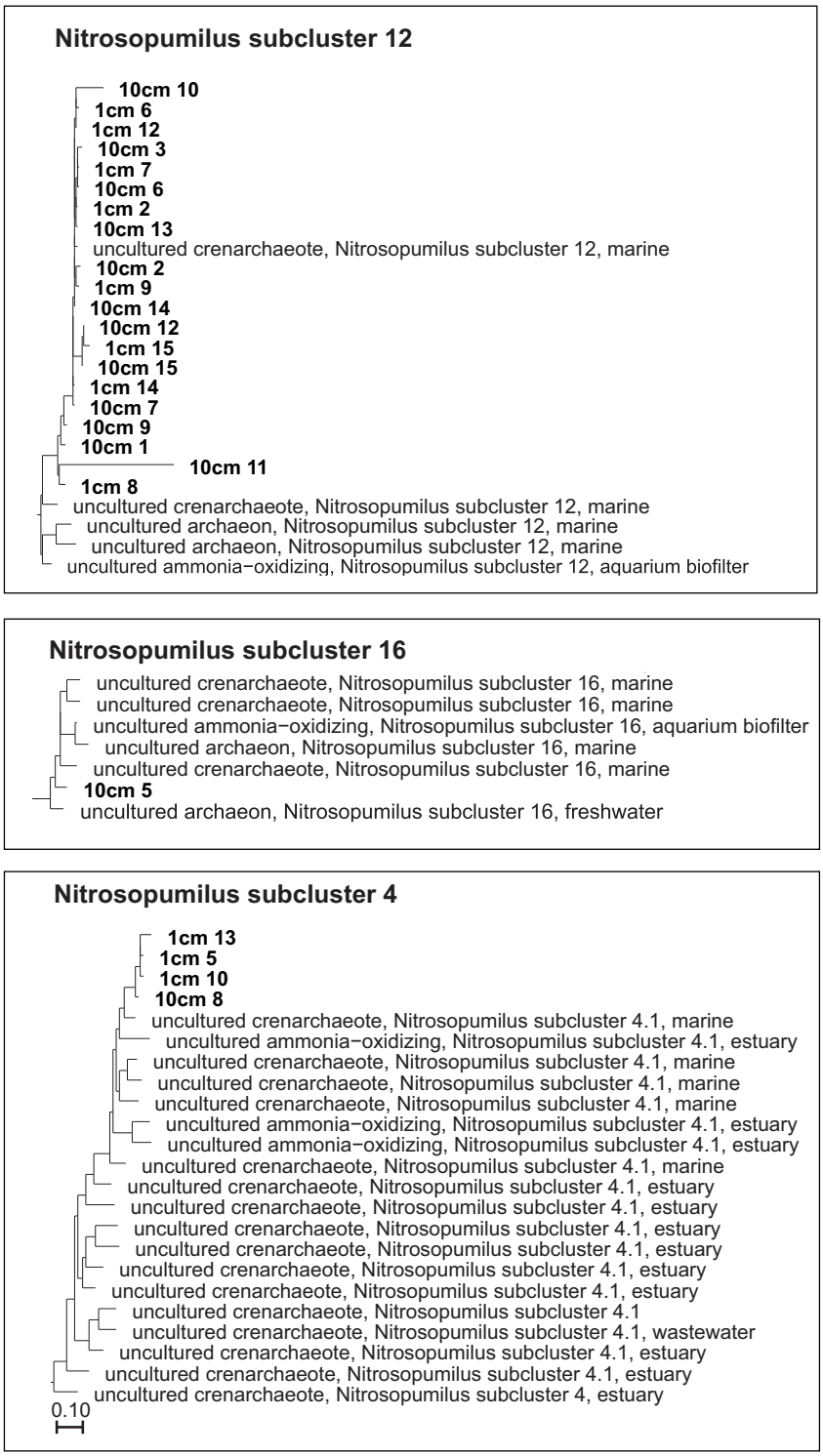

Figure S2B: Phylogenetic tree of AOA *amoA* gene sequences retrieved in this study and closest relatives (Bold: sequences retrieved at 1cm sediment depth and sequences retrieved at 10 cm sediment depth; August 2011) the scale bar indicates 10% sequence divergence.

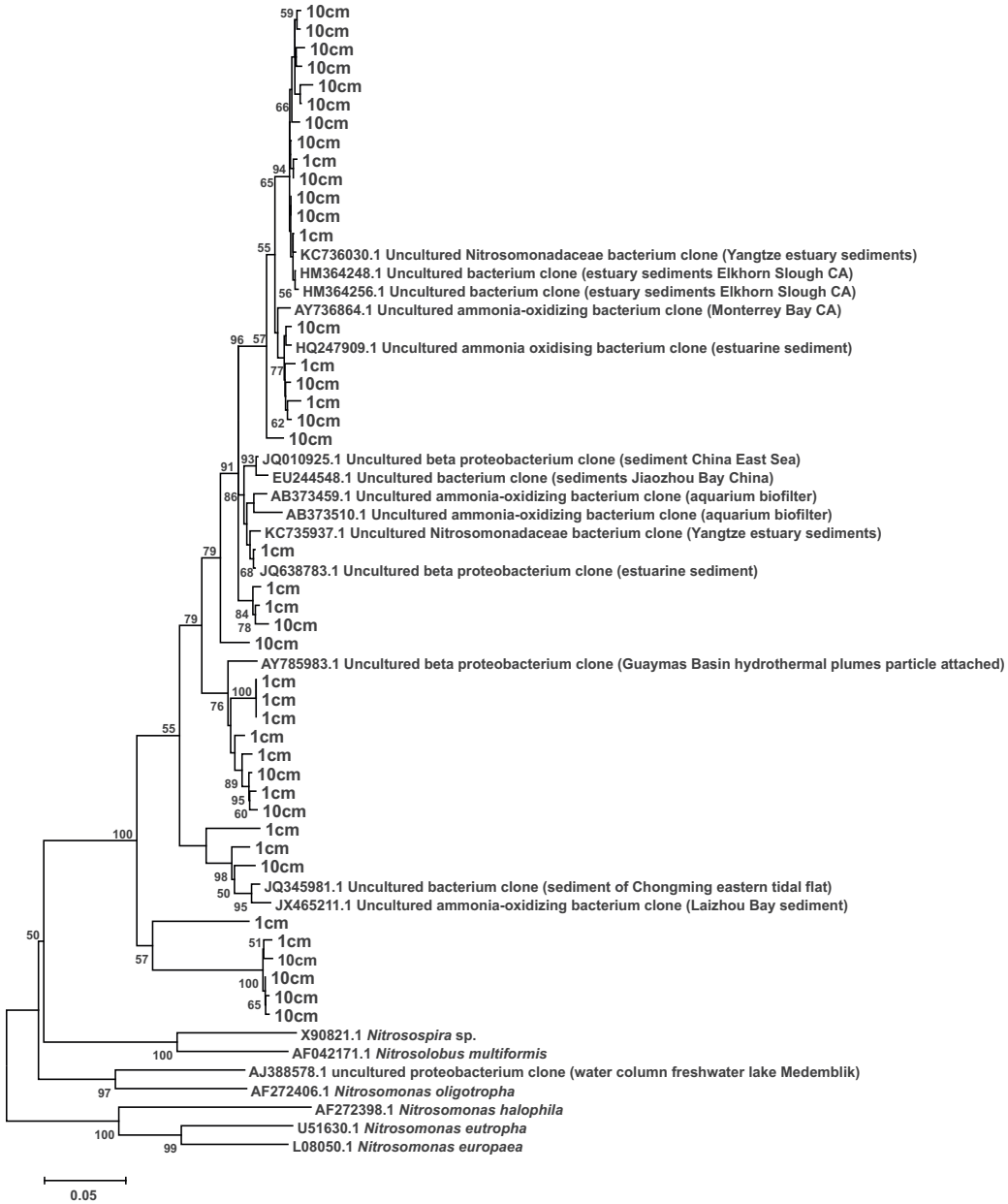

Figure S3: Phylogenetic tree of AOB *amoA* gene sequences retrieved in this study; the scale bar indicates 5% sequence divergence.

Figure S4: Phylogenetic tree of anammox bacteria 16S rRNA gene sequences retrieved in this study; the scale bar indicates 0.2% sequence divergence.

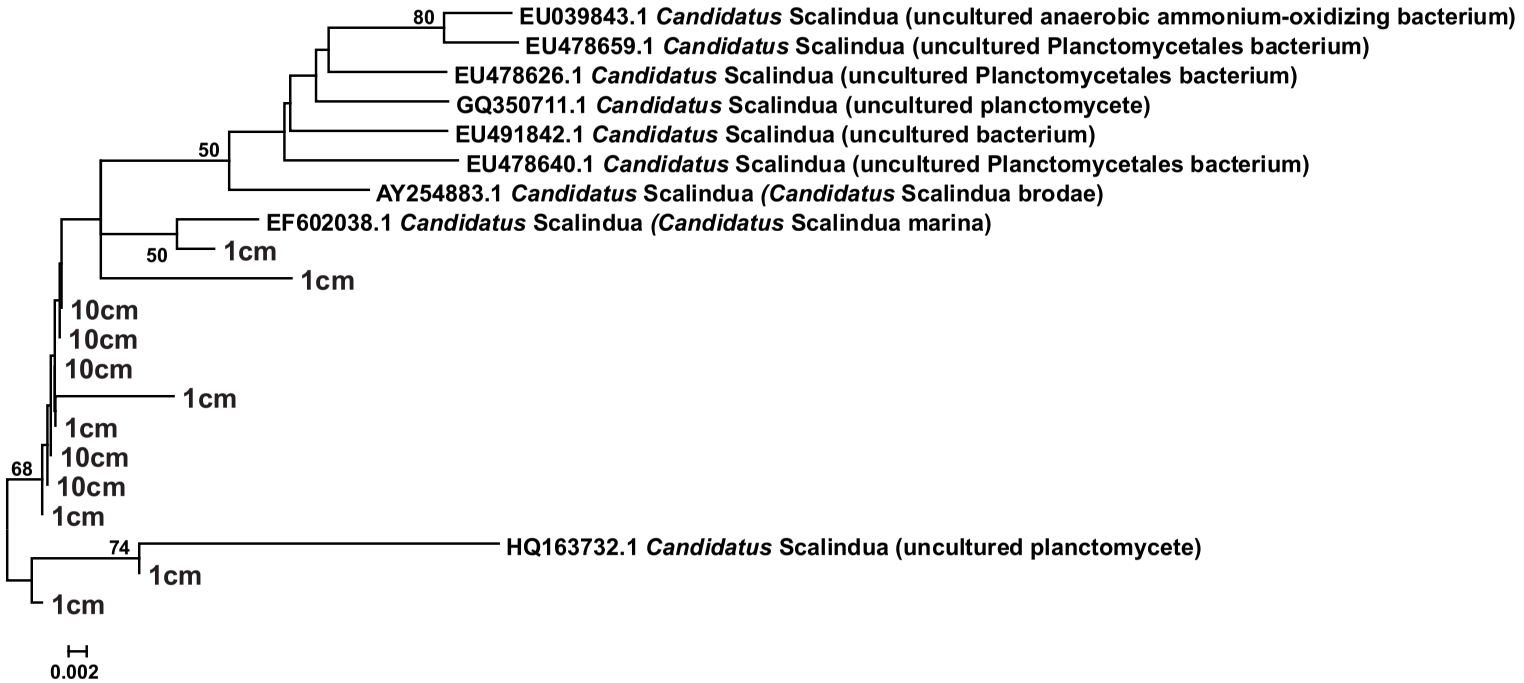

Table S1: Primer pairs described in the text, polymerase chain reaction (PCR) conditions and amplicon size used in this study.

Assay	Target	Primer pair	T_m	Amplicon size [bp*]	Reference
qPCR	AOA 16S rRNA gene	MCGI-391F (5'-AAGGTTARTCCGAGTGRTTTC-3') MCGI-554R (5'-TGACCACTTGAGGTGCTG-3')	qPCR 61°C	163	Coolen et al. (2007)
qPCR + PCR/ cloning	AOA *amoA* gene	AmoA-ModF (5'-GGTCTGGYTWAGACGATG-3') AmoA-ModR (5'-GCCATCCATCTGTAWGTC-3')	qPCR 59.5°C PCR 55°C	626	Yakimov et al. (2011)
qPCR + PCR/ cloning	Anammox bacteria 16S rRNA gene	Brod541F (5'-GAGCACGTAGGTGGGTTTGT-3') Amx820R (5'-AAAACCCCTCTACTTAGTGCCC-3')	qPCR 59°C PCR 58°C	279	Li et al. (2010)
qPCR	Anammox bacteria *hzsA* gene	hzsA_1597F (5'-WTYGGKTATCARTATGTA-3') hzsA_1857R (5'-AAABGGYGAATCATARTGGC-3')	qPCR 51°C	260	Harhangi et al. (2012)
qPCR	AOB 16S rRNA gene	CTO189F_A (5'-GGAGRAAAGCAGGGGATCG-3')† CTO189F_C (5'-GGAGGAAAGTAGGGGATCG-3')† CTO654R (5'-CTAGCYTTGTAGTTTCAAACGC-3')	qPCR 60°C	465	Kowalchuk et al. (1997)
qPCR + PCR/ cloning	AOB *amoA* gene	AOB-amoAF (5'-GGGGTTTCTACTGGTGGT-3') AOB-amoAR new (5'-CCCCTCBGSAAAVCCTTCTTC-3')	qPCR 61.6°C PCR 58°C	491	Rotthauwe et al. (1997), Hornek et al. (2006)

PCR conditions: 95°C 5 min; 40 × [95°C 1 min, melting temperature (T_m) 40 s, 72°C 1 min]; 72°C 5 min; qPCR conditions: 95°C 4 min; 40 × [95°C 30 s, T_m 40 s, 72°C 30 s]; 80°C 25 s; *base pairs (bp); † The forward primer contains an equimolar mixture of the reported forward primers.

Table S2: Quantitative polymerase chain reaction (qPCR) efficiencies (E) and correlation coefficients (R^2).

		February			May			August		
qPCR assay	**target molecule**	**T_m [°C]**	**E [%]**	**R^2**	**T_m [°C]**	**E [%]**	**R^2**	**T_m [°C]**	**E [%]**	**R^2**
AOA 16S rRNA gene	DNA	61.0	100.0	0.998	61.0	93.5	0.988	61.0	94.3	0.999
(MCGI-391F/MCGI-554R)	cDNA	61.0	96.1	1.000	61.0	98.8	0.998	61.0	97.2	0.999
AOA *amo*A gene	DNA	59.5	100.7	0.998	59.5	99.7	0.988	59.5	97.8	0.994
(AmoA_modF/AmoA_modR)	cDNA	59.5	99.9	0.983	59.5	107.6	0.984	59.5	96.4	0.995
Anammox bacteria 16S rRNA gene	DNA	59.0	99.0	0.998	59.0	96.4	0.995	59.0	98.9	0.993
(Brod541F/Amx820R)	cDNA	59.0	98.4	0.999	59.0	93.7	0.996	59.0	106.8	0.989
Anammox bacteria *hzs*A gene	DNA	51.0	82.0	0.997	51.0	81.1	0.997	51.0	80.1	0.997
(hzsA_1597F/hzsA_1857R)	cDNA	51.0	80.0	0.998	51.0	80.2	0.997	51.0	80.4	0.996
AOB 16S rRNA gene	DNA	60.0	86.6	0.998	60.0	86.6	0.998	60.0	86.6	0.998
(CTO189F/CTO654R)	cDNA	60.0	84.0	0.998	60.0	84.0	0.998	60.0	84.0	0.998
AOB *amo*A gene	DNA	61.6	85.4	0.994	61.6	85.4	0.994	61.6	85.4	0.994
(AOB-amoAF/AOB-amoAR new)	cDNA	61.6	91.0	0.993	61.6	91.0	0.993	61.6	91.0	0.993

qPCR conditions: 95°C 4 min; 40 × [95°C 30 s, melting temperature (T_m) 40 s, 72°C 30 s]; 80°C 25 s

Table S3: Physical (CTD) and chemical (nutrient) properties of the bottom water (BW).

Parameter/ Sediment dbsf [cm]	Ammonium [µM]	Nitrite [µM]	Nitrate [µM]	Phosphate [µM]	Temperature [°C]	Salinity [psu]	water depth [m]
Feb-11							
BW	3.0	0.6	6.8	1.0	5.0	34.4	43.0
May-11							
BW	1.9	0.1	2.0	0.7	8.6	34.3	44.0
Aug-11							
BW	1.6	0.1	0.5	0.5	15.4	34.7	45.0

Table S4: Total organic carbon (TOC) and total organic nitrogen (TON).

Parameter/ Sediment dbsf [cm]	TOC [%]	TON [%]
Feb-11		
0.5	0.22	0.02
1.5	0.22	bdl*
2.5	0.21	0.04
3.5	0.22	0.04
4.5	0.26	0.03
6.5	0.20	0.02
8.5	0.28	0.03
11.5	0.37	0.08
May-11		
0.5	0.20	0.02
1.5	0.30	0.05
2.5	0.25	0.04
3.5	0.27	0.03
4.5	0.38	0.08
6.5	0.25	0.05
8.5	0.15	0.03
11.5	0.21	0.02
Aug-11		
0.5	0.26	0.03
1.5	0.27	0.05
2.5	0.23	0.02
3.5	0.20	0.02
4.5	0.31	0.06
6.5	0.25	0.03
8.5	0.28	0.03
11.5	0.22	0.04

*bdl: below detection limit

Table S5a: Spearman rank order correlation of all parameters observed in this study.

Parameter	NH_4^+ [µM]	NO_3^- [µM]	PO_4^+ [µM]	TON [%]	AOA 16S rRNA gene (RNA:DNA ratio)	AOA *amoA* gene (DNA) [copies g^{-1}]
Sediment depth [cm]	0.822***		0.801***			
NH_4^+ [µM]		0.734***				
NO_3^- [µM]						
PO_4^+ [µM]						
AOA 16S rRNA gene (DNA) [copies g^{-1}]						0.694***
AOA 16S rRNA gene (cDNA) [copies g^{-1}]					0.846***	
AOA *amoA* gene (DNA) [copies g^{-1}]						
AOA *amoA* gene (cDNA) [copies g^{-1}]						
AOA *amoA* gene (RNA:DNA ratio)						
Crenarchaeol IPLs total [area g^{-1}]						
HPH-crenarchaeol [area g^{-1}]						
TOC [%]				0.657***		
Anammox bacteria 16S rRNA gene (DNA) [copies g^{-1}]						
Anammox bacteria 16S rRNA gene (cDNA) [copies g^{-1}]						
Anammox bacteria 16S rRNA gene (RNA:DNA ratio)						
Anammox bacteria *hzsA* gene (DNA) [copies g^{-1}]						
Anammox bacteria *hzsA* gene (cDNA) [copies g^{-1}]						
Anammox *hzsA* gene (RNA:DNA ratio)						
PC Monoether ladderane [ng g^{-1}]						
AOB 16S rRNA gene (cDNA) [copies g^{-1}]						
AOB *amoA* (DNA) gene [copies g^{-1}]						
AOB *amoA* (cDNA) gene [copies g^{-1}]						
AOB *amoA* gene (RNA:DNA ratio)						

Correlation coefficients $r_s \geq 0.6$ and $r_s \leq -0.6$ with *P*-values ≤ 0.05 were reported. The degree of significance was indicated by using a single asterisk for *P*-values ≤ 0.05 and ≥ 0.01, two asterisks for *P*-values ≤ 0.01 and three asterisks for *P*-values ≤ 0.005.

Table S5b: Spearman rank order correlation of all parameters observed in this study.

Parameter	AOA *amoA* gene (cDNA) [copies g^{-1}]	AOA *amoA* gene (RNA:DNA ratio)	Anammox 16S rRNA gene (DNA) [copies g^{-1}]	Anammox 16S rRNA gene (cDNA) [copies g^{-1}]
Sediment depth [cm]				
NH_4^+ [µM]				
NO_3^- [µM]			-0.647***	-0.743***
PO_4^+ [µM]	-0.745***	-0.739***		
AOA 16S rRNA gene (DNA) [copies g^{-1}]				
AOA 16S rRNA gene (cDNA) [copies g^{-1}]				
AOA *amoA* gene (DNA) [copies g^{-1}]			0.725***	
AOA *amoA* gene (cDNA) [copies g^{-1}]		0.994***		0.629***
AOA *amoA* gene (RNA:DNA ratio)				
Crenarchaeol IPLs total [area g^{-1}]				
HPH-crenarchaeol [area g^{-1}]				
TOC [%]				
Anammox bacteria 16S rRNA gene (DNA) [copies g^{-1}]				0.797***
Anammox bacteria 16S rRNA gene (cDNA) [copies g^{-1}]				
Anammox bacteria 16S rRNA gene (RNA:DNA ratio)		0.717***		
Anammox bacteria *hzsA* gene (DNA) [copies g^{-1}]				
Anammox bacteria *hzsA* gene (cDNA) [copies g^{-1}]				
Anammox *hzsA* gene (RNA:DNA ratio)				
PC Monoether ladderane [ng g^{-1}]				
AOB 16S rRNA gene (cDNA) [copies g^{-1}]				
AOB *amoA* (DNA) gene [copies g^{-1}]				
AOB *amoA* (cDNA) gene [copies g^{-1}]				
AOB *amoA* gene (RNA:DNA ratio)				

Table S5c: Spearman rank order correlation of all parameters observed in this study.

Parameter	Anammox 16S rRNA (RNA:DNA ratio)	Anammox *hzsA* (DNA) [copies g^{-1}]	Anammox *hzsA* (cDNA) [copies g^{-1}]	Anammox *hzsA* (RNA:DNA ratio)	PC-ME ladderane [ng g^{-1}]
Sediment depth [cm]					-0.662***
NH_4^+ [µM]					
NO_3^- [µM]					
PO_4^+ [µM]					
AOA 16S rRNA gene (DNA) [copies g^{-1}]					
AOA 16S rRNA gene (cDNA) [copies g^{-1}]					
AOA *amoA* gene (DNA) [copies g^{-1}]		0.662***			
AOA *amoA* gene (cDNA) [copies g^{-1}]	0.708***				
AOA *amoA* gene (RNA:DNA ratio)					
Crenarchaeol IPLs total [area g^{-1}]				0.797***	
HPH-crenarchaeol [area g^{-1}]				0.760***	
TOC [%]					
Anammox bacteria 16S rRNA gene (DNA) [copies g^{-1}]		0.848***		-0.751***	
Anammox bacteria 16S rRNA gene (cDNA) [copies g^{-1}]	0.748***	0.729***			
Anammox bacteria 16S rRNA gene (RNA:DNA ratio)					
Anammox bacteria *hzsA* gene (DNA) [copies g^{-1}]					
Anammox bacteria *hzsA* gene (cDNA) [copies g^{-1}]					
Anammox *hzsA* gene (RNA:DNA ratio)					
PC Monoether ladderane [ng g^{-1}]					
AOB 16S rRNA gene (cDNA) [copies g^{-1}]					
AOB *amoA* (DNA) gene [copies g^{-1}]					
AOB *amoA* (cDNA) gene [copies g^{-1}]			0.713***		
AOB *amoA* gene (RNA:DNA ratio)					

Table S5d: Spearman rank order correlation of all parameters observed in this study.

Parameter	AOB 16S rRNA (DNA) [copies g^{-1}]	AOB 16S rRNA (cDNA) [copies g^{-1}]	AOB 16S rRNA (RNA:DNA ratio)	AOB *amoA* (cDNA) [copies g^{-1}]	AOB *amoA* (RNA:DNA ratio)
Sediment depth [cm]					-0.646***
NH_4^+ [µM]					
NO_3^- [µM]					
PO_4^+ [µM]					
AOA 16S rRNA gene (DNA) [copies g^{-1}]					
AOA 16S rRNA gene (cDNA) [copies g^{-1}]					
AOA *amoA* gene (DNA) [copies g^{-1}]					
AOA *amoA* gene (cDNA) [copies g^{-1}]					
AOA *amoA* gene (RNA:DNA ratio)					
Crenarchaeol IPLs total [area g^{-1}]					
HPH-crenarchaeol [area g^{-1}]					
TOC [%]					
Anammox bacteria 16S rRNA gene (DNA) [copies g^{-1}]					
Anammox bacteria 16S rRNA gene (cDNA) [copies g^{-1}]					
Anammox bacteria 16S rRNA gene (RNA:DNA ratio)					
Anammox bacteria *hzs*A gene (DNA) [copies g^{-1}]					
Anammox bacteria *hzs*A gene (cDNA) [copies g^{-1}]		0.656***			
Anammox *hzsA* gene (RNA:DNA ratio)					
PC Monoether ladderane [ng g^{-1}]					
AOB 16S rRNA gene (cDNA) [copies g^{-1}]			0.620***		
AOB *amoA* (DNA) gene [copies g^{-1}]	0.617***				
AOB *amoA* (cDNA) gene [copies g^{-1}]		0.702***			
AOB *amoA* gene (RNA:DNA ratio)				0.887***	

3. Lack of ^{13}C-label incorporation suggests low turnover rates of thaumarchaeal intact polar tetraether lipids in sediments from the Iceland Shelf

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Thaumarchaeota are amongst the most abundant microorganisms in aquatic environments, however, their metabolism in marine sediments is still debated. Labeling studies in marine sediments have previously been undertaken, but focused on complex organic carbon substrates which Thaumarchaeota have not yet been shown to take up. In this study, we investigated the activity of Thaumarchaeota in sediments by supplying different ^{13}C-labeled substrates which have previously been shown to be incorporated into archaeal cells in water incubations and/or enrichment cultures. We determined the incorporation of ^{13}C-label from bicarbonate, pyruvate, glucose and amino acids into thaumarchaeal intact polar lipid-glycerol dibiphytanyl glycerol tetraethers (IPL-GDGTs) during 4–6 day incubations of marine sediment cores from three sites on the Iceland Shelf. Thaumarchaeal intact polar lipids, in particular crenarchaeol, were detected at all stations and concentrations remained constant or decreased slightly upon incubation. No ^{13}C incorporation in any IPL-GDGT was observed at stations 2 (clay-rich sediment) and 3 (organic-rich sediment). In bacterial/eukaryotic IPL-derived fatty acids at station 3, contrastingly, a large uptake of ^{13}C label (up to + 80‰) was found. ^{13}C was also respired during the experiment as shown by a substantial increase in the ^{13}C content of the dissolved inorganic carbon. In IPL-GDGTs recovered from the sandy sediments at station 1, however, some enrichment in δ^{13}C (1–4‰) was detected after incubation with bicarbonate and pyruvate. The low incorporation rates suggest a low activity of Thaumarchaeota in marine sediments and/or a low turnover rate of thaumarchaeal IPL-GDGTs due to their low degradation rates. Cell numbers and activity of sedimentary Thaumarchaeota based on IPL-GDGT measurements may thus have previously been overestimated.

Biogeosciences, 11, 201–216, 2014

3.1. Introduction

Thaumarchaeota are ubiquitous microorganisms (Hatzenpichler, 2012 and references cited therein) which have recently been discovered to form a separate phylum, the Thaumarchaeota (Brochier-Armanet et al., 2008; Spang et al., 2010). They were initially discovered as the Marine Group I Crenarchaeota in the coastal and open ocean (DeLong, 1992; Fuhrman et al. 1992; Karner et al., 2001) where they are abundantly present in the epipelagic zone (e.g. Francis et al., 2005; Beman et al., 2008) but also in deep water (Fuhrman and Davis, 1997; Herndl et al., 2005; Agogué et al., 2008) where they can be as abundant as the total bacterial population. Thaumarchaeota have also been detected in marine sediments (Hershberger et al., 1996; Francis et al., 2005).

Before enrichment cultures became available, the metabolism of Thaumarchaeota was unclear. Hoefs et al. (1997) suggested, based on the ^{13}C-enrichment of thaumarchaeal lipids in comparison to algal biomarkers, the possibility of autotrophic assimilation of dissolved inorganic carbon (DIC) using a different pathway than via Rubisco, or, alternatively, the heterotrophic uptake of small organic molecules. Indeed, ^{13}C-labeling studies with bicarbonate showed the incorporation of ^{13}C-labelled DIC into thaumarchaeal membrane lipids and thus their autotrophic metabolism (Wuchter et al., 2003). Unambiguous proof for the growth of Thaumarchaeota on inorganic carbon came with the first enrichment culture of *Nitrosopumilus maritimus*, which was growing chemolithoautotrophically on bicarbonate, and gaining energy by the oxidation of ammonia to nitrite (Könneke et al., 2005). It was suggested that 3-hydroxypropionate/4-hydroxybutyrate pathway was used by these microorganisms (Berg et al., 2007), which was supported by genetic analyses of *C. symbiosum* (Hallam et al., 2006) and *N. maritimus* (Walker et al., 2010). However, in these two species, also genes involved in heterotrophic metabolisms were detected, which implies their potential for mixotrophy. Furthermore, the uptake of pyruvate into cell material in cultures of a Thaumarchaeote enriched from soil, *Nitrososphaera viennensis*, was demonstrated (Tourna et al., 2011).

Indeed, there is some environmental evidence for a mixotrophic metabolism of Thaumarchaeota. Ouverney and Fuhrman (2000) as well as Herndl et al. (2005) showed, via MICRO-CARD-FISH using ^{3}H-labeled amino acids, that thaumarchaeal cells present in sea water take up amino acids. There is also circumstantial evidence for mixotrophy of Thaumarchaeota, such as the radiocarbon values of archaeal lipids in the deep ocean (Ingalls et al., 2006), thaumarchaeal activity (as quantified by uptake of substrate measured by MAR-FISH) being correlated to the presence of urea (Alonso-Sáez et al., 2012), and the possession of genes for urea transporters (Baker et al., 2012). Mußmann et al. (2011) found thaumarchaeal cell numbers in waste reactors that were too high to be supported by the rates of ammonia oxidation, and a lack of incorporation of ^{13}C-labelled DIC into thaumarchaeal lipids during growth, also suggesting heterotrophy of Thaumarchaeota. Circumstantial evidence, based on the correlation of organic carbon concentration with archaeal biomarker lipids, suggests that Archaea in deep subsurface marine sediments (<1 m) use organic carbon (Biddle et al., 2006; Lipp et al., 2008; Lipp and Hinrichs, 2009), however, this empirical correlation could also be due to preservation factors affecting biomarkers and total organic carbon in a similar way (Hedges et al., 1999; Schouten et al., 2010). These studies thus illustrate the potential for diversity of thaumarchaeal metabolisms.

Cell membranes of Thaumarchaeota consist of glycerol dibiphytanyl glycerol tetraether lipids (GDGT) in the form of intact polar lipids (IPL; Schouten et al., 2008; Pitcher et al., 2011a;

Sinninghe Damsté et al., 2012; Fig. 1a). They possess GDGTs which are also present in other Archaea (Koga and Nakano, 2008), such as GDGT-0, -1, -2 and -3 (Fig. 1b), but also a specific lipid named crenarchaeol, which up to now has only been found in (enrichment) cultures of Thaumarchaeota (Sinninghe Damsté et al., 2002; de la Torre et al., 2008; Schouten et al., 2008; Pitcher et al., 2011b; Sinninghe Damsté et al., 2012). Stable isotope probing (SIP) experiments targeting the biphytanyl chains contained in crenarchaeol (Fig. 1c), have been undertaken in order to determine the uptake of different substrates. This revealed the incorporation of ^{13}C from bicarbonate into crenarchaeol (Wuchter et al., 2003). Pitcher et al. (2011c) showed that this uptake was dependent on ammonia oxidation in North Sea water, as addition of inhibitors for ammonia oxidation, nitrapyrine (N-serve) or chlorate, resulted in a decreased incorporation of ^{13}C-labelled DIC. Other SIP experiments carried out with organic substrates were less successful: Lin et al. (2012) achieved only a 2‰ enrichment in one of the biphytanyl chains of crenarchaeol, but 2‰ depletion in the other biphytanyl chain when adding ^{13}C -labeled phytodetrital organic carbon to sediment slurries. Takano et al. (2010) and Nomaki et al. (2011) also carried out benthic *in situ*-labeling experiments and did not find incorporation of ^{13}C in the biphytanyl chains in an incubation experiment with ^{13}C-labeled glucose in sediment from Sagami Bay, Japan, after 405 days, although they noted an initial apparent increase in $\delta^{13}C$ of one biphytane by ~10‰. This was in spite of an increase in molecular thaumarchaeal biomarkers within 9 days. Remarkably, however, they did report labeling of the glycerol moieties of the GDGT molecules after 405 days. Thus, not much evidence for substantial incorporation of carbon from organic substrates into thaumarchaeal lipids is observed in SIP experiments performed with sediments. It is possible that the types of substrate used in these studies were not suitable, i.e. not readily taken up by the Thaumarchaeota, and that other organic compounds would result in higher incorporation. Interestingly, none of the previously conducted labeling studies in sediments used ^{13}C-labelled DIC. Bicarbonate had previously unambiguously been shown to be incorporated by pelagic Thaumarchaeota (Wuchter et al., 2003; Pitcher et al. 2011c) and by Thaumarchaeota enriched from sediments (Park et al., 2010) and soils (Jung et al., 2011; Kim et al., 2012).

To shed further light on the metabolism of sedimentary Thaumarchaeota, we performed labeling experiments using ^{13}C-labeled substrates, i.e. bicarbonate, pyruvate, amino acids and glucose in sediment cores from the Iceland shelf and determined the degree of ^{13}C incorporation into the most abundant GDGT-lipids, i.e. crenarchaeol which is specific for Thaumarchaeota and GDGT-0, which can also be produced by other Archaea. All these compounds have previously been shown to be taken up by Thaumarchaeota either in the environment (Ouverney and Fuhrman, 2000; Wuchter et al. 2003; Herndl et al., 2005; Takano et al., 2010; Pitcher et al. 2011c) or enrichment cultures (Park et al., 2010; Jung et al., 2011; Tourna et al., 2011; Kim et al., 2012) and were thus deemed the most suitable substrates and most likely to result in uptake by sedimentary Thaumarchaeota. The results are discussed with respect to the uptake of substrates as well as the turnover rates of thaumarchaeal GDGTs.

3.2. Material and Methods

3.2.1. Sampling stations

Sediment cores were sampled at three stations, located on the Iceland Shelf, during the 'Long Chain Diols' cruise on the R/V Pelagia in July 2011 (Fig. 2). Station 1 (63°21'N 16°38'W), south of Iceland, was at 240 m water depth and consisted of dark, sandy sediment.

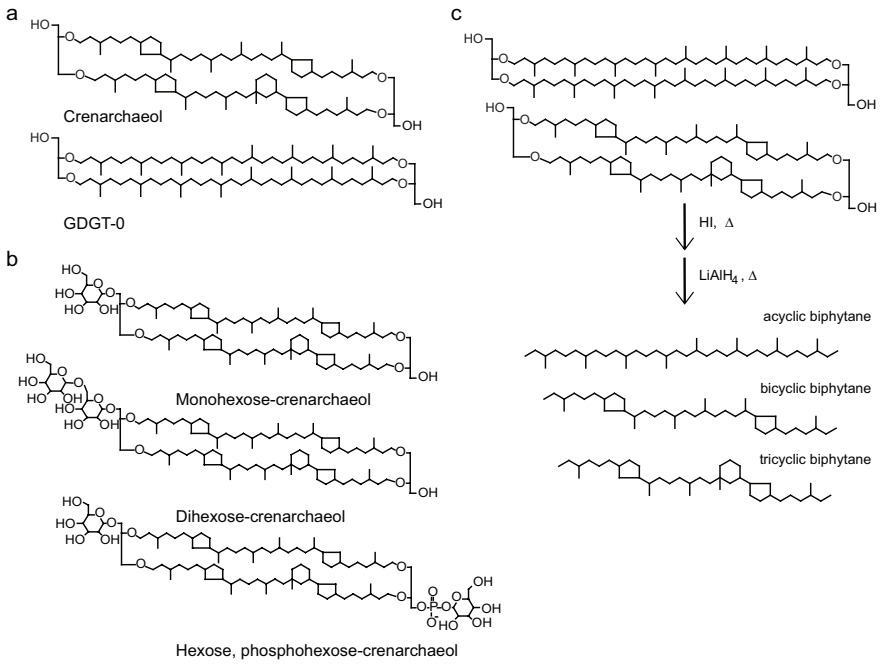

Figure 1: Structures of crenarchaeol and GDGT-0 (a), IPL-crenarchaeol species (b) and biphytanyl chains released from crenarchaeol and GDGT-0 by chemical degradation (c).

Station 2 (66°18'N 13°58'W) is located north-east of Iceland, at 261 m water depth, and the sediment was sandy-clayish. Sediment at Station 3 (67°13'N 19°07'W), north of Iceland, at 503 m water depth, and further offshore than the other two stations, consisted of dark, soft, black mud. From each station, multicores were retrieved in polycarbonate core tubes. Ten cores from each station were retrieved in tubes of 10 cm diameter with drilled holes downwards, spaced 1 cm apart, and used for incubation. Cores sampled with tubes of 5 cm diameter were used for oxygen microprofiling, pore-water extraction, and four cores from each station were sliced and stored for density and total organic carbon (TOC) analysis.

3.2.2. Core characterization

For each station, control cores were sliced into 1 cm thick layers from 0–10 cm core depth. The water content of each sediment was determined by weighing prior to and after freeze-drying. Total organic carbon was determined from freeze-dried sediments following overnight acidification with 2N HCl, subsequent washes with bidistilled H_2O and water removal by freeze-drying. The decalcified sediments were measured on a Flash EA 1112 Series (Thermo Scientific, Waltham, MA) analyzer coupled via a Conflo II interface to a Finnigan Delta^{plus} mass spectrometer. Standard deviations from three measurements ranged from 0.0 to 0.6% TOC.

Two cores (5 cm diameter) from each cast were used for oxygen microprofiling with an OX-100 Unisense oxygen microelectrode (Unisense, Aarhus, DK; Revsbech, 1989). A two-point

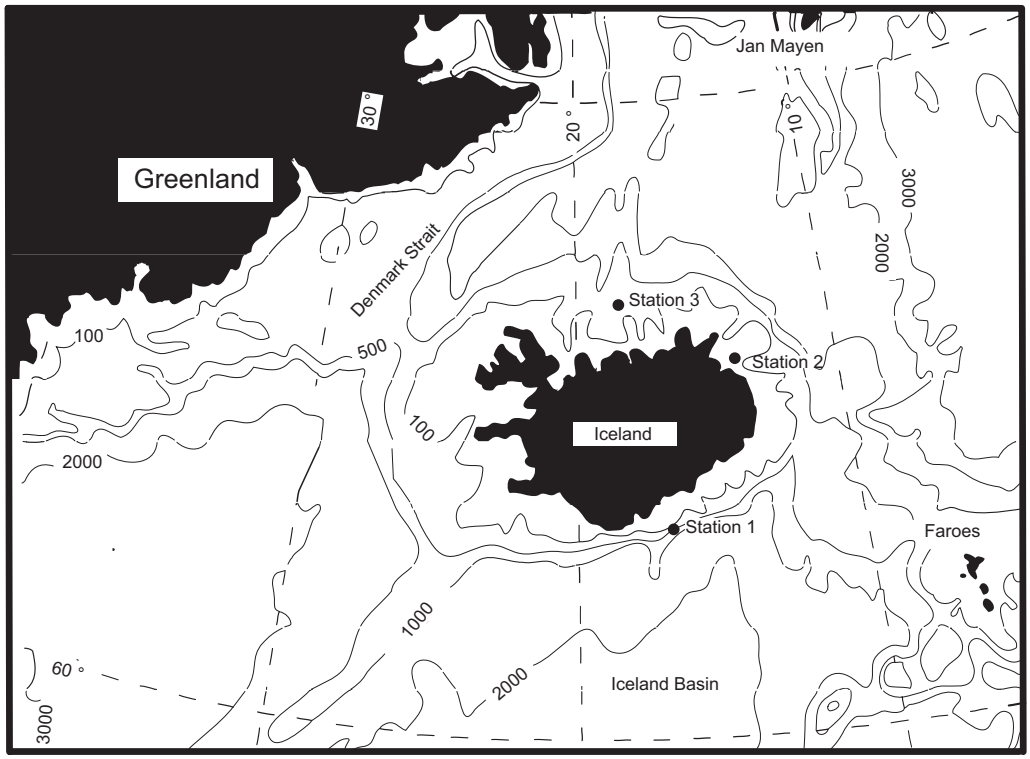

Figure 2: Map of the sampling stations (1, 2 and 3) on the Iceland shelf.

calibration was carried out with bottom water from the same station bubbled with air at 4°C for at least 5 min (100% saturation) and a solution of 0.1 M sodium ascorbate in 0.1 N NaOH in Milli-Q Water (0% saturation) and the electric current measured in pA was converted to mg L^{-1} O_2. The sensor was moved with a micromanipulator one mm at a time.

Pore water was extracted on board from slices of several cores by centrifugation. Five mL from each depth was preserved air-free with added $HgCl_2$ for analysis of the $\delta^{13}C$ of the dissolved inorganic carbon (DIC) at 4°C, and 3 mL were preserved at –20°C for analysis of phosphate, ammonia, nitrite and nitrate.

Dissolved inorganic phosphate, ammonia, nitrite and nitrate were measured on a Traacs 800 Autoanalyzer (Seal Analytical, Fareham, UK). Ortho-phosphate was measured using potassium antimonyltartrate as a catalyst and ascorbic acid as a reducing reagent which resulted in formation of a blue (880 nm) molybdenum phosphate-complex at pH 0.9–1.1 (Murphy and Riley, 1962). Ammonia was measured via formation of the indo-phenol blue-complex (630 nm) with phenol and sodium hypochlorite at pH 10.5 in citrate (Helder and de Vries, 1979). Dissolved inorganic nitrite concentrations were measured after diazotation of nitrite with sulphanilamide and N-(1-naphtyl)-ethylene diammonium dichloride to form a reddish-purple dye measured at 540 nm. Nitrate was first reduced in a copperized Cd-coil using imidazole as a buffer, measured as nitrite and concentrations were calculated by subtraction of nitrite (Grasshoff et al., 1983).

$\delta^{13}C$ of DIC was measured in pore water from all stations, and bottom water samples from the experiments, on a Thermo GasBench II coupled to a Thermo Delta Plus and is reported in

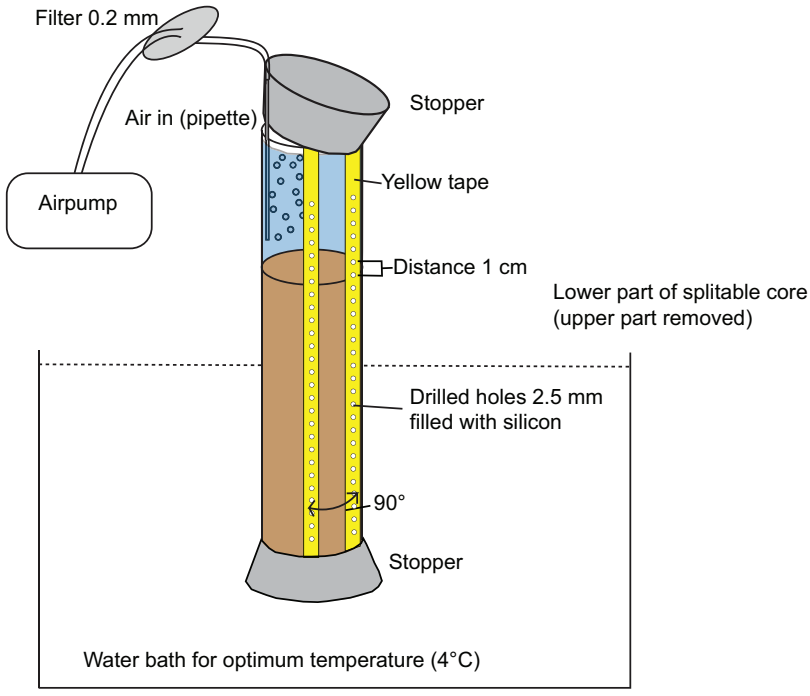

Figure 3: Scheme of the incubation set-up of the cores.

the delta notation against the Vienna Pee-Dee Belemnite (V-PDB) standard. To this end, 0.5 mL of each sample was injected into gastight exetainers, containing 0.1 mL of 80% H_3PO_4, that had been flushed for 15 min each with He. Performance was monitored by analysis of an in-house laboratory standard of Na_2CO_3 with a $\delta^{13}C$ of –0.57‰ after every 12 measurements and an in-house laboratory standard of $CaCO_3$, with a $\delta^{13}C$ value of –24.2‰ at the start and the end of every series. Measured values of these standards were usually not deviating by more than 0.5‰ from the calibrated (on NBS-19) values.

3.2.3. Incubation conditions

Ten cores from each station were used for incubations (Table 2). For this, splitable polycarbonate core tubes with 10 cm diameter were prepared by drilling two rows of 1 mm holes spaced at distances of 1 cm. The two rows were 90° contorted; holes were filled with silicon and covered with plastic non-permeable tape (see setup in Fig. 3). The cores were, after recovery from the multicorer, transferred as quickly as possible to a temperature-controlled room kept at 4°C, and the bottom water was sampled with a sterile pipette for determination of pH, $\delta^{13}C$ of DIC and nutrients as described above. Four whole cores at each station were incubated with ^{13}C-labelled substrates, i.e. bicarbonate, pyruvate, amino acids and glucose, and one whole core ('background') was injected with MilliQ water only. Four more cores were incubated with solutions containing one of the substrates and a nitrification inhibitor, and one ('background + inhibitor') with MilliQ water and the nitrification inhibitor. The incubation solutions were injected into

the seven injection ports from the surface downwards, i.e. the first 8 cm were supplied with the labeled substrate. Injection solutions contained 99% ^{13}C-labelled substrates as supplied by Cambridge Isotope Laboratories (Andover, MA, USA). The substrates were dissolved in MilliQ-H_2O in concentrations of 3.7 mg mL^{-1} for $NaHCO_3^-$ (Bic), 1.01 mg mL^{-1} for Glucose (Glu), 0.89 mg mL^{-1} for a mix of 16 algal amino acids (AA; 97–99% labeled; CLM 1548) and 1.24 mg mL^{-1} of sodium pyruvate (Pyr). 200 µL were delivered to each slice by injection through the two 90° contorted injection ports, which corresponded to a total addition of 1.6 g ^{13}C m^{-3} sediment for the bicarbonate, and 1.2 g ^{13}C m^{-3} for the organic compounds. The nitrification inhibitor was Nitrapyrine (N-Serve, Pestanal) at 11.5 µg mL^{-1}, which is known to inhibit archaeal ammonia oxidation (Park et al., 2010; Pitcher et al., 2011c). As Nitrapyrine cannot be dissolved in pure water, 5% of EtOH was added and the solutions were kept at 30°C to prevent precipitation of the Nitrapyrine. Nitrapyrine-free incubation solutions were kept at the same conditions.

After injection, the cores were stored in the dark at 4°C in a water bath - boxes with core holders filled with water for 62 h (38 h for Station 3), after which the bottom water was sampled for ^{13}C-DIC and N- and P-nutrients as described above and re-injected with a fresh solution of the substrates in water, with or without nitrapyrine added. During the incubation time, the cores were covered lightly with a rubber stopper, so that the escape of air was possible, and aerated through a sterile plastic pipette, with air pumped by an aquarium pump and filtered through a 0.2 mm filter for sterilization purposes. After 144 h (96 h for Station 3), bottom water samples for ^{13}C-DIC (dissolved inorganic carbon) and N- and P-nutrients were taken again, and oxygen microprofiles were measured as described above on all cores (except for Station 2, where cores were too short and thus surfaces too low in the core tubes, to allow measurements in more than three cores). Subsequently, the upper 10 cm of the sediment were sliced in 1 cm resolution and each slice mixed thoroughly, split in half and both parts were frozen at –80°C immediately. The incubations at station 3 were shorter due to time constraints.

3.2.4. Lipid extraction, separation and measurements

The freeze-dried sediments of 0–1 cm, 1–2 cm and 7–8 cm depth intervals of the incubation and background cores were ground and extracted by a modified Bligh-Dyer extraction method (Pitcher et al., 2009). Briefly, they were extracted ultrasonically three times in a mixture of methanol/dichloromethane (DCM)/phosphate buffer (2:1:0.8, v:v:v), centrifuged and the solvent phases were combined. The solvent ratio was then adjusted to 1:1:0.9, v:v:v, which caused the DCM to separate. Liquid extraction was repeated two more times, the DCM fractions were combined, the solvent was evaporated and bigger particles were removed over cotton wool. An aliquot of the extracts was stored for high performance liquid chromatography /electron spray ionization tandem mass spectrometry (HPLC/ESI-MS^2) analysis, and another aliquot was separated into core lipid (CL)- and intact polar lipid (IPL)-GDGTs by silica column separation with hexane/ethyl acetate (1:2, v:v) for the CL-fraction and MeOH to elute the IPL-fraction. 0.1 mg C_{46} internal standard (Huguet et al., 2006) were added to the CL-fractions. The IPL-fraction was split in three aliquots: 5% were used to determine the carry-over of CL-GDGTs into the IPL-fraction and measured directly via HPLC/APCI-MS, 60% were kept for ether cleavage in order to determine the ^{13}C incorporation into the biphytanes (see below), and 35% were subjected to acid hydrolysis under reflux for 2 h with 5% HCl in MeOH, followed by addition of water and extraction (3 ×) of the aqueous phase at an adjusted pH of 4–5 with DCM. This yielded the IPL-derived GDGTs which were measured via HPLC/APCI-MS. The C_{46} internal standard was

used to quantify the IPL-derived GDGTs and the carry over, but was added after the aliquot for ^{13}C analysis was taken in order to prevent any possible interference of alkyl chains released from the internal standard molecule.

3.2.5. $\delta^{13}C$ analysis of biphytanes

Aliquots (60%) of the IPL-fractions were subjected to chemical treatment in order to cleave GDGTs, releasing the biphytanyl chains (Fig. 1c) according to Schouten et al. (1998). For this, the IPL fraction was refluxed in 57% HI for 1 h to break the ether bonds and produce alkyliodides and subsequently extracted three times with hexane. The hexane phase was washed with 5% NaS_2O_7 and twice with water. The alkyliodides were purified over Al_2O_3 with hexane/DCM (9:1, v:v), hydrogenated with H_2/PtO_2 in hexane for 1 h (Kaneko et al., 2011) and eluted over Na_2O_3 with DCM. As the H_2/PtO_2 hydrogenation-procedure has never been tested for stable carbon isotope analysis, we used four CL-fractions of extracts from Station 1 and Station 2, split them in half and hydrogenated one half with the conventional $LiAlH_4$ reduction method described by Schouten et al. (1998), the other half with the method described here and by Kaneko et al. (2011). The conventional method consisted of $LiAlH_4$ in 1,4-dioxane for 1 h under reflux, then the remaining $LiAlH_4$ was reacted with ethyl acetate, bidistilled H_2O was added and the biphytanes were extracted with DCM from the dioxane/H_2O mixture. Additional purification was achieved by elution over an Al_2O_3 column using hexane. The results showed that the $\delta^{13}C$ values of the biphytanes were not significantly different when H_2/PtO_2 treatment and $LiAlH_4$ hydrogenation were compared (Table 1). However, when yields were compared, it was obvious that hydrogenation with H_2/PtO_2 gave better results. This is probably due to the fact that the H_2/PtO_2 treatment requires fewer workup procedures after hydrogenation (only one drying step) than the $LiAlH_4$ treatment, which requires two column separations in addition to a drying step.

3.2.6. $\delta^{13}C$ of phospholipid-derived fatty acids

In order to demonstrate that the incubation experiments did result in label uptake by the sedimentary microbial community, we determined the uptake of ^{13}C-label by bacteria and eukaryotes by isotopic analysis of polar lipid-derived fatty acids (PLFA). For this, one third of the extract of the 0–1 cm sediment slice from Station 3, from the cores incubated with bicarbonate, glucose, amino acids and the background core (all without nitrapyrine), were used and analyzed for PLFA according to Guckert et al. (1985). Briefly, the aliquots were separated over a silica column with DCM, acetone and methanol, respectively, with the methanol-fraction containing the PLFA. PLFA fractions were saponified in methanolic KOH (2N) and, after adjusting to pH 5, the resulting fatty acids were extracted with DCM and methylated with BF_3-MeOH (with a $\delta^{13}C$ of -25.4 ± 0.2 ‰V-PDB) before measurements by gas chromatography – isotope ratio mass spectrometry (GC-irMS). The $\delta^{13}C$ values for the fatty acids were corrected for the added carbon.

3.2.7. HPLC-APCI-MS and HPLC-ESI-MS2

The CL-fractions, hydrolyzed IPL- fractions and the IPL-fractions themselves (to determine the carry-over of CL into the IPL fraction) were analyzed by HPLC-APCI-MS as described previously (Schouten et al., 2007). HPLC-ESI-MS^2 in selected reaction monitoring (SRM) mode, as described by Pitcher et al. (2011b), was used to analyze selected IPL-GDGTs. IPL-GDGTs monitored were monohexose (MH)-crenarchaeol, dihexose (DH)-crenarchaeol and hexose, phosphohexose (HPH)-crenarchaeol. Performance was monitored using a lipid extract of sed-

Table 1: $\delta^{13}C$ values of the acyclic, bicyclic, and tricyclic biphytanes obtained via H_2/PtO_2 and $LiAlH_4$ reduction of alkyliodides formed via HI ether cleavage.

	$\delta^{13}C$ (‰)					
	acyclic		bicyclic		tricyclic	
Sample	H_2/PtO_2	$LiAlH_4$	H_2/PtO_2	$LiAlH_4$	H_2/PtO_2	$LiAlH_4$
Station 1 0–1 cm	–20.3 ± 0.4	–20.2 ± 0.0	–19.7 ± 0.7	–20.0 ± 0.6	–19.5 ± 1.1	–21.2 ± 0.8
Station 1 1-–2 cm	–19.9 ± 0.6	–22.0 ± 0.2	–18.4 ± 0.2	–20.2 ± 1.6	–19.0 ± 0.2	–19.8 ± 0.6
Station 2 0–1 cm	–22.7 ± 0.7	–22.0 ± 0.4	–24.2 ± 1.0	–22.4 ± 0.3	–22.6 ± 1.6	–22.0 ± 0.4
Station 2 1–2 cm	–24.0 ± 1.0	–25.6 ± 0.2	–22.3 ± 1.4	–25.6 ± 0.8	–22.6 ± 0.2	–25.0 ± 0.6

	Total amount / µg					
	acyclic		bicyclic		tricyclic	
Sample	H_2/PtO_2	$LiAlH_4$	H_2/PtO_2	$LiAlH_4$	$H_2/PtO2$	$LiAlH_4$
Station 1 0–1 cm	0.79	0.28	0.27	0.16	0.31	0.18
Station 1 1–2 cm	0.42	0.16	0.14	0.07	0.15	0.08
Station 2 0–1 cm	0.45	0.34	0.17	0.16	0.23	0.23
Station 2 1–2 cm	0.13	0.05	0.04	0.0	0.06	0.0

iment known to contain the monitored GDGTs that was injected every 8 runs. Response areas per mL sediment (mL sed) are reported, as absolute quantification was not possible due to a lack of standards. The values reported are the averages of duplicate injections and their standard deviations.

3.2.8. GC-MS and GC-irMS

GC-MS was used to identify the biphytanes and phospholipid-derived fatty acid methyl esters (FAME) using a TRACE GC with a DSQ-MS. The gas chromatograph was equipped with a fused silica capillary column (25 m, 0.32 mm internal diameter) coated with CP Sil-5 (film thickness 0.12 mm). The carrier gas was helium. The compound specific isotope composition of the biphytanes and fatty acids was measured with an Agilent 6800 GC, using the same GC column conditions, coupled to a ThermoFisher Delta V isotope ratio monitoring mass spectrometer. The isotopic values were calculated by integrating the mass 44, 45 and 46 ion currents of the peaks and that of CO_2-spikes produced by admitting CO_2 with a known ^{13}C-content into the mass spectrometer at regular intervals. The performance of the instrument was monitored by

Table 2: The bottom water NO_3^- concentrations and $\delta^{13}DIC$ changes over the incubation time (Δt–t0), as well as oxygen penetration depths measured after the incubations. In brackets, the average values of oxygen penetration depth for the cores before incubation. BG: background, Bic: bicarbonate, Pyr: pyruvate, +Inh: nitrification inhibitor nitrapyrine added.

Station	Incubation	NO_3^- [mM]			$\Delta\delta^{13}DIC_{t-t0}$ [‰]		O_2 Pen depth [mm]
		t=0h	t=62 h	t=144h	t=62 h	t=144h	t=144h
							(before: 17)
1	BG	17.9	19.1	4.1	0.6	2.8	11
	Bic	15.9	16.2	8.2	66.5	156.2	16
	Pyr	18.1	19.2	5.9	109.1	457.0	16
	BG+Inh	15.5	16.3	13.9	–0.5	2.6	20
	Bic+Inh	11.5	12.0	3.8	135.3	275.5	20
	Pyr+Inh	25.4	25.6	14.3	72.7	671.1	16
							(before: 13)
2	BG	12.14	11.37	0.09	–0.5	2.9	9
	Bic	5.55	4.05	0.07	336.5	606.0	6
	Pyr	13.77	12.48	0.12	366.6	1570.7	6
	BG+Inh	10.72	10.45	0.19	0.1	3.5	5
	Bic+Inh	7.22	6.79	0.09	255.4	570.1	6
	Pyr+Inh	5.01	3.17	0.06	511.3	1136.4	16
							(before: 16)
3	BG	13.84	0.00	0.13	1.5	–2.3	n.d.
	Bic	13.83	12.60	0.08	558.2	814.3	n.d.
	Pyr	6.45	4.23	0.12	1108.9	1496.3	n.d.
	Glu	14.14	12.03	0.11	90.4	194.2	n.d.
	AA	15.34	13.55	0.15	82.7	201.9	n.d.
	BG+Inh	16.06	13.39	0.11	0.3	4.9	8
	Bic+Inh	15.23	14.05	0.18	244.8	520.3	n.d.
	Pyr+Inh	8.92	6.68	0.10	682.7	1644.5	8
	Glu+Inh	5.86	4.77	0.03	110.8	301.2	10
	AA+Inh	14.36	13.41	0.09	62.2	368.2	n.d.

n.d. - could not be determined.

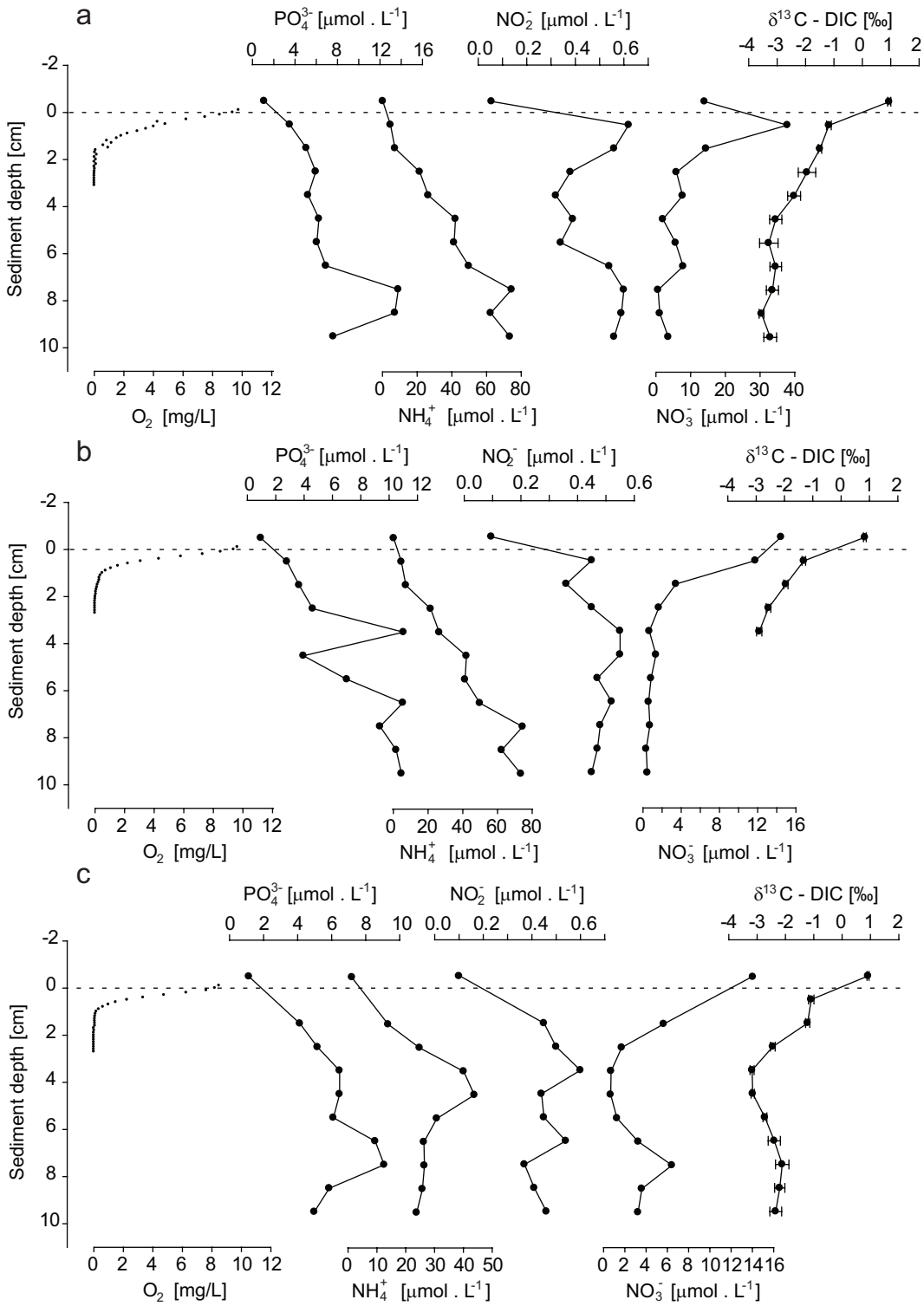

Figure 4: Oxygen microprofiles and pore water results vs. depth at station 1 (a), 2 (b) and 3 (c).

daily injections of a standard mixture of a C_{20} and a C_{24} perdeuterated n-alkane. Two replicate analyses (one in the case of the fatty acids) were carried out and the results were averaged in order to obtain the average and standard deviations. The stable carbon isotope compositions are reported in the delta notation against the V-PDB standard. Some values of the biphytanes could not be determined due to low concentrations, mainly in the background cores at station 2. For calculations of incorporation, the background values from station 1 were used.

3.3. Results

3.3.1. Sediment characteristics

The sediments at stations 1–3 consisted of sand, sandy clay, and dark and soft mud, respectively. Oxygen penetration depth was on average 17 mm at station 1, while oxygen penetrated slightly less deep at station 2 (13 mm on average) and station 3 (15.5 mm on average; Fig. 4). At station 1, organic carbon contents were low with 0.6 % at 0–1 cm depth and 0.3% at 1–2 cm and 6–7 cm depth. Organic carbon contents were higher at station 2 and amounted to 1.4–1.7% and were highest at station 3 (2.0–2.5%). The pH of the bottom water hardly differed between stations and was typically marine, i.e. 8.2 at station 1, 8.0 at station 2, and 7.9 at station 3.

Pore water concentrations of nutrients, as shown in Fig. 4, showed similar trends with depth at the three stations. Inorganic phosphate concentrations were 1 μM in the bottom water and generally increased with depth up to 7–14 μM. Ammonia concentrations increased as well with sediment depth, from 0.19 to 1.3 μM in bottom water to 44–70 μM at depth. Nitrite concentrations were an order of magnitude higher in the pore water (0.32–0.62 μM) than in the bottom water (0.055 μM), and increased with sediment depth. Nitrate concentrations were high in the bottom water, 14 μM, and generally decreased quickly with depth in the sediment to values between 0.6 and 3 μM. The $\delta^{13}C$ of the DIC showed also similar profiles at the three stations. It decreased from 1‰ in the bottom water to –3‰ in the pore water. At station 2, $\delta^{13}C$ of the DIC could only be measured in the bottom water and the four uppermost pore water samples, but, in these samples, showed the same decrease with depth as observed at Station 1, from –1.3 ‰ to –2.9 ‰.

3.3.2. GDGT-abundance

GDGT-concentrations averaged over all cores from the incubations (10 per station) are shown with standard deviation in order to give an impression of the range of concentrations found (Fig. 5). GDGT-0 and crenarchaeol were present as a core lipid in concentrations of 0.4 to 0.8 μg mL sed^{-1}, and 0.08 to 0.12 μg mL sed^{-1} for the minor isoprenoid GDGTs (i-GDGTs; GDGT-1, -2 and -3). Concentrations were similar at all stations and did not show substantial changes with sediment depth. IPL-derived GDGTs were present in lower concentrations and amounted to 0.09 to 0.3 (GDGT-0), 0.07 to 0.18 (crenarchaeol) μg mL sed^{-1}. Concentrations were generally lower at station 1 compared to stations 2 and 3. Direct analysis of individual IPL-crenarchaeol showed that the head groups mainly consisted of monohexose (MH)- and hexose, phosphohexose (HPH)- headgroups, with only a small contribution of dihexose (DH)- head groups (Fig. 5c). The proportion of HPH- to the total peak area of IPL-crenarchaeol was ca. 80–90% in the surface sediments (0–1 and 1–2 cm) of all stations. At station 1 and 2, this decreased to 10–25% in the deepest sediment slice analyzed, 6–7 cm. At station 3, HPH-crenarchaeol dominated at all sediment depths (Fig. 4).

Table 3: Background (BG) values of$\delta^{13}C$ of the biphytanes (BP) measured in the sediment (i.e. $\delta^{13}C$ of the biphytanes that were incubated just with water, but under the same conditions). These values were used for determination of the $\Delta^{13}C$: BG values were subtracted from the ^{13}C of the corresponding value measured from the cores incubated with label (Fig. 6). Numbers indicate the depth interval the sample was coming from, + : nitrification inhibitor nitrapyrine added for incubation, - : no inhibitor added. Values at station 2 could not be determined due to low concentrations.

		Station 1 $\delta^{13}C$ [‰]			Station 2 $\delta^{13}C$ [‰]			Station 3 $\delta^{13}C$ [‰]		
	Sediment (cm)	acyclic BP	bicyclic BP	tricyclic BP	acyclic BP	bicyclic BP	tricyclic BP	acyclic BP	bicyclic BP	tricyclic BP
–	0–1	–22.0 ± 0.1	–20.5 ± 1.1	–19.9 ± 0.3	n.d.*	n.d.*	n.d.*	–20.4 ± 0.2	–20.2 ± 1.2	–19.8 ± 0.8
	1–2	–21.4 ± 0.5	–20.7 ± 0.1	–20.2 ± 0.0	n.d.*	n.d.*	n.d.*	–19.6 ± 1.2	–18.3 ± 1.4	–17.7 ± 1.9
	6–7	–21.4 ± 0.2	–22.4 ± 1.9	–21.5 ± 2.3	n.d.*	n.d.*	n.d.*	–20.6 ± 0.4	–20.1 ± 0.4	–19.2 ± 0.6
+	0–1	n.d.*	n.d.*	n.d.*	n.d.*	n.d.*	n.d.*	–19.3 ± 0.6	–19.7 ± 0.6	–19.4 ± 0.3
	1–2	–22.0 ± 0	–21.2 ± 0.4	–21.3 ± 0	–22.9 ± 0.9	–21.4 ± 1.4	–20.3 ± 0.9	–19.4 ± 0.1	–19.9 ± 0.4	–19.2 ± 0.2
	6–7	–22.4 ± 0	–20.0 ± 0.6	–22.2 ± 0	–22.0 ± 0.0	–21.0 ± 0.4	–20.0 ± 0.4	–22.4 ± 0.0	–21.4 ± 0.4	–20.7 ± 0.1

*n.d. – could not be determined.

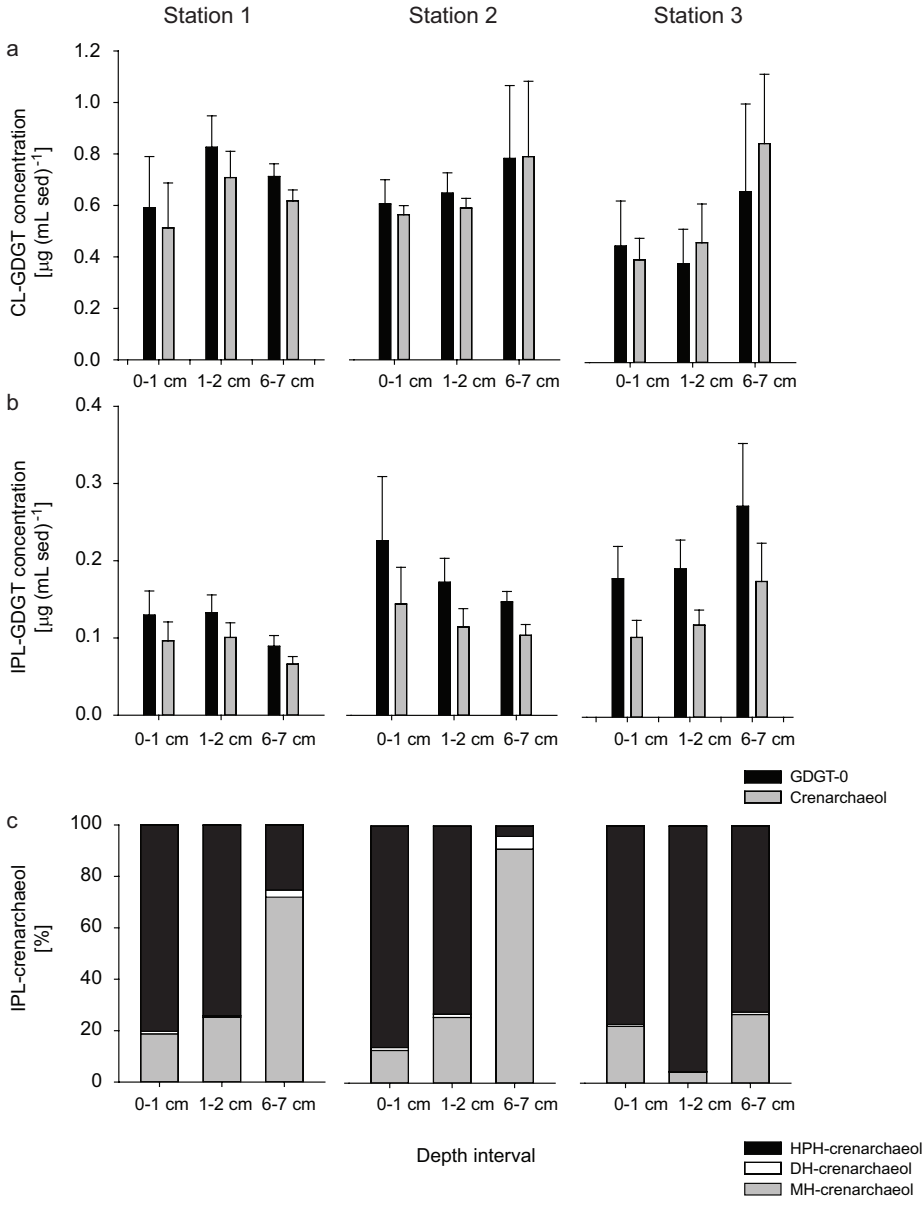

Figure 5: GDGT-0 and crenarchaeol concentrations (averages), for CL-GDGTs (a) and IPL-derived GDGTs (b) at all three stations and core depths. Proportions of head groups of IPL-crenarchaeol at all three stations (c).

3.3.3. Incubation experiments

Incubations experiments were started directly after core recovery and initial sampling by injection of solutions, which contained ^{13}C-bicarbonate, pyruvate, glucose , amino acids or just water (incubated for comparison and further called the "background core") over the first 7 cm of the cores. The cores were incubated at *in situ* temperatures (4°C) for 6 days (4 days for station 3) and subsequently sliced and stored for analysis of ^{13}C-enrichment in lipids. Oxygen penetration depths were measured and bottom water was sampled at the start of the experiment, after 48 hours (36 for Station 3) and at the end of the incubations. At all stations, nutrient concentrations of the bottom water did not change over the 6 days (5 days for station 3) of incubation, except for nitrate, which generally showed a decrease (Table 2). The bottom water pH remained mostly constant, except at station 1, where it changed during the incubation from 8.2 to 8.0 in all incubations, similar to the other sediment cores and perhaps indicating that the initial value of 8.2 might have been due to an error in the measurement. The δ^{13}C of the DIC in the bottom water substantially increased in the cores where ^{13}C-labeled compounds and bicarbonate was added, but not in the background cores (Table 2). In cores from station 2, oxygen penetration depths decreased to 6–9 mm in all incubation experiments except for the core incubated with pyruvate and inhibitor, as well as in cores from station 3, where a decrease in oxygen penetration depth to 8–10 mm was observed (Table 2). In all cores from the three stations, no major differences in IPL-crenarchaeol concentrations upon incubation could be detected (Figs. S1–S3).

The δ^{13}C-values of the biphytanes released from the IPL-GDGTs of the background cores were ranging from –17 to –24‰ at all stations (Table 3), which is within the natural variation observed for biphytanes sourced by Thaumarchaeota in marine sediments (Hoefs et al., 1997; Könneke et al., 2012; Schouten et al., 2013). No substantial ^{13}C enrichment of biphytanes was observed in nearly all of the incubation experiments (Fig. 6). The only exception was the tricyclic biphytane in some of the incubations at station 1 (with ^{13}C-labelled substrates bicarbonate, bicarbonate and inhibitor, and pyruvate and inhibitor at nearly all depths) and one incubation (with pyruvate and inhibitor at 6–7 cm depth) at station 2 with enrichments ranging from 2 to 4‰. However, due to the large standard deviations in the δ^{13}C values of the background cores, only the enrichment of 2–5 ‰ in the 1–2 cm slices of station 1 is statistically significant at a 95% confidence level (t(2)= 6.6–9.0, P=0.05). To determine whether the added ^{13}C-labelled substrate was incorporated into sedimentary microbial biomass at all, phospholipid-derived fatty

Table 4: δ^{13}C values of fatty acids measured in the 0-1 cm slice in cores from station 3, incubated without inhibitor, for 5 days.

	δ^{13}C [‰]			
Fatty acid	**BG**	**Bic**	**Glu**	**AA**
$C_{16:0}$	–25.5 ± 0.2	–25.3 ± 0.6	20.6 ± 0.5	30.0 ± 3.9
$C_{18:0}$	–24.6*	–24.5 ± 0.2	66.2 ± 0.4	63.1 ± 2.1

* Single measurement

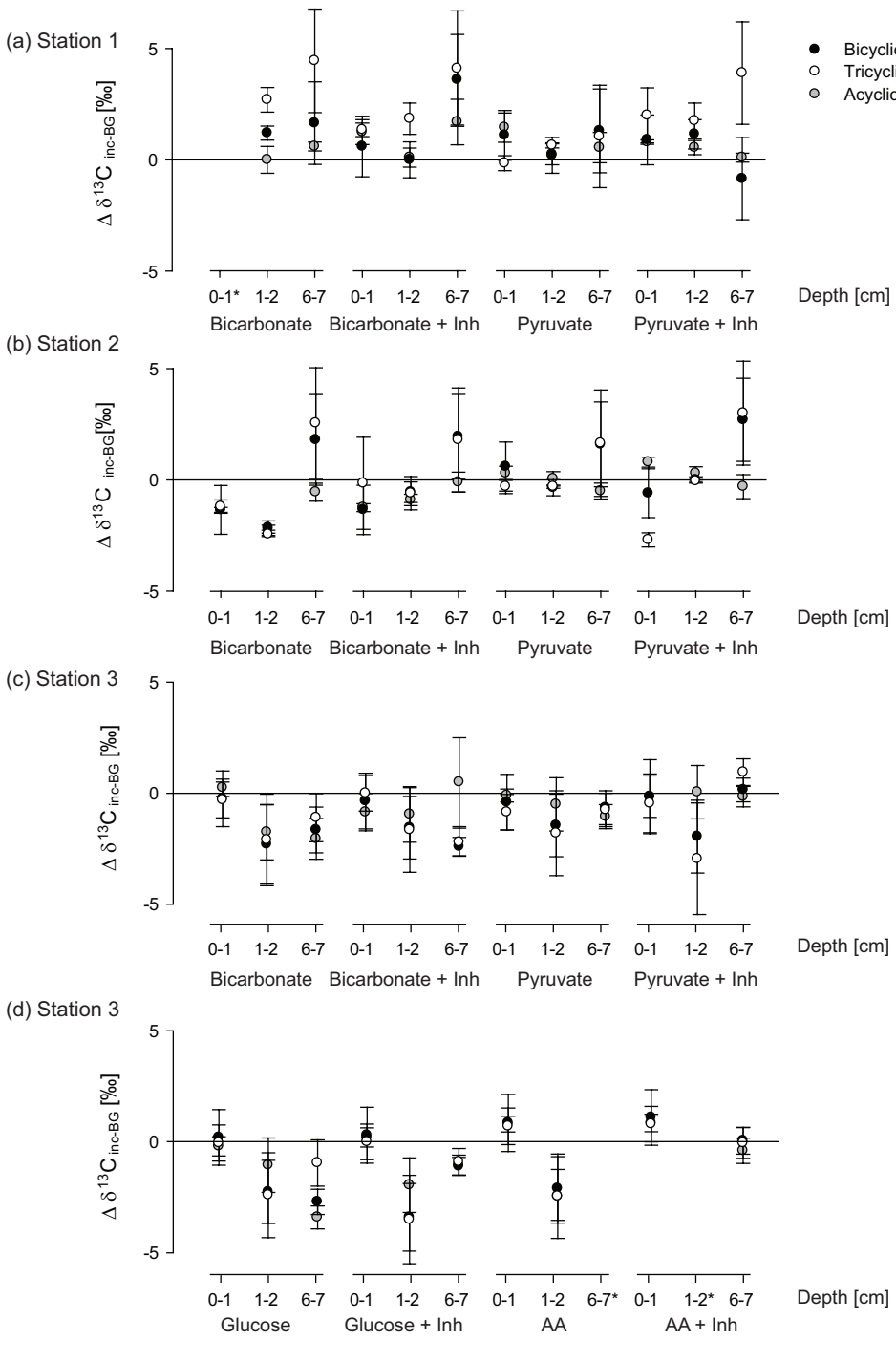

Figure 6: Difference in $\delta^{13}C$-values ($\Delta\delta^{13}C_{inc\text{-}bg}$) of the acyclic, bicyclic and tricyclic biphytanes in ‰ in the cores incubated with labeled substrate versus the ^{13}C-values in the background cores (incubated with and without inhibitor) at station 1 (a), station 2 (b) and station 3 (c) from the cores incubated with bicarbonate and pyruvate, with and without inhibitor, and the cores incubated with glucose and amino acids with and without inhibitor, at station 3 (d).

acids, stemming from bacteria or eukaryotes, were analyzed from the surface sediments of the bicarbonate, amino acids and glucose incubation experiments. $\delta^{13}C$ values of the most common fatty acids, $C_{16:0}$ and $C_{18:0}$ were enriched in ^{13}C by up to 80‰ in the amino acid and glucose incubation experiments compared to the background (Table 4). The cores incubated with ^{13}C-labeled bicarbonate that were analyzed showed no ^{13}C-enriched fatty acids.

3.4. Discussion

3.4.1. Biomarkers indicative for live sedimentary Thaumarchaeota

The only biomarker lipids up to now which are probably indicative for live Thaumarchaeota are crenarchaeol IPLs (Pitcher et al., 2011b). Of these, the ones with a glycosidic head group likely contain a substantial fossil component, while the hexose, phosphohexose (HPH)-crenarchaeol is more labile and its concentration corresponds well to DNA-based thaumarchaeal abundance (Pitcher 2011a and 2011b; Schouten et al., 2012; Lengger et al., 2012a). The presence and dominance of HPH-crenarchaeol in the sediments strongly suggests that live Thaumarchaeota were present in sediments at all the stations (Fig. 5). This was expected for the surface sediment, as oxygen and ammonium were present (Martens-Habbena et al., 2009; Erguder et al., 2009; Hatzenpichler, 2012). The ammonium concentration profiles, with concentrations decreasing upwards, agree with aerobic ammonia oxidation proceeding in the oxygenated parts, using the ammonium diffusing from below. However, HPH-crenarchaeol was present down to 7 cm depth, below the oxygen penetration depth, which could indicate that either live Thaumarchaeota were present at depth, using a metabolic process different from aerobic ammonia oxidation, or that this HPH-crenarchaeol is not stemming from live Thaumarchaeota at depth and was partly preserved. In any case, it is not yet known what exactly the preferred environment for Thaumarchaeota is, as they have been found to be active in low and high ammonia and low and high oxygen environments (Martens-Habbena et al., 2009; Erguder et al., 2009; Hatzenpichler, 2012).

3.4.2. Activity indicators and label uptake during incubation

Several parameters can be monitored during the incubation experiments to cast a light on sedimentary microbial activities stimulated by the addition of substrates. During incubation, a decrease of oxygen penetration depths would indicate an increased aerobic metabolism at the surface, utilizing oxygen and preventing it from penetrating deeper, which was expected in the slices where organic carbon was added. However, at station 1, this was not observed and oxygen penetration depths only decreased in the background core (Table 2). This could be due to the aeration carried out by bubbling air into the bottom water of the core, and oxygen penetrating deeper into the sandy sediment. However, the cores from station 2 showed a decrease of oxygen penetration depth after incubation, except for the core incubated with pyruvate and inhibitor. Also the cores from station 3, i.e. those where oxygen penetration depth could be measured, showed a decrease, indicating that the oxygen was consumed upon incubation, independent of the type of substrate added to the cores. Ammonia and nitrite concentrations in the bottom water showed no changes during the incubations. No significant differences can be seen if the cores incubated with and without nitrification inhibitor are compared, indicating that either the communities are not affected by it, or potentially that the inhibitor was not as effective in a sedimentary environment as in water incubations (e.g. due to adherence to particles). Nitrate concentrations were, at station 2 and station 3, below detection limit after 6 and 5 days, respec-

tively, suggesting consumption of nitrate (Table 2). This may indicate a strong contribution of denitrification to organic carbon processing in these sediments in the sections below oxygen penetration depth. The increase in $\delta^{13}C$ of the DIC in the bottom water of all cores compared to the background cores (Table 2) clearly showed that the organic substrates added were respired. Previous labeling experiments with similar substrates have been successful in detecting uptake of ^{13}C-label into bacterial PLFA (e.g. Guilini et al., 2010), resulting in a highly labeled $C_{16:0}$, among other PLFAs. Indeed, the strong labeling of the bacterial PLFA $C_{16:0}$ at station 3 indicated that the organic substrates were readily taken up by bacteria, and potentially non-photosynthetic eukaryotes (PLFA $C_{16:0}$, $C_{18:0}$), and incorporated into their biomass. Intriguingly, no incorporation was observed upon labeling with bicarbonate, which is in contrast to other studies with oligotrophic sediment cores who used similar concentrations (e.g. Guilini et al., 2010). The amount of PLFA generated by chemoautotrophs present might have been too low to allow unambiguous detection of the incorporation in this case.

The most abundant archaeal lipids were crenarchaeol, a lipid specific for Thaumarchaeota (Sinninghe Damsté et al., 2002; de la Torre et al., 2008; Schouten et al., 2008; Pitcher et al., 2011b; Sinninghe Damsté et al., 2012), and GDGT-0, a lipid produced by Thaumarchaeota as well as some other Archaea (cf. Koga and Nakano, 2008). Their concentrations did not substantially change upon incubation, suggesting that Thaumarchaeota abundances did not change (Fig. 5 showing an average of all incubated cores, with standard deviations). However, this could also indicate that the majority of IPLs in these sediments were fossil (cf. Schouten et al., 2010; Logemann et al., 2011; Lengger et al., 2012a) and not part of living Thaumarchaeota, and thus not affected at all by the incubation. This could include HPH-GDGTs as well, though it is also possible that the bias during analysis, i.e. loss during silica column chromatography (cf. Lengger et al., 2012b), resulted in an underrepresentation of HPH- derived GDGTs.

The biphytanes analyzed included a-, bi- and tricyclic biphytane, which are mainly derived from the two most abundant GDGTs: GDGT-0 (acyclic; general archaea), and crenarchaeol (bi- and tricyclic; Thaumarchaeota). Importantly, in nearly all of the incubation experiments, the $\delta^{13}C$ values of all measured biphytanes, including those derived from crenarchaeol, changed by less than 2‰ and sometimes even decreased in ^{13}C-content compared to the background values (Fig. 6). In some of the experiments, mainly at station 1, a slight enrichment in the tricyclic biphytane, derived from crenarchaeol, of up to 4 ± 2‰ was observed though this difference is not statistically significant for individual experiments. Furthermore, the bicyclic biphytane, also predominantly derived from crenarchaeol, shows, in general, lower ^{13}C-enrichments than the tricyclic biphytane and also no statistically significant enrichments for individual experiments. Thus, there is no evidence for substantial uptake of ^{13}C-label in archaeal GDGTs, contrary to what is observed for the PLFA.

This apparent lack of uptake could be due to three reasons. Firstly, it is possible that the Thaumarchaeota were active, but the substrates were not taken up. As the metabolism of the different Thaumarchaeota could be diverse, this is possible, but, in case of the bicarbonate incubations, unlikely, as in sea water incubations as well as enrichment cultures – including those of sedimentary Thaumarchaeota – ^{13}C from bicarbonate has been shown to be readily incorporated into biphytanes (Wuchter et al., 2003; Park et al., 2010; Pitcher et al., 2011c). Secondly, it is possible that most of the IPLs measured (i.e. MH- and DH-GDGTs, assuming the chromatographic discrimination against the HPH-GDGTs) are fossil, and thus only a small amount of these IPLs

are part of live and active sedimentary Thaumarchaeota. Finally, it is also possible that a higher proportion of the IPLs are part of sedimentary Thaumarchaeota, but that their metabolism and growth rates are so slow (as opposed to Thaumarchaeota in sea water and enrichment cultures), that no incorporation could be detected over the time scales investigated. Thus, our incubation times of 96–144 h may have been too short. However, longer incubation times inevitably result in spreading of the label over the whole sedimentary food web as e.g. discussed by Radajewski et al. (2003) for stable isotope probing of nucleic acids. This is why this study, and nearly all other incubation studies (e.g. Boschker et al. 1998, Guilini et al. 2010, cf. Middelburg 2013 and references cited therein), including those for sediments (e.g. Middelburg et al. 2000, Moodley et al. 2002), use only a short time interval (mostly just a few days) to avoid this label scrambling. Indeed, our results show that the uptake in bacteria is occurring within a few days suggesting that, if active, microbes rapidly take up label.

3.4.3. Estimating growth rates of Thaumarchaeota and turnover rates of IPL-GDGTs

To investigate whether growth rates as well as degradation rates of IPL-GDGTs were responsible for the apparent lack of substantial ^{13}C-labeling, we used data from our experiments to estimate minimal growth rates and the turnover time of IPL-GDGTs. The greatest label incorporation was found at station 1 in the sandy sediment, indicating that Thaumarchaeota may have been active there. If all IPL-GDGT biphytanes are stemming from living cells, and an enrichment of 2‰ of biphytanes is assumed, this can be converted to growth rates by using a formula used for growth of phytoplankton based on the isotope dilution theory of Laws (1984; Eq. (1)), with μ representing the growth rate, t_{inc} the incubation time, P^* the atom%-excess of the product, in our case the biphytane, and A^* the atom%-excess of the substrate added (cf. alkenone lipids for eustigmatophyte algae; Popp et al., 2006):

$$\mu = -\frac{1}{t_{inc}} \cdot \ln\left(1 - \frac{P^*}{A^*}\right) \quad (1)$$

Unfortunately, as natural concentrations of the added substrates were not determined, A* could not be calculated for the incubation experiments with pyruvate, glucose and amino acids. In the bicarbonate experiments, we calculated A* based on the amount of added 99%-labeled DIC and natural δ^{13}C and concentration of DIC (Table 2). For P* we used the highest enrichment observed at 6–7 depth with no inhibitor, i.e. 4‰. This gives an estimate of the maximum μ as 6.3×10^{-4} d^{-1}, corresponding to a generation time of 3 yr. Clearly, for the other bicarbonate experiments which resulted in even less label incorporation, generation times will be much higher (e.g. for a 2 ‰ enrichment, 9 yr). Interestingly, growth rates as low as estimated here have been predicted by Valentine (2007), who stated that the archaeal domain, other than bacteria and eukaryotes, has adapted to low energy conditions and is thus characterized by the exceptional ability to live in low energy environments. They are thus not fast in adapting to settings with higher concentrations of available carbon and redox substrates. However, this has not been observed for pelagic Thaumarchaeota as SIP experiments showed a substantial enrichment in ^{13}C in IPL-GDGTs (Wuchter et al., 2003; Pitcher et al., 2011c) in incubation experiments of 168 and 24 h duration, respectively. Indeed, the generation times calculated from enriched North Sea water labeling experiments conducted by Wuchter et al. (2003; A^*=5%; P^*=0.46% – 400 ‰ enrichment) and Pitcher et al. (2011c; A^*=9%; P^*=0.07% – 44‰ enrichment) were at least an order of magnitude lower than observed here – 50 and 88 d, respectively. Also sedimentary

Thaumarchaeota have been shown to incorporate ^{13}C from bicarbonate in enrichment cultures (Park et al., 2010). The growth rates reported here might be an underestimation if a large proportion of archaeal cells is fossil; and the use of lipid turnover times might be more suitable, as discussed below. Slow growth could potentially be due to the experiments not being carried out *in situ* on the sea floor. However, incubations were carried out in whole, intact sediment cores, in the dark, immediately after recovery, and at *in situ* temperatures in order to minimize the deviations from *in situ* conditions, although, admittedly, the pressure was at atmospheric conditions. Therefore, this is unlikely to explain the low growth rates.

The calculations above assume exponential growth of sedimentary Thaumarchaeota and an immediate translation of this signal into the lipid pool. If, on the other hand, steady state conditions are assumed, i.e. cell numbers and lipid concentrations are not increasing but remain constant, the turnover time for the sedimentary lipid pool t_{to} can be calculated according to Lin et al. (2012) :

$$t_{to} = \frac{A^* \cdot t_{inc}}{P^*} \quad (2)$$

Using Eq. (2), the turnover time of IPL-crenarchaeol in sediments would thus be at least ~4 yrs based on the 4‰ enrichment of the tricyclic biphytane in the bicarbonate incubation experiment. Since labeling was not detected in the other sediments, this turnover time estimate is likely to be a minimal estimate. This large turnover time is not an unusual postulate for thaumarchaeal lipids in sediments, as also Lin et al. (2012) found very low ^{13}C incorporation rates of labeled glucose into sugar moieties of glycosidic GDGTs over >400 d in slurry incubations of subsurface sediments and used these to estimate turnover times of diglycosyl-lipids of 1,700–20,500 years. Similarly, recently published degradation experiments by Xie et al. (2013) using ^{14}C-labeled IPL-diether lipids found very high turnover times and estimated half-lives of up to 310 kyr under energy-deprived subsurface conditions. This is in strong contrast to GDGTs in the water column: Using the SIP data of Wuchter et al. (2003) and Pitcher et al. (2011c) from incubations of North Sea water, we obtained t_{to} of 76 and 128 d, respectively, for the thaumarchaeal GDGTs.

Our results are similar to those obtained by *in situ*-incubations carried out by Takano et al. (2010) and Nomaki et al. (2011), i.e. they did not observe significant incorporation after 405 days into the biphytanyl chains either (even though one single data point after 9 days does show a 10‰ enrichment), though they did observe incorporation into the glycerol moiety. A possibility, as suggested earlier, is that biphytanyl chains are not or hardly produced by sedimentary Thaumarchaeota, but recycled, and the sugar headgroups and/or the glycerol are only newly generated and ^{13}C-labeled. In this case, isotope ratios of whole ether lipids instead of the biphytanes would enable detection of the label, but this involves laborious clean-up of the isolated ether lipids (Takano et al., 2010). However, as also the incorporation into the sugar headgroups of GDGTs, as reported by Lin et al. (2012), is similarly low, it suggests that there is indeed a lack of, or only a very slow ^{13}C-uptake into IPL-GDGTs.

It seems that stable carbon isotope probing using biphytanes is most likely not the method of choice for thaumarchaeal activity measurements in sediments, where conditions are favorable for preservation of fossil GDGTs, thus creating a large background signal. In the water column, however, where growth of Thaumarchaeota is faster and lipids are likely degraded on shorter time scales, due to the abundant presence of oxygen and lack of mineral matrix protection (Keil

et al., 1994; Hedges and Keil, 1995), stable isotope incubations are useful for determining activities of Thaumarchaeota. In sediments that contain a high proportion of fossil IPL-GDGTs which are not part of live Archaea, the incorporation of ^{13}C into the lipids of the few active Thaumarchaeota is probably not enough to change the $\delta^{13}C$ of the IPL-GDGTs to a significant degree over the commonly used time scales of SIP experiments.

3.5. Conclusion

Incubations of sediment cores from the Iceland Shelf were used in order to determine the metabolism of sedimentary Thaumarchaeota. Thaumarchaeal IPLs were present in sediment cores recovered from the Iceland Shelf and the presence of labile IPL-biomarkers for Thaumarchaeota, such as HPH-crenarchaeol, suggested the presence of living Thaumarchaeota. However, incubations with ^{13}C-labeled substrates bicarbonate, pyruvate, glucose and amino acids did not result in any substantial incorporation of the ^{13}C into the biphytanyl chains of these lipids. Turnover times and generation times of thaumarchaeal lipids were estimated to be at least several years, suggesting slow growth of sedimentary Thaumarchaeota in contrast to other sedimentary organisms, and/or a slow degradation of IPL-GDGTs, in contrast to bacterial or eukaryotic PLFAs.

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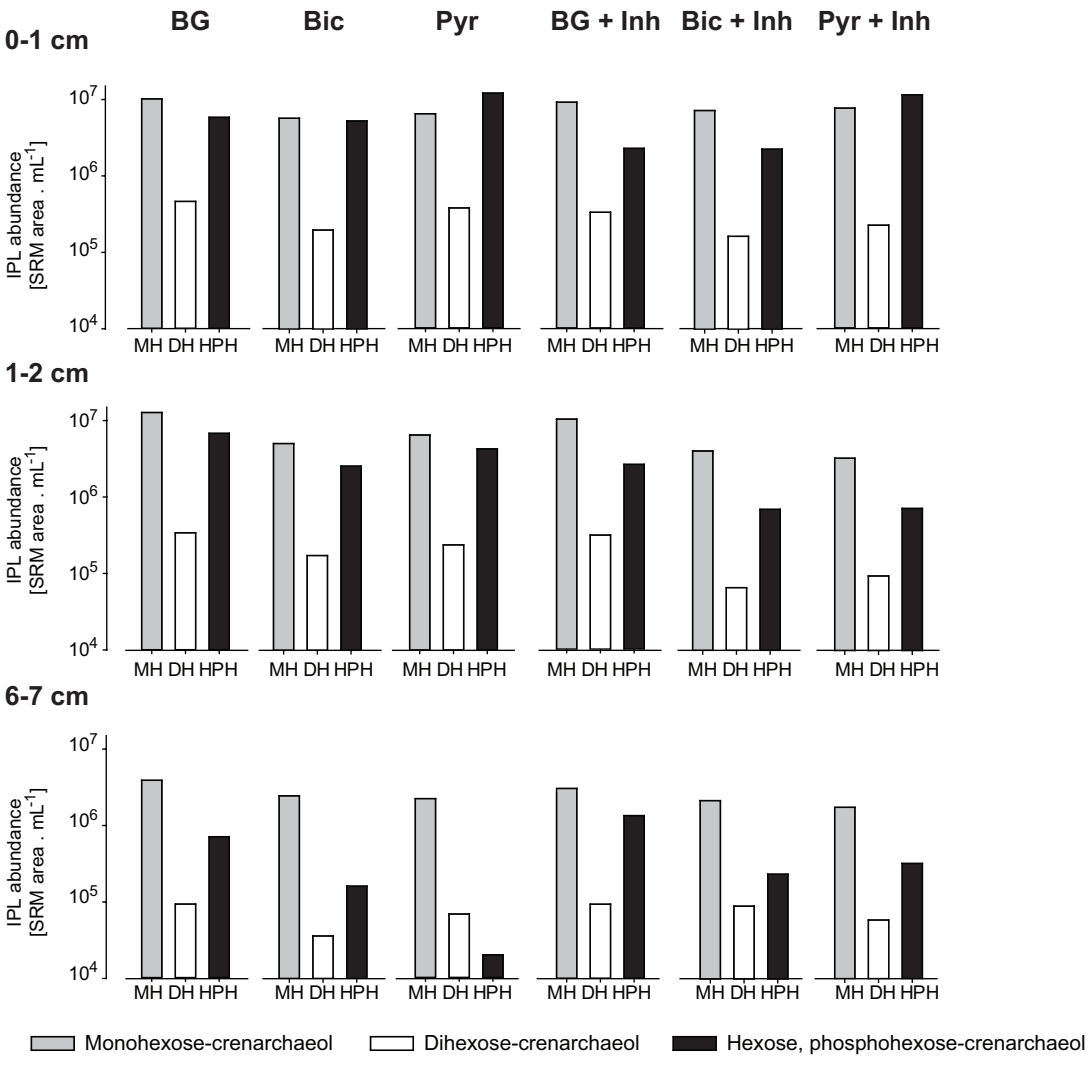

Figure S1: IPL-crenarchaeol profiles of the incubated cores (station 1) - background (BG), bicarbonate (Bic) and pyruvate (Pyr) without (left plot) and with nitrification inhibitor (right plot). Top graphs shows the values in SRM area . mL^{-1} from 0–1 cm depth, graphs in the middle the profiles from 1–2 cm depth and at the bottom the profiles from 6–7 cm depth

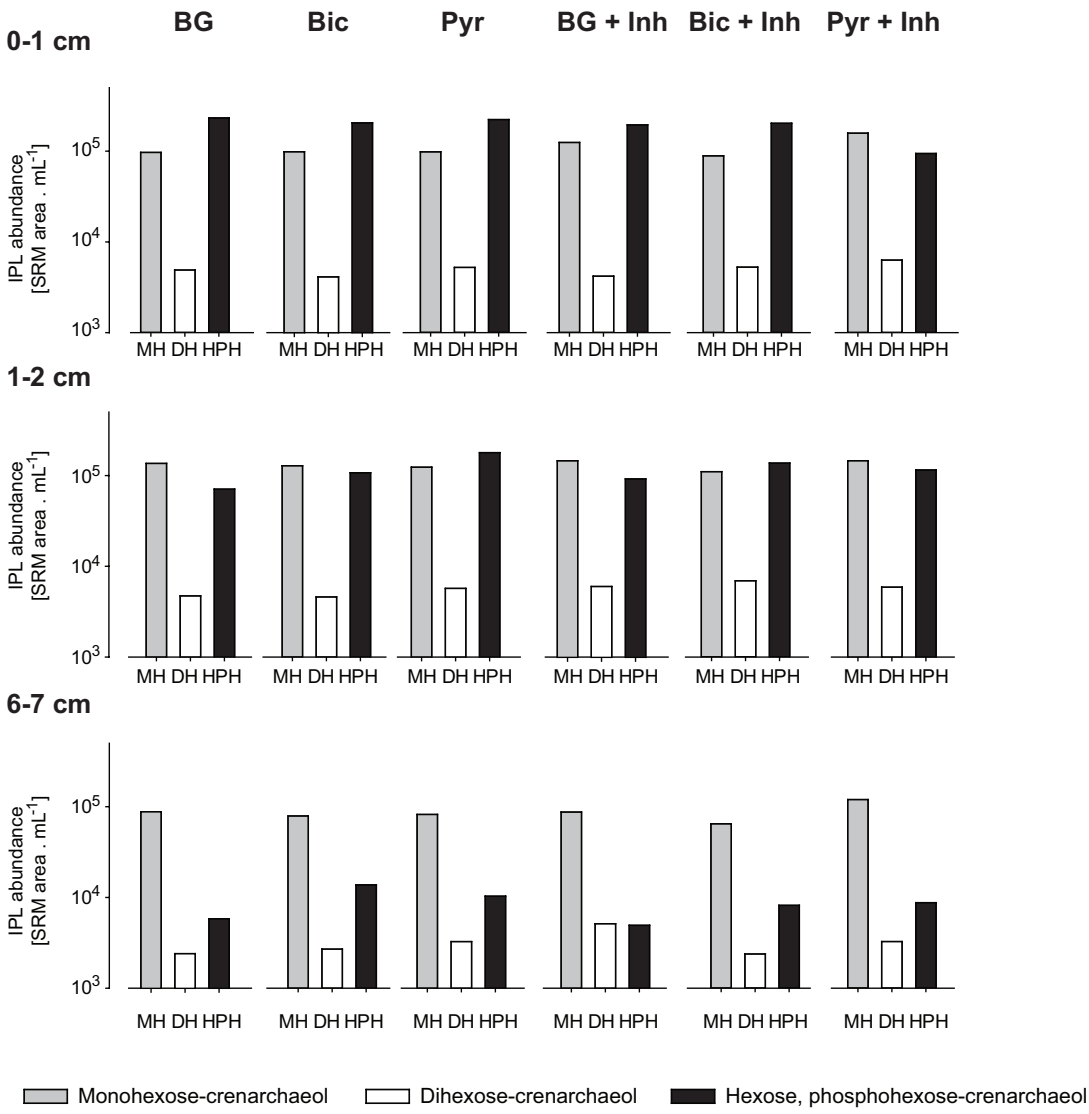

Figure S2: IPL-crenarchaeol profiles of the incubated cores (station 2) - background (BG), bicarbonate (Bic) and pyruvate (Pyr) without (left plot) and with nitrification inhibitor (right plot). Top graphs shows the values in SRM area. mL^{-1} from 0–1 cm depth, graphs in the middle the profiles from 1–2 cm depth and at the bottom the profiles from 6–7 cm depth.

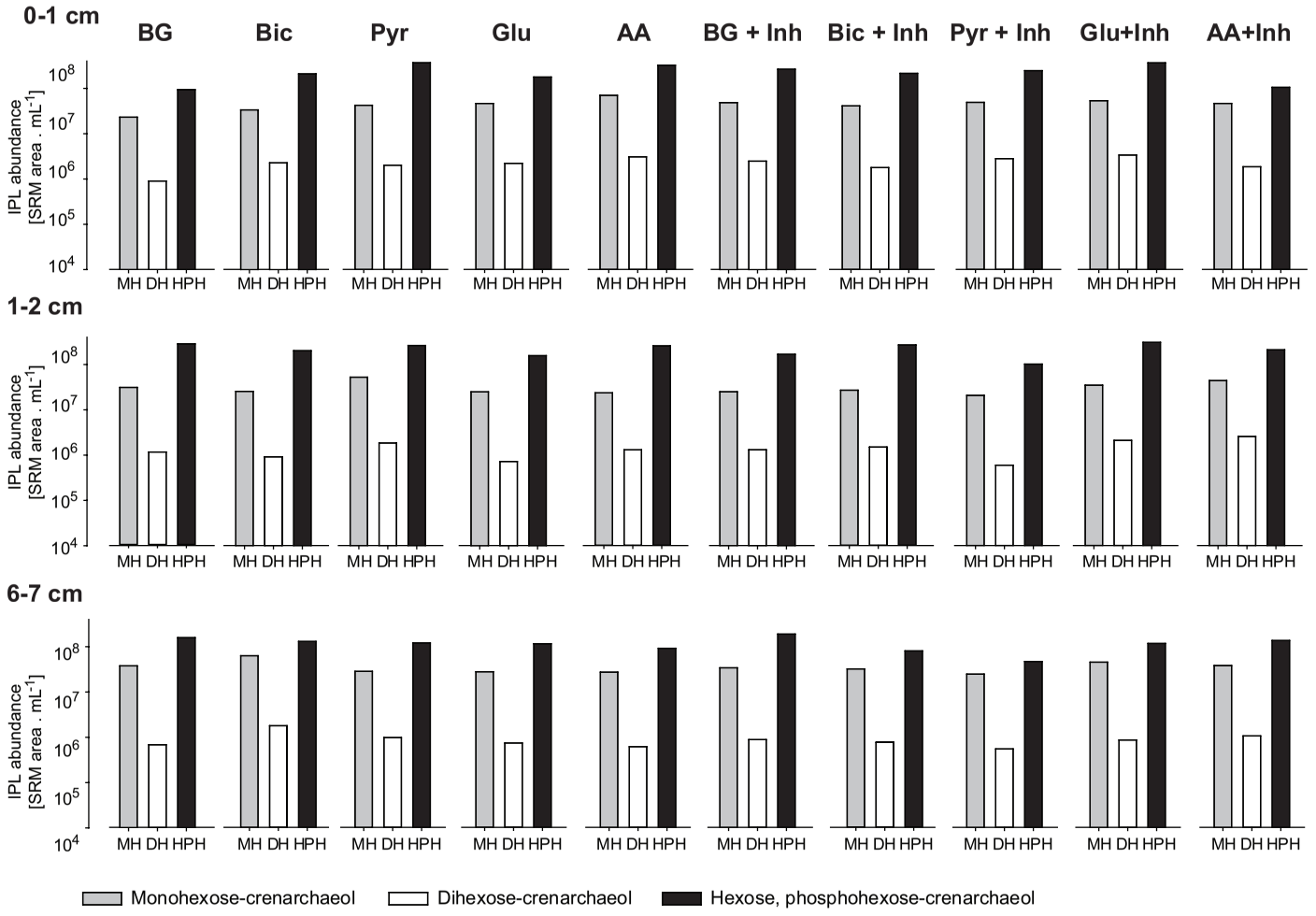

Figure S3: IPL-crenarchaeol profiles of the incubated cores (station 3) - background (BG), bicarbonate (Bic), pyruvate (Pyr), glucose (Glu) and amino acids (AA) without (left plot) and with nitrification inhibitor (right plot). Top graphs shows the values in SRM area. mL^{-1} from 0–1 cm depth, graphs in the middle the profiles from 1–2 cm depth and at the bottom the profiles from 6–7 cm depth.

4. Abundance and diversity of denitrifying and anammox bacteria in seasonally hypoxic and sulfidic sediments of the saline Lake Grevelingen

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Denitrifying and anammox bacteria are involved in the nitrogen cycling in marine sediments but the environmental factors that regulate the relative importance of these processes are not well constrained. Here, we evaluated the abundance, diversity and potential activity of denitrifying, anammox, and sulfide-dependent denitrifying bacteria in the sediments of the seasonally hypoxic saline Lake Grevelingen, known to harbor an active microbial community involved in sulfur oxidation pathways. Depth distributions of 16S rRNA gene, *nirS* gene of denitrifying and anammox bacteria, *aprA* gene of sulfur-oxidizing and sulfate-reducing bacteria, and ladderane lipids of anammox bacteria were studied in sediments impacted by seasonally hypoxic bottom waters. Samples were collected down to 5 cm depth (1 cm resolution) at three different locations before (March) and during summer hypoxia (August). The abundance of denitrifying bacteria did not vary despite of differences in oxygen and sulfide availability in the sediments, whereas anammox bacteria were more abundant in the summer hypoxia but in those sediments with lower sulfide concentrations. The potential activity of denitrifying and anammox bacteria as well as of sulfur-oxidizing, including sulfide-dependent denitrifiers and sulfate-reducing bacteria, was potentially inhibited by the competition for nitrate and nitrite with cable and/or Beggiatoa-like bacteria in March and by the accumulation of sulfide in the summer hypoxia. The simultaneous presence and activity of organoheterotrophic denitrifying bacteria, sulfide-dependent denitrifiers and anammox bacteria suggests a tight network of bacteria coupling carbon-, nitrogen- and sulfur cycling in Lake Grevelingen sediments.

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4.1. Introduction

Nitrogen availability is a major factor controlling primary production in temperate coastal marine environments with high anthropogenic nitrogen input (Herbert, 1999). Denitrification is a key process in the nitrogen cycle of coastal sediments releasing gaseous end products, nitric oxide (NO), nitrous oxide (N_2O) and dinitrogen gas (N_2) to the atmosphere. This nitrogen removal can result in a decrease of nitrogen availability for primary producers and thereby controlling the rate of eutrophication in coastal marine systems (Seitzinger 1988; Herbert 1999). Multiple microbial mediated pathways result in the removal of nitrogen in anoxic sediments (see Devol, 2015 for a detailed review). Here we focus on (1) denitrification, the stepwise conversion of nitrate/nitrite to dinitrogen gas which is mainly performed by facultative organoheterotrophic anaerobic bacteria and some archaea (Zumft, 1997); (2) anaerobic ammonium oxidation (anammox), the oxidation of ammonium with nitrite to dinitrogen gas carried out by anammox bacteria (Kuypers et al., 2003); and (3) sulfide-dependent denitrification, the oxidation of sulfide with nitrate performed by autotrophic members of α-, β-, γ- and ε-proteobacteria, which could contribute to denitrification and to the removal of sulfide in the oxygen transition zone of coastal marine sediments (Shao et al., 2010).

Numerous environmental factors, such as the availability of nitrogen speciation and concentration, temperature, oxygen concentrations, organic matter quality and quantity, bioturbation and other sediment characteristics have been suggested to affect the distribution and abundance of denitrifying and anammox bacteria (Thamdrup and Dalsgaard 2002; Meyer et al., 2005; Jensen et al., 2008; Dang et al., 2010; Laverock et al., 2013; Prokopenko et al., 2013; Babbin et al., 2014; Zhang et al., 2014). In this study, we assessed the effects of hypoxia and elevated sulfide concentration on the abundance and activity of denitrifiers and anammox bacteria in marine sediments as these factors have been previously suggested as potential limiting factors in denitrification processes (Brunet and Garcia-Gil, 1996; Burgin and Hamilton, 2007; Aelion and Warttinger, 2010; Neubacher et al., 2011; 2013; Bowles et al., 2012). Seasonal hypoxia is an increasing phenomenon that occurs in coastal areas causing a decrease in the electron acceptors (O_2, NO_3^-) in the bottom waters (Diaz & Rosenberg 2008). Besides, sulfide inhibition can decrease denitrification rates as the enzyme catalyzing the reduction of nitrous oxide to N_2 is sensitive to sulfide (e.g. Porubsky et al., 2009). In addition, several studies have provided putative evidence indicating that sulfide inhibits the anammox reaction. For example, Dalsgaard et al. (2003) reported a decrease in anammox activity in the sulfidic waters of the anoxic basin of Golfo Dulce (Costa Rica). Also Jensen et al. (2008) showed that sulfide had a direct inhibiting effect on the activity of anammox bacteria in the Black Sea.

In this study, we evaluated the impact of environmental factors, such as oxygen and free sulfide concentrations in the diversity, abundance, activity, and spatial distribution of bacteria involved in N_2 removal pathways in sediments of Lake Grevelingen (The Netherlands), a seasonally hypoxic saline reservoir. Here, we determined the diversity, abundance and potential activity of denitrifying bacteria, anammox bacteria and sulfur-oxidizing bacteria (SOB), including sulfide-dependent denitrifiers, and sulfate-reducing (SRB), in three different stations within the lake both in March (before hypoxia) and August (i.e. during hypoxia). In order to determine changes in the diversity, abundance and activity of denitrifiers, we targeted the *nirS* gene, encoding for the cytochrome cd_1 nitrite reductase, catalyzing nitrite reduction to nitric oxide (NO) (Braker et al., 1998; Smith et al., 2007; Huang et al., 2011). Here, we focused on the cytochrome

*cd*1-containing nitrite reductase (*nirS* gene), as it has been found to be more widespread in the bacterial communities compared to the copper-containing nitrite reductase (*nirK*) in various sediments (Braker 1998; Priemé et al. 2002; Liu et al. 2003; Throbäck et al. 2004; Tiquia et al. 2006; Oakley et al. 2007; Dang et al. 2009; Huang et al. 2011). For anammox bacteria, the *nir*S gene was also quantified as it has been recently suggested to be a functional biomarker anammox bacteria (Li et al., 2011). Both, denitrifying and anammox bacteria harbor one copy of the *nirS* gene, indicating that the *nirS* gene might be a suitable marker to compare the abundance and distribution of *nirS*-type denitrifiers and anammox bacteria in coastal sediments. The potential activity of denitrifying and anammox bacteria was also determined by estimating the gene expression of those metabolic genes as in previous studies (Smith et al., 2007; Lam et al., 2009; Bale et al., 2014; Bowen et al., 2014; Zhang et al., 2014; Chapter 2). Besides functional genes, the abundance of anammox bacteria was also determined by the quantification of ladderane lipids, which are specific lipid biomarkers for this microbial group (Sinninghe Damsté et al., 2002; Jaeschke et al., 2009; Russ et al., 2013). Finally, the diversity and abundance of sulfur-oxidizing and sulfate-reducing bacteria in marine sediments was estimated by targeting the *aprA* gene encoding the adenosine-5'-phosphosulfate (APS) reductase (Lenk et al., 2011; Blazejak and Schippers, 2011; Dyksma et al., 2016). The APS reductase is operating in in SOB, oxidizing sulfite to APS, as well as in SRB, operating in reverse direction, converting APS to adenosine monophosphate (AMP) and sulfite (SO_3^{2-}) (Meyer and Kuever, 2007b).

Our starting hypothesis is that the abundance and potential activity of organoheterotrophic denitrifiers and anammox bacteria would decrease upon increase of the sulfide concentration found in the sediments during the summer hypoxia. Moreover, recent studies point out that sulfide-dependent denitrifiers play a relevant role in the sulfide transition zone of intertidal sediments (Dyksma et al., 2016). Therefore, we hypothesize that their abundance and activity would be higher in hypoxic and sulfidic conditions, thus contributing more to the general N_2 removal in comparison to organoheterotrophic denitrifying and anammox bacteria and eventually being involved in sulfide detoxification which could promote anammox activity.

4.2. Material and Methods

4.2.1. Study site and sampling

Lake Grevelingen is a former estuary within the Scheld- Rhine- Meuse delta, and was formed by the construction of the Grevelingendam on the landside in 1964 and the Brouwersdam on the seaward side in 1971. The lake (surface area: 108 km^2, mean water depth 5.3 m) mainly consists of shallow water areas with the exception of the former tidal gullies, that have a water depth of up to 48 m (Keldermann 1984; Nienhuis and De Bree, 1984). After its closure, the lake transformed into a freshwater body, but in 1979, the connection with the North Sea was partially re-established, and since then, Lake Grevelingen has a high and relatively constant salinity (29−32). Within Lake Grevelingen, the Den Osse basin forms a deeper basin within the main gully, and experiences a regular seasonal stratification leading to oxygen depletion in the bottom water (summer hypoxia) (Wetstejn, 2011). Due to sediment focusing, the Den Osse basin (maximum water depth 34 m) also experiences a rapid accumulation of fine-grained, organic rich sediments (sediment accumulation rate ~2 cm yr^{-1}; Donders, 2012).

Sediment cores were collected along a depth gradient in Den Osse basin during two cruises March and August 2012 on board of the *RV* Luctor. Sampling took place at three different sta-

tions: S1 was located in the deepest point of the basin at 34 m water depth (51.747°N, 3.890°E), S2 at 23 m (51.749°N, 3.897°E) and S3 at 17 m (51.747°N, 3.898°E). Sediment was collected with single core gravity corer (UWITEC) using transparent PVC core liners (6 cm inner diameter, 60 cm length). Four sediment cores were collected at each station in March and in August. The cores were sliced with a 1 cm resolution until 5 cm depth, and sediment samples were collected for lipid and DNA/RNA analysis and kept at –80ºC until further processing. In each sampling campaign, a water column depth profile of temperature, salinity and oxygen (O_2) concentration was recorded at S1 using an YSI 6600 CTD instrument (for details see Hagens et al., 2015). Oxygen concentrations recorded by the CTD instrument were calibrated based on discrete water samples using an automated Winkler titration procedure (Knap et al., 1996). Bottom water concentrations of ammonium (NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-) were measured colometrically on a SEAL QuAAtro segmented flow nutrient analyzer. Monitoring data at Lake Grevelingen (Wetstejn, 2011) shows that the water column is laterally homogenous over the Den Osse basin scale, which allows estimation of the bottom water parameters at S2 and S3 from corresponding depths in the measured CTD profiles at station S1.

4.2.2. Sediment geochemistry

High-resolution depth profiles of O_2 and sulfide (H_2S) were measured in intact sediment cores to determine the oxygen penetration depth (OPD) and the sulfide appearance depth (SAD), using commercial micro-electrodes (Unisense A.S., Denmark) operated with a motorized micromanipulator (for details on the procedure see Malkin 2014). The oxygen penetration depth (OPD) is operationally defined as the depth below which $[O_2] < 1$ μM, while the sulfide appearance depth (SAD) is operationally defined as the depth below which $[H_2S] > 1$ μM (Seitaj et al., 2015).

Sediment cores were sectioned in increments of 0.5 cm from the sediment-water interface to 5 cm depth, and pore water was extracted by centrifugation, and analyzed following the procedure of (Sulu-Gambari et al., 2016). After filtration through 0.22 μm cellulose filters (Chromafil Xtra), pore water samples were analyzed for total free sulfide ($\sum H_2S$) via spectrophotometry (Cline, 1969; standard deviation ± 0.4 μM), whereas ammonium (NH_4^+) was determined by a SEAL QuAAtro segmented flow analyzer (Aminot et al., 2009) after a 25 times dilution with a low nutrient seawater matrix solution (standard deviation ± 3.5%).

The free sulfide and ammonium concentration depth profiles of the sediment pore water were averaged to provide a 1 cm resolution down to 5 cm sediment depth to enable a direct comparison with results of the DNA/RNA and lipid analysis. The total organic carbon (TOC) content of the sediment was determined on sediment samples that were freeze-dried, ground to a fine powder and analyzed by an a Thermo Finnigan Delta plus isotope ratio monitoring mass spectrometer (irmMS) connected to a Flash 2000 elemental analyzer (Thermo Fisher Scientific, Milan). Before the analysis, samples were first acidified with 2N hydrogen chloride (HCl) to remove the inorganic carbon (Nieuwenhuize et al., 1994). Concentrations of TOC are expressed as mass % of dry sediment.

4.2.3. DNA/RNA extraction

DNA and RNA from sediments (previously centrifuged to remove excess of water thus values are given as grams of wet weight; S1, S2 and S3; 0–5 cm sediment depth; 1 cm resolution) were extracted by using the DNA and RNA PowerSoil® Total Isolation Kit, respectively (Mo Bio Laboratories, Inc., Carlsbad, CA). Nucleic acid concentrations were quantified spectrophoto-

metrically (Nanodrop, Thermo Scientific, Wilmington, DE) and checked by agarose gel electrophoresis for integrity. Extracts were kept frozen at –80°C. The RNA extracts were treated with RNase-free DNase (DNA-*free*™, Ambion Inc., Austin, TX), and RNA quality and concentration were estimated by the Experion RNA StdSens Analysis Kit (Bio-Rad Laboratories, Hercules, CA). DNA contamination was checked by PCR using RNA as a template. Reverse transcription was performed as specified in Chapter 2.

4.2.4. PCR amplification and cloning

Amplifications of the *nirS* gene of denitrifying bacteria (S1, S2, S3, March and August, 0–1 cm), and anammox bacteria 16S rRNA gene (S2, March, 1–2 cm), specific *nirS* genes of *Scalindua* sp. (S3, August, 0–1 cm) and the *aprA* gene (S2, August, 0–1 cm) were performed with the primer pairs specified in Table S1. The PCR reaction mixture consisted of (final concentration): Q-solution (PCR additive, Qiagen, Valencia, CA) 1 ×; PCR buffer 1 ×; BSA (200 μg mL^{-1}); dNTPs (20 μM); primers (0.2 pmol μL^{-1}); $MgCl_2$ (1.5 mM); 1.25 U Taq polymerase (Qiagen, Valencia, CA). PCR conditions for these amplifications were: 95°C, 5 min; 35 × [95°C, 1 min; Tm, 1 min; 72°C, 1 min]; final extension 72°C, 5 min. PCR products were gel purified (QIAquick gel purification kit, Qiagen, Valencia, CA) and cloned in the TOPO-TA cloning® kit (Life Technologies, Carlsbad, CA) and transformed in *E. coli* TOP10 cells following the manufacturer's recommendations. In addition, in order to test the specificity of the quantitative PCR reaction we repeated the reactions of anammox bacteria 16S rRNA gene (Broc541F - Amx820R) with DNA extract of S2, March, 0–1 cm, *nirS* gene of heterotrophic denitrifying bacteria (nirS1F - nirS3R) with cDNA of S2, March, 0–1 cm, and *aprA* gene (Apr1F- Apr5R) with cDNA of S2, August, 0–1 cm, which were then treated to add 3'-A-overhangs and then cloned with the TOPO-TA cloning® kit as indicated above. Recombinant plasmid DNA was sequenced using the M13R primer by Macrogen Inc. (Amsterdam, The Netherlands).

4.2.5. Phylogenetic analysis

Sequences were analyzed for the presence of chimeras using the Bellerophon tool at the GreenGenes website (http://greengenes.lbl.gov/). Sequences were aligned with MEGA6 software (Tamura, 2013) by using the alignment method ClustalW. The phylogenetic trees of the *nirS* and *aprA* genes were computed with the Neighbour-Joining method (Saitou and Nei, 1987) using the Poisson model with a bootstrap test of 1,000 replicates. The phylogenetic affiliation of the partial anammox bacteria 16S rRNA gene sequences was compared to release 123 of the SILVA NR SSU Ref database (http://www. arb-silva.de/; Quast et al., 2013) using the ARB software package (Ludwig et al., 2004). Sequences were added to the reference tree supplied by the SILVA database using the ARB Parsimony tool. Sequences were deposited in NCBI with the following accession numbers: KP886533–KP886678 for *nirS* gene sequences of denitrifiers, KP886679–KP8866700 for 16S rRNA gene sequences of anammox bacteria, KP886701–KP886721 for *nirS* gene sequences of anammox bacteria and KP886722–KP886804 for *aprA* gene sequences of SOB and SRB.

4.2.6. Quantitative PCR (qPCR) analysis

qPCR analyses were performed on a Biorad CFX96™ Real-Time System/C1000 Thermal cycler equipped with the CFX Manager™ software for sediment DNA/RNA extracts (S1, S2 and S3; 0–5 cm sediment depth; 1 cm resolution). Detailed information about the primers used in this

study are summarized in Table S1. The abundance of denitrifying bacteria specific *nirS* gene was quantified using the primer set nirS1F/nirS3R as described by Braker et al. (1998). The abundance of anammox bacteria 16S rRNA gene was estimated using primers Brod541F/Amx820R as described by Li et al. (2010). Additionally, a fragment of the *Scalindua* sp. specific *nirS* gene, which codes for the cytochrome *cd*1-containing nitrite reductase, was quantified using the primer combination Scnir372F/Scnir845R as described by Lam et al. (2009). The abundance of SOB and SRB including sulfide dependent denitrifiers was estimated by targeting the dissimilatory adenosine-5'-phosphosulfate (APS) reductase (*aprA* gene) involved in the APS reduction of sulfur-oxidizing bacteria and in the sulfite oxidation of sulfate-reducing bacteria by using the primer combination Apr-1-FW/Apr-5-RW as described by Meyer and Kuever (2007a) (see Table S1 for details). Gene abundances are expressed as copies g^{-1} sediment of wet weight.

All qPCR amplifications were performed in triplicate with standard curves ranging from 10^0 to 10^7 molecules per microliter. Standard curves and qPCR amplifications were performed as previously described in Chapter 2. Coefficients of determination (R^2) for standard curves ≥ 0.998 and qPCR efficiencies (E) ≥ 80% were accepted.

4.2.7. Anammox bacteria ladderane lipid analysis

Intact polar lipids (IPLs) were extracted with the Bligh and Dyer extraction method (Bligh 1959; mod. by Pitcher et al., 2011) as described in detail by Bale et al. (2014). Intact ladderane phospholipids specific for anammox bacteria, the C_{20}-[3]-monoether ladderane attached to a phosphatidylcholine (PC) headgroup (PC-monoether ladderane) was analyzed by HPLC-MS/MS following Jaeschke et al. (2009) and quantified using an external standard consisting of isolated PC-monoether ladderane. Sediment samples between 0–5 cm depth (1 cm resolution) were analyzed and PC-monoether ladderane lipid concentrations were expressed per nanogram of dry weight sediment (ng g^{-1}). In order to determine the fatty acid composition of the ladderane lipids, aliquots of the Bligh and Dyer extracts (BDE) obtained from the 0–1 and 4–5 cm sediment layers were saponified by reflux with aqueous KOH (in 96% MeOH) for 1 h. Fatty acids were obtained by acidifying the saponified samples to a pH of 3 with 1N HCl in MeOH and extracted using dichloromethane (DCM). The fatty acids were converted to their corresponding fatty acid methyl esters (FAMEs) by methylation with diazomethane (CH_2N_2) as described by Rush et al. (2012a). Polyunsaturated fatty acids (PUFAs) were removed by eluting the sample over a silver nitrate ($AgNO_3$) (5 %) impregnated silica column with DCM and air-dried at room temperature. The fatty acid fractions were dissolved in acetone, filtered through a 0.45 μm polytetrafluoroethylene (PTFE) filters (4 mm diameter), and analyzed by high performance liquid chromatography coupled to positive ion atmospheric pressure chemical ionization tandem mass spectrometry (HPLC/APCI-MS/MS) in selective reaction monitoring (SRM) mode following Hopmans et al. (2006) including the recent modifications described by Rush et al. (2012b). Ladderane lipids were quantified using external calibration curves of three standards of isolated methylated ladderane fatty acids (C_{20}-[3]-ladderane fatty acid, and C_{20}-[5]-ladderane fatty acid) (Hopmans et al., 2006; Rush et al., 2011).

4.3. Results

Sediment samples were collected along a depth gradient in Den Osse basin (Lake Grevelingen) during two cruises March (before summer hypoxia) and August (during summer hypoxia) 2012. Sampling took place at three different stations: S1 was located in the deepest point of the basin

at 34 m water depth, S2 at 23 m and S3 at 17 m.

4.3.1. Environmental conditions

The temperature of the bottom water at station S1 (Fig. S1) showed a regular seasonal cycle with lowest values in late winter (1.5 ˚C in February) and highest values in late summer (16.9 ˚C in September). During the spring campaign (March 2012), the water column was only partially stratified and showed a limited surface-to-bottom temperature gradient. In contrast, during the summer campaign (August 2012), the water column was thermally stratified. The yearly pattern of the bottom water oxygenation at station S1 was inversely correlated the temperature, with greatest oxygenation levels in winter and fall, and lowest concentrations in summer (Fig. S1). In March 2012, the bottom water oxygenation was similar for all three stations, whereas in August 2012, the bottom water at S1 and S2 were anoxic (< 1 μM), while the bottom water at S3 still had 88 μM of O_2 (36% air saturation). Bottom water ammonium (NH_4^+) concentrations in station S1 ranged from 3 μM in March to 11.5 μM in August; nitrite (NO_2^-) concentrations were relatively constant (0.7–1 μM) in March and August. Nitrate concentrations ranged from 28 μM in March to <2 μM in August in station S1 (Fig. S1) whereas in stations S2 and S3 values varied between 28 μM in March to ~10 μM in August.

The OPD in the sediment was seasonally variable and increased from S1 to S3, i.e. in S1 in March OPD was 1.5 mm and in August the sediment was completely anoxic. In S2, OPD was between 1.7–2.5 mm in March and in August ca. 0.5 mm (hypoxic) and in S3, the OPD was between 1.5–2.2 mm in March and ca. 1.0 mm (hypoxic) in August. (Table 1). The sulfide appearance depth (SAD) varied between March and August in all stations. The SAD moved towards the sediment surface between March and August in all stations. In March, the SAD in stations S1 and S2 was at 18.4 and 21.3 mm respectively, whereas in S3, the SAD was detected at 41.8 mm sediment depth. However, in August, SAD in stations S1 and S2 was at 0.4 and 0.6 mm, respectively. In station S3, the sulfide was detected at 4.2 mm sediment depth (Table 1). At station S2, white mats of *Beggiatoa* sp.-like microorganisms covered the sediment surface in March. Small polychaetes were observed in the sediment at station S3 in March, suggesting some bioturbation. Sulfide ($\sum H_2S$) concentrations in the sediment pore water were low in March in all three stations, i.e. ranging from 0 to 0.007 mM, whereas in comparison, all three stations showed high sulfide concentrations in August, i.e. 0.15 to 1.6 mM (Table 1) (see Seitaj, 2015 for detailed geochemical profiles of station S1). Ammonium concentrations were low in March, i.e. ranging from 0.24 to 0.63 mM on average, in comparison with August when NH_4^+ concentrations reached higher values, i.e. 0.7 to 1.2 mM on average (Table 1). The TOC content of the sediments varied slightly between stations and seasons, ranging between 1.8 and 4.4% (Table S2).

4.3.2. Diversity, abundance and potential activity of *nirS*-type denitrifiers

In our study, we focused on heterotrophic and autotrophic bacteria that are able to perform the dissimilatory reduction of nitrite to nitric oxide. This reaction forms an intermediate step in the complete denitrification of nitrate to N_2 and is catalyzed by the cytochrome *cd*1-containing nitrite reductase encoded by the *nirS* gene (Braker et al., 2000). The diversity of *nirS*-type denitrifiers was evaluated for the surface sediment layer (0–1 cm) at the three stations in March and August by phylogenetic analysis targeting the *nirS* gene (Figs. 1 A, B). In general, the *nirS* sequences obtained (147 sequences in total) were closely related to *nirS* sequences of uncultured organisms found in coastal marine environments with a high input of organic matter such as

Table 1: Sediment porewater ammonia (NH_4^+) and sulfide (HS^-) concentrations (1 cm resolution), oxygen penetration depth (OPD) and sulfide appearance depth (SAD) determined by micro-sensor profiling.

Station	Sediment depth [cm]	HS^- [µM]		NH_4^+ [µM]		OPD [mm]		SAD [mm]	
		March	August	March	August	March	August	March	August
1	0–1	0	810	279	656	1.5	0	18.4	0.4
	1–2	0	1503	410	1071				
	2–3	0	1639	636	1322				
	3–4	0	1962	833	1567				
	4–5	33	2063	979	1768				
2	0–1	0	1157	165	550	1.7	2.5	21.3	0.6
	1–2	0	802	455	836				
	2–3	0	1008	526	1027				
	3–4	2	1238	592	1138				
	4–5	27	1190	671	1228				
3	0–1	0	211	73	537	1.5	2.2	41.8	4.2
	1–2	0	177	154	694				
	2–3	0	146	236	736				
	3–4	0	109	333	749				
	4–5	0	93	408	746				

*Data are averaged to reach a 1 cm resolution; data provided by Fatimah Sulu-Gambari (2016; unpublished data), **data provided by Dorina Seitaj, 2015; unpublished data).

estuarine sediments and eutrophic bay sediments (Braker et al., 2000, Zhang et al., 2014). The phylogenetic analysis of protein sequences of the *nirS* gene revealed two distinct clusters (Fig. 1A; cluster 1 and 2), where most (ca. 95%) of the sequences clustered in cluster 1 (ca. 95%). Within cluster 1, approximately 90% of the *nirS* gene sequences were grouped into subcluster 1.1 and 10% into subcluster 1.2 (Figs. 1 A, B; Fig. S2). Within subcluster 1.1 sequences were affiliated to *nirS* sequences of members of α-, β- and γ-proteobacteria able to perform autotrophic denitrification coupled to sulfide oxidation (*Thiobacillus denitrificans*) and heterotrophic denitrification (*Azospirillum brasilense*, *Marinobacter hydrocarbonoclasticus, Kangiella aquamirina*, *Halomonas* sp.). Sequences grouped in subcluster 1.2 were affiliated to the *nirS* gene sequences of divers α- proteobacteria able to perform autotrophic denitrification coupled to sulfide or iron oxidation (*Paracoccus denitrificans, Sideroxidans lithotrophicus*), as well as heterotrophic denitrification (e.g. *Aromatoleum aromaticum, Azocarus toluclasticus, Acidovorax delafieldii*). Sequences grouped into cluster 2 were affiliated to *nirS* gene sequences of uncultured bacteria detected in coastal marine and estuarine sediments (Braker et al., 2000; Zhang et al., 2014). In order to determine the specificity of the qPCR assay, sequences of *nirS* cDNA (complementary DNA of *nirS* mRNA) generated during the qPCR reaction were cloned and sequenced (28 sequences in total) and also added to the protein-coding *nirS* sequences phylogenetic tree showing that those sequences were grouped in the clusters described before (Figs. 1 A, B).

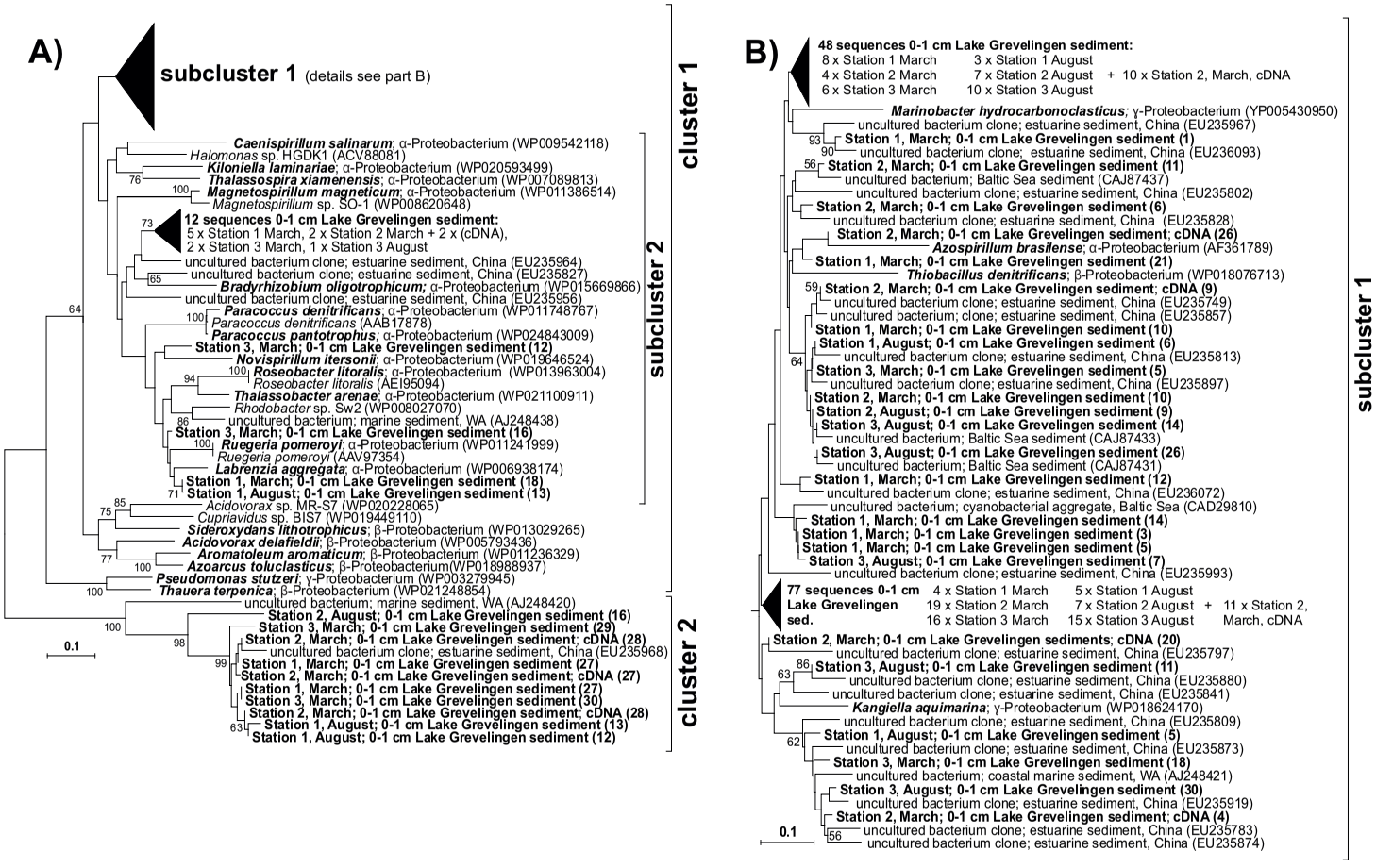
The abundance and distribution of *nirS*-type denitrifiers was estimated through quantification of the *nirS* gene copy number in the upper 5 cm (1 cm resolution) of the sediments at the three sampling sites in March and August (Fig. 2). The *nirS* gene abundance was relatively stable with depth with slightly higher values in station S1 (6.4×10^7 copies g^{-1} on average) compared to stations S2 and S3 (5.7×10^7 and 5.2×10^7 copies g^{-1} on average, respectively). Overall, *nirS* gene abundance was slightly higher in March compared to August in S2 and S3, and this difference was especially evident in station S1.

To estimate the potential transcriptional activity of *nirS*-type denitrifiers, *nirS* transcripts (mRNA copy numbers) were quantified (data reported in Table S3) and the RNA:DNA ratio was calculated. In station S1, transcripts were only detectable in March in the upper 2 cm of the sediment, 5.6×10^3 copies g^{-1}on average, whereas in August, *nirS* gene transcripts could only be detected within the 2–3 cm depth layer (10^2 copies g^{-1}). In station S2, *nirS* gene transcripts were only detectable in March in the upper 3 cm and numbers varied between 1.9×10^2–1.5×10^3 copies g^{-1} with the highest value within the 1–2 cm zone. In station S3, the *nirS* gene transcripts could be detected in March in the upper cm of the sediment (1.5×10^2 copies g^{-1}) and in August for 1–2 cm and for 3–4 cm sediment (1.1×10^2 and 1.8×10^3 copies g^{-1}, respectively) (Table S3). The ratio of *nirS* gene and transcript copies (RNA:DNA ratio) was ≤ 0.00013 in all stations in March and August.

4.3.3. Diversity, abundance and potential transcriptional activity of anammox bacteria

Most of the anammox bacterial *nirS* gene sequences obtained in this study (20 sequences out of 21) were part of one cluster (cluster 1, Fig. 3) closely related to *nirS* sequences of "*Candidatus* Scalindua profunda" (Van de Vossenberg et al., 2013), and of uncultured bacteria obtained from continental margin sediment of the Arabian Sea (Sokoll et al., 2012) and surface sediments of the South China Sea (Li et al., 2013).

Figure 1: Phylogenetic tree of partial *nirS* gene sequences of denitrifying bacteria retrieved in this study (147 DNA sequences recovered from stations S1, S2 and S3 between 0–1 cm sediment depth, in March and August by amplification (PCR) and cloning and 28 cDNA sequences obtained from station S2 between 0–1 cm sediment depth in March recovered by amplification (qPCR) and cloning) and closest relatives (bold: our sequences and closest known relatives); the scale bar indicates 10 % sequence divergence.



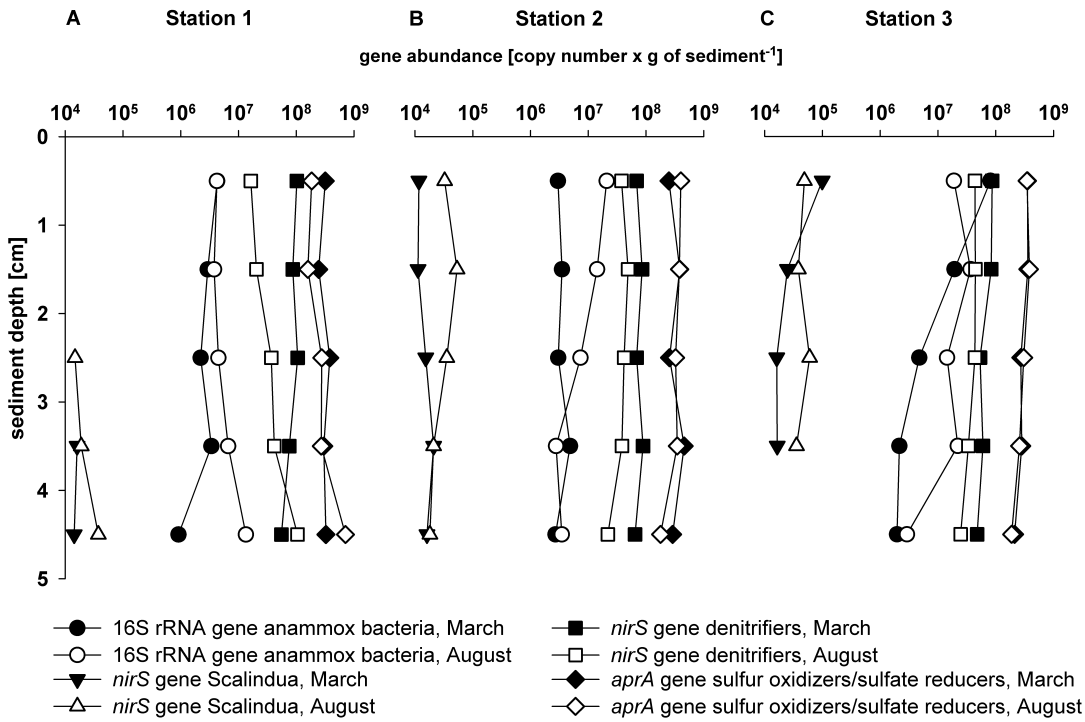


Figure 2: Depth profiles of anammox bacteria 16S rRNA gene abundance [copies g^{-1}] (circle); anammox bacteria *nirS* gene abundance [copies g^{-1}] (triangle), denitrifying bacteria *nirS* gene abundance [copies g^{-1}] (square); SOB and SRB *aprA* gene abundance [copies g^{-1}] (diamond); A) Station 1; B) Station 2; C) Station 3; black symbol: March; white symbol: August.

The diversity of the 16S rRNA gene sequences of anammox bacteria (Fig. S3) obtained from surface sediments (0–1 cm) revealed that most of the sequences (21 sequences derived from PCR plus cloning, and 23 gene sequences obtained from the qPCR assay and further cloning to check the specificity of the qPCR assay) were closely related to "*Candidatus* Scalindua" brodae and "*Candidatus* Scalindua marina" obtained from surface sediments of the Gullmar Fjord (Brandsma et al., 2011), and to sequences detected in OMZ waters of the Arabian Sea, Peru and Namibia (Woebken et al., 2008) and Peruvian coastal margin (Henn, unpublished). Two 16S rRNA sequences were more distantly related to the other sequences, and were closely related to the 16S rRNA sequence of "*Candidatus* Scalindua wagneri" obtained from a bioreactor (Woebken et al., 2008) (Fig. S3).

We also quantified the C_{20}-[3]-ladderane monoether-PC (for 0–5 cm sediment depth) and ladderane core lipids (for 0–1 and 4–5 cm sediment depth) (Table S4 and S5) as markers for the presence of anammox bacteria. Abundance of PC-monoether ladderane ranged between 0.9–20.4 ng g^{-1} with highest values in station S1 in March (between 3.4–20.4 and 2.4–7 ng g^{-1}, respectively). PC-monoether ladderane values were relatively constant with depth in August in all stations (between 0.9–7 ng g^{-1}; Table S4). On the other hand, PC-monoether ladderane abundance was always higher in March in the upper 2 cm of the sediment and decreased four-fold between 2 and 5 cm in all stations (on average from 10.7 to 2.5 ng g^{-1}). The summed concentration of the ladderane fatty acids was on average lowest in station S1 (13 ng g^{-1}), with slightly

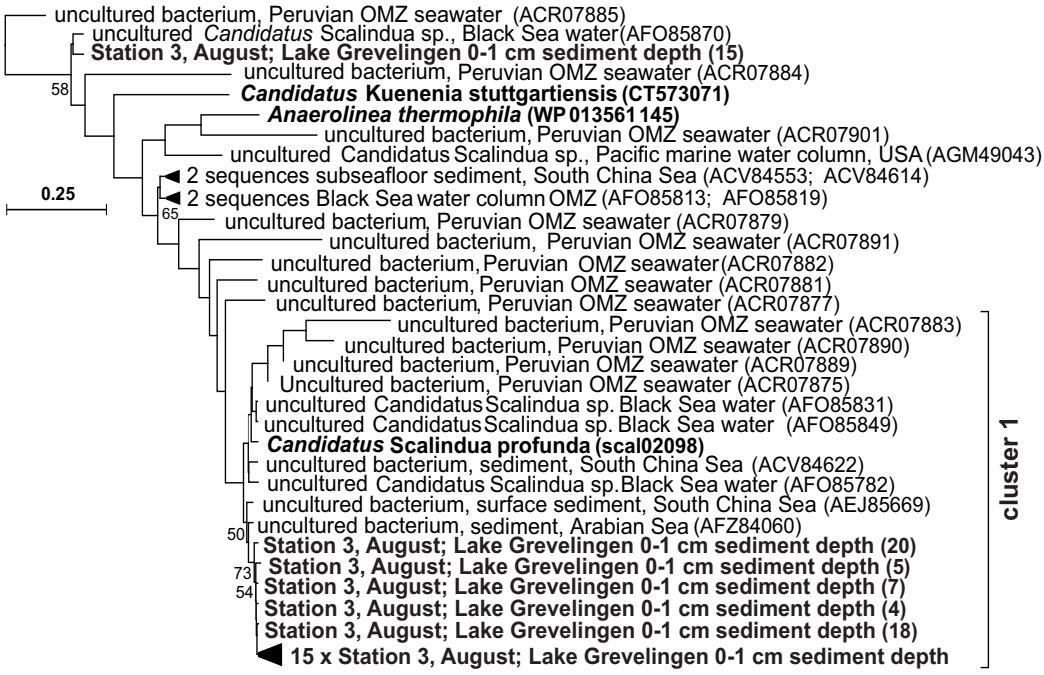


Figure 3: Phylogenetic tree of partial *nirS* gene sequences of anammox bacteria retrieved in this study and closest relatives (S3; 0–1 cm Lake Grevelingen sediment; bold: sequences retrieved in March and sequences of known relatives); the scale bar indicates 25% sequence divergence.

higher average values in S2 (27 ng g^{-1}), and the highest average values in S3 (41 ng g^{-1}) (Table S5). The summed ladderane fatty acid concentrations were comparable to the abundance of anammox bacteria determined by the 16S rRNA gene quantification in the first centimeter of the sediment obtained in different stations and seasons, whereas the PC-monoether ladderane revealed a different trend compared to the 16S rRNA gene abundance, i.e., was more abundant in March compared to August (Table S4).

The abundance of anammox bacteria was determined by the quantification of the 16S rRNA gene copy number of anammox bacteria as well as of the *nirS* gene copy number of members of the genus "*Candidatus* Scalindua" (Figure 2) (Strous et al., 2006; Li et al., 2011). Copy numbers of the anammox bacteria 16S rRNA gene ranged between 9.5×10^5 and 8.1×10^7 copies g^{-1} with highest values in S3 (between 2×10^6 and 8.1×10^7 copies g^{-1}). The abundance of the anammox bacterial 16S rRNA gene was slightly higher in August compared to March in all stations. The "*Candidatus* Scalindua" *nirS* gene abundance followed the same depth trend but values were 2 – 3 orders of magnitude lower (between 1.1×10^4 and 9.9×10^4 copies g^{-1}) compared to the anammox bacteria 16S rRNA gene copy numbers.

The anammox bacterial 16S rRNA transcript, used as a proxy for the potential transcriptional activity, varied between $1.3 \times 10^3 – 5.1 \times 10^6$ copies g^{-1} of sediment (Table S3). The anammox 16S rRNA transcript copy number varied slightly and reached highest values in S2 and S3 in August between 2–4 cm sediment depth (2.7×10^6 copies g^{-1} on average). The ratio between 16S rRNA gene and transcript (RNA:DNA ratio) was ≤ 0.32 in all stations in March and August.

Anammox bacteria *nirS* transcript copies were below detection level of the qPCR assay.

4.3.4. Diversity, abundance and potential transcriptional activity of bacteria involved in sulfur cycling

Here, we focused on bacteria involved in sulfur cycling, performing dissimilatory sulfur oxidation or sulfate reduction. The dissimilatory APS reductase encoded by the *aprA* gene is operating in SRB, converting APS to adenosine monophosphate (AMP) and sulfite (SO_3^{2-}), as well as in SOB, operating in reverse direction, oxidizing sulfite to APS (Meyer and Kuever, 2007b). The diversity of the microorganisms harboring the *aprA* gene was evaluated in the first centimeter of the sediment core at S2 in August as the observation of *Beggiatoa* mats on top indicated the occurrence of sulfide oxidation at this station. The protein sequences (86 sequences PCR + cloning) coded by the *aprA* gene grouped in two clusters (Fig. 4; cluster I (66%) and II (34%)). Within cluster I, sequences clustered in three distinct subclusters. Sequences of the *aprA* gene included in subcluster 1.1 (36%) were affiliated to heterotrophic SRB of the δ-proteobacteria class (e.g. *Desulfosarcina* sp., *Desulfofaba gelida*, *Desulfobulbus propionicus*) and to *aprA* gene sequences of uncultured bacteria found in environments such as in Black Sea sediments and associated with benthic organisms (Blazejak and Schippers, 2011; Ruehland et al., 2008). In subcluster 1.2 (21%), sequences were closely affiliated to β- and γ-proteobacteria involved in autotrophic sulfur-dependent denitrification (*Thiobacillus denitrificans* and *Sulfuricella denitrificans*) or in phototrophic sulfur oxidation (*Lamprocystis purpurea*) and to the *aprA* gene sequence of an uncultured bacterium associated with the sea urchin *Asterechinus elegans* (Quast et al., 2009). Sequences clustering in subcluster 1.3 (9%) were affiliated with *aprA* gene sequences of bacteria of the phylum Firmicutes (i.e. *Desulfotomaculum* sp.) known to perform heterotrophic sulfate reduction, and to sequences of uncultured bacteria obtained in various sediments such as hydrothermal seep sediments, Peru margin sediments and salt lake sediments (Blazejak and Schippers, 2011; Meyer and Kuever, 2007c; Kleindienst et al., 2012). Cluster II contained sequences (34%) closely related to obligately chemolithoautotrophic members of the β-proteobacteria class (*Thiobacillus thioparus*) able to perform nitrate reduction to nitrite with thiocyanate, and to *aprA* gene sequences of uncultured bacteria retrieved in salt lake sediments or associated with benthic organisms (Becker et al., 2009). In addition, to assess the specificity of the *aprA* gene qPCR assay, *aprA* gene transcripts (cDNA of *aprA* mRNA) generated during the qPCR assay (21 sequences in total) were cloned, sequenced and added to the *aprA* phylogenetic tree, where they were classified in the clusters previously described (Fig. 4).

The abundance and the potential activity of SOB including sulfide-dependent denitrifiers and SRB were determined by the quantification of the *aprA* gene and its gene transcripts (Fig. 2, Table S3). The copy number of the *aprA* gene was relatively constant with varying sediment depth and season. Gene copy numbers of the *aprA* gene were of the same order of magnitude as observed for the denitrifiers *nirS* (on average 3.1×10^8 copies g^{-1}) and reached highest values in S2 in March and August (on average 3.3×10^8 copies g^{-1}). At station S1, the *aprA* gene transcript (Table S3) was detectable at 2–3 cm sediment depth in March (5.4×10^2 copies g^{-1} on average), whereas in August the *nirS* transcript was detectable at 0–1 cm (3.8×10^3 copies g^{-1}) and 2–4 cm (1.8×10^4 copies g^{-1} on average) sediment depth. In S2 and S3, *aprA* gene transcripts were detectable at 1–4 cm sediment depth in March and August (on average 4.7×10^3 copies g^{-1} in March and 4.4×10^3 copies g^{-1} in August). The ratio between *aprA* gene and transcript (RNA:DNA ratio) was ≤ 0.000088 in all stations in March and August.

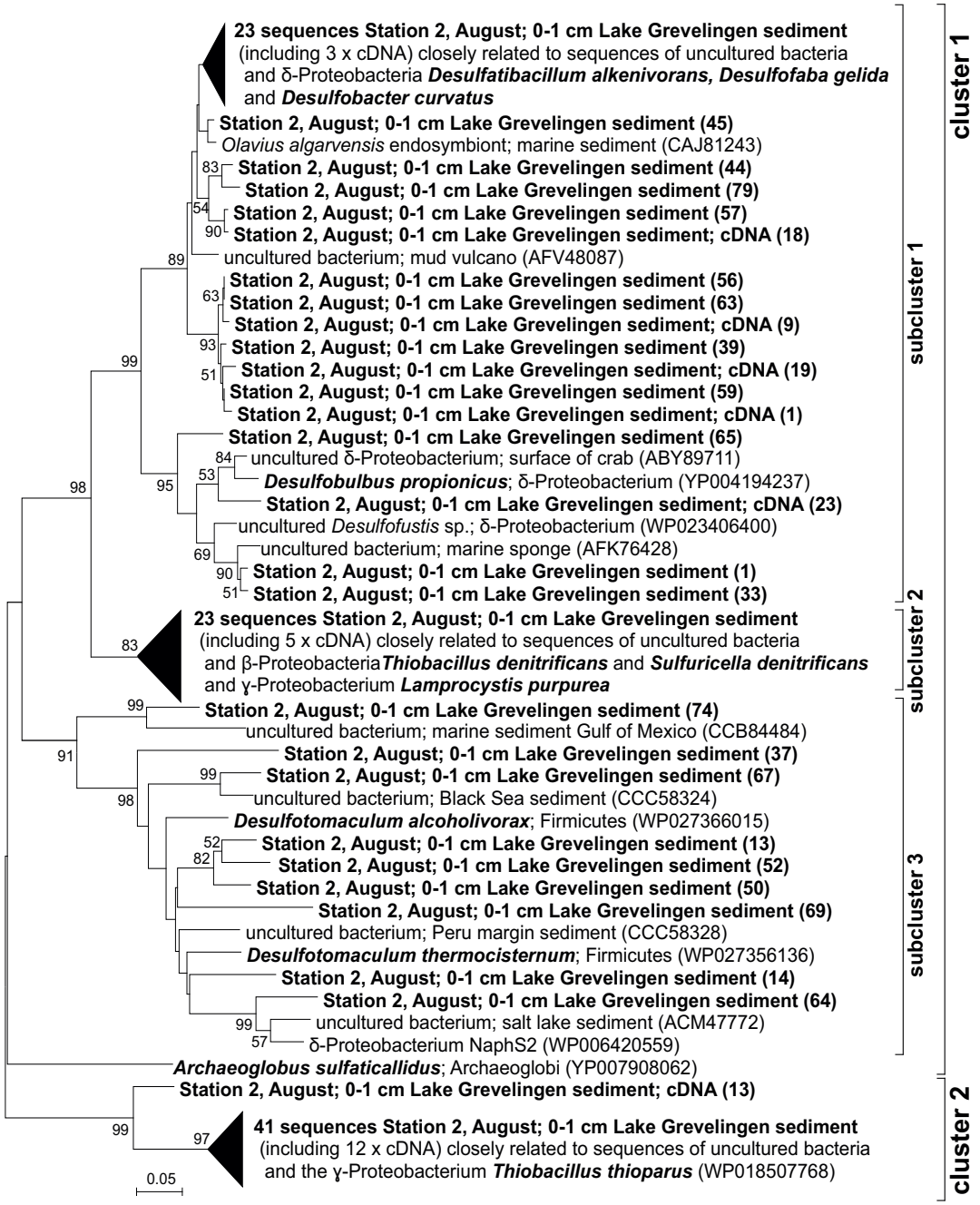


Figure 4: Phylogenetic tree of partial *aprA* gene sequences of sulfur-oxidizing and sulfate-reducing bacteria (SOB and SRB) retrieved in this study (86 DNA sequences recovered by amplification (PCR) and 21 cDNA sequences recovered by amplification (qPCR) and cloning from sediments (0–1 cm) of station S2 in August) and closest relatives (bold: our sequences and closest known relatives); the scale bar indicates 5% sequence divergence.

4.4. Discussion

The phylogenetic analysis of the *nirS* gene sequences amplified from Lake Grevelingen sediments revealed a substantial diversity of autotrophic and heterotrophic denitrifiers (Fig. 1). Most of the sequences obtained in this study were affiliated to α-, β- and γ-proteobacteria able to perform denitrification coupled to the oxidation of reduced sulfur compounds. Sequences of the *nirS* gene (~30%) and the *aprA* gene (21%) (Fig. 4) were closely related to sequences of *Thiobacillus denitrificans*, able to couple the oxidation of inorganic sulfur species such as sulfide with denitrification (Shao et al., 2010). Sulfide-dependent denitrifiers couple nitrogen and sulfur cycling and their presence point to an important denitrification potential in these sediments. In order to determine differences in abundance, and therefore of denitrification potential, between sediments and with depth, in our study we quantified the abundance of the *nirS* gene. The *nirS* gene abundance was relatively constant with increasing sediment depth, suggesting a stable community of heterotrophic and autotrophic denitrifying bacteria in Lake Grevelingen sediments. *NirS* gene abundance (in the order of 10^7 copies g^{-1}) was slightly higher in March in all three stations, but the difference was especially notable in station S1, most likely explained by the lower free sulfide concentrations in March compared to August (Table 1). Lower sulfide concentrations in March could favor the proliferation of heterotrophic denitrifiers not coupled with sulfide oxidation as sulfide has been seen to impair denitrification by inhibition of NO and N_2O reductases (Sørensen et al., 1980). In addition, the *nirS* gene abundance (Fig. 2) determined in Lake Grevelingen sediments (between 10^7–10^8 copies g^{-1}) was one to three orders of magnitude higher compared to the values previously detected in other marine sediments such as in sediments of open estuaries with permanently oxygenated bottom water (10^4–10^7 copies g^{-1} on average) (Smith et al., 2007; Zhang et al., 2013). The *nirS* gene abundance has previously been shown to correlate positively with potential denitrification rates (Mosier and Francis, 2010), and therefore, we can conclude that the sediments of the marine Lake Grevelingen harbor a stable community of heterotrophic and autotrophic denitrifiers, which can be actively involved in the denitrification process independent of season and sediment depth.

Besides quantifying the *nirS* gene as an indicator of the denitrification potential of these sediments, we also estimated the presence of *nirS* gene transcripts as a measure of potential activity (Table S3). Unfortunately, there is a lack of *in situ* studies correlating *nirS* gene expression with denitrification rates, and some studies have even shown a lack of direct relationship between *nirS* gene expression and modeled rates of denitrification in marine surface sediments (Bowen et al., 2014). On the other hand, other studies have observed a correlation between a decrease of *nirS* gene transcripts and reduction of denitrification rates together with declining concentrations of nitrate in estuarine sediment (Dong et al., 2000, 2002; Smith et al., 2007), which would justify the use of *nirS* gene transcripts as a proxy for potential denitrification performed by *nirS*-type denitrifiers. In this study, the copy number of denitrifier *nirS* transcripts was two to three orders of magnitude lower compared to values previously reported for estuarine sediments (Smith et al., 2007), suggesting a low denitrification rate in surface sediments of all stations in March and in deeper sediment layers in August. Gene expression of the *nirS* gene was detected in surface sediments in March (all stations) and in deeper sediment layers in August (stations S1 and S3), which is most likely explained by the availability of nitrate/nitrite in the water-sediment interface (bottom water).

Apart from *nirS*-containing denitrifiers, also anammox bacteria were detected in Lake Grevelingen sediments by applying both DNA and lipid-based biomarkers. The phylogenetic analysis of the anammox 16S rRNA gene amplified from the sediments pointed to an anammox community dominated by "*Candidatus* Scalindua" (Fig. S3). In order to estimate the abundance of anammox bacteria in the sediments and their potential role in the nitrogen cycle in this system, we estimated the abundance of the "*Candidatus* Scalindua" *nirS* gene. In contrast to *nirS*-type denitrifiers, anammox bacteria showed a clear seasonal contrast in their *nirS* gene abundance with higher copy numbers in August (summer hypoxia) compared to March (Fig. 2). However, this seasonal trend was not reflected in the abundance of the anammox bacteria lipid biomarker PC-monoether ladderane lipid (Table S4). Previous studies have suggested that this biomarker lipid could be partly preserved in the sediment due to a relatively low turnover rate (Brandsma et al., 2011; Bale et al., 2014; Chapter 2). On the other hand, the concentration of ladderane lipid fatty acids (Table S5) correlated with the anammox bacteria abundance given by 16S rRNA gene quantification, i.e. with the lowest values in station S1 and highest values in station S3. This suggests that the abundance of ladderane lipid fatty acids could be interpreted as a proxy for anammox bacteria abundance together with specific gene quantification. It is also worth noticing that a difference of three to four orders of magnitude was detected between abundances of anammox 16S rRNA gene and the "*Candidatus* Scalindua" *nirS* gene (Fig. 2). This discordance between anammox 16S rRNA genes and functional genes has been previously observed and is possibly linked to primer biases attributed to the anammox bacteria 16S rRNA gene primers that would amplify 16S rRNA gene fragments of bacteria other than anammox bacteria (Li et al., 2010; Harhangi et al., 2012; Bale et al., 2014; Chapter 2). However, in our study we have ruled out the possibility of the anammox bacteria 16S rRNA gene primers amplifying bacteria other than anammox by cloning and sequencing of the product generated during the qPCR reaction as shown in Fig. S3. Therefore, further studies should address the causes of this discordance.

Factors other than hypoxia and sulfide concentration could have also contributed to the differences in abundance and activity of anammox bacteria. For example, both the anammox bacteria 16S rRNA and *nirS* gene abundances were higher in August in comparison to March (Fig. 2). This may indicate that anammox bacteria are more abundant at higher temperatures (15°C), which is the anammox bacteria temperature optimum in temperate shelf sediments (Dalsgaard et al., 2005). This seasonality of anammox bacteria abundance has been reported before in sandy and as well in muddy, organic rich sediments of the North Sea (Bale et al., 2014; Chapter 2). Additionally, oxygen penetration depth in the sediments decreased and the sediment even became entirely anoxic during summer stratification (Table 1), which would also favor the anaerobic metabolism of anammox bacteria (Dalsgaard and Thamdrup, 2002). Anammox bacterial abundance was highest in station S3, which could also be related to elevated bioturbation activity that was observed in this station, as bioturbation and mixing extends the area of nitrate reduction, which might fuel the anammox process (Meyer et al., 2005; Laverock et al., 2013). Another factor determining anammox and denitrification pathways in sediments is the organic carbon content. Anammox and denitrification have been suggested to be more important nitrogen removal pathways in sediments of low carbon input compared to sulfide-dependent denitrification which seem to be more important in sulfidic sediments with high carbon input (Burgin and Hamilton, 2007). The TOC content in Lake Grevelingen sediments is high, i.e. in the order of 2.5–4.5% (Malkin et al., 2014), which is hence consistent with the low activity of anammox bacteria. More-

over in station S1, fresh organic-rich sediment rapidly accumulates (> 2 cm yr^{-1}; Malkin et al., 2014), which might inhibit the activity of anammox bacteria and denitrifying bacteria.

Overall, the *nirS* gene abundance of denitrifiers was three to four orders of magnitude higher compared to the anammox bacteria 'Scalindua' *nirS* gene values, suggesting that *nirS*-type denitrifiers have a more prominent role in the overall denitrification activity in Lake Grevelingen sediments in comparison with anammox bacteria (Fig. 2). This is also supported by the anammox bacteria *nirS* gene transcript abundance, which remained below detection limit. On the other hand, anammox bacteria 16S rRNA gene transcripts were detected throughout the sediment (Table S3). A recent study by Bale et al. (2014) observed a good correlation between the 16S rRNA gene abundance, 16S rRNA gene transcript abundance and anammox rates in North Sea sediments, which suggests that the transcriptional activity of anammox bacteria 16S rRNA gene is a suitable proxy of anammox bacteria activity. However, the anammox bacteria 16S rRNA gene RNA:DNA ratio was low (0.01–1) compared to the values previously reported in sediments of the southern North Sea (1.6–34.6) (Chapter 2), further supporting a low anammox activity in the Lake Grevelingen sediments.

Although denitrification was more relevant than anammox process as suggested by the gene abundance and potential activity (Fig. 2, Table S3), the measured denitrification rates (between 36–96 μM m^{-2} d^{-1}; D. Seitaj, personal communication) in Lake Grevelingen are low in comparison with other marine sediments. For example, denitrification rates were reported to vary between 30–270 μM m^{-2} d^{-1} in marine Arctic sediments (Rysgaard et al., 2004), while denitrification rates of 270±30 μmol N_2 m^{-2} d^{-1} were measured in sediments of the Lower St. Lawrence Estuary (Crowe et al., 2012). The lower denitrification and anammox potential in Lake Grevelingen sediments could be explained by the effect of high concentrations of sulfide (potentially inhibiting both heterotrophic denitrification and anammox). In our study, the abundance of *nirS*-type denitrifiers did not vary substantially for the different stations however the highest anammox bacterial abundance was observed in station S3 where lowest sulfide concentration was reported (Fig. 2, Table 1). Besides, these sediments harbor an important population of sulfide-dependent denitrifiers (as indicated by *aprA* gene values comparable to those found in sediments of the Black Sea; Blazejak and Schippers, 2011), which could alleviate the inhibitory effect of sulfide on other microbial groups such as anammox bacteria. For example, a study by Russ et al. (2014) observed the coexistence and interaction of sulfide-dependent denitrifying and anammox bacteria in a co-culture in which anammox bacteria remained active. Also a study by Wenk et al (2013) provided evidence for the coexistence of anammox bacteria and sulfide-dependent denitrifiers in the stratified water column of Lake Lugano, and reported that the addition of sulfide in incubation studies enhanced both processes. They speculated that anammox bacteria in this system would rely on nitrite released as intermediate during sulfide-dependent denitrification and that they would overcome inhibiting or toxic effects of sulfide by creating a sulfide-free microenvironments in aggregates as previously proposed by Wenk et al (2013). However, in the case of Lake Grevelingen sediments the potential detoxification of sulfide and a source of nitrite by sulfide-dependent denitrifiers did not translate in significant anammox potential. Another explanation for the low denitrification potential of these sediments can be found in the interactions of anammox and heterotrophic denitrifiers with sulfur oxidizers also involved in the nitrogen

cycle. For example in Lake Grevelingen, cable bacteria have been detected in stations S1 and S3 in March, whereas station S2 was dominated by Beggiatoaceae down to ~3 cm, but both groups were hardly detectable in subsurface sediments (0.5 cm downwards) during summer hypoxia (Seitaj et al., 2015; Seitaj, unpublished data). In fact, the analysis of *aprA* gene of SOB/SRB in our study showed that up to 36% of the *aprA* gene sequences were closely related to the *aprA* sequences of *Desulfobulbus propionicus* (Fig. 4), which has been identified as closest known relative of cable bacteria (Pfeffer et al., 2012). Cable bacteria perform a novel 'electrogenic' form of sulfur oxidation, in which the oxidation of the electron donor and the reduction of the electron acceptor are separated over centimeter-scale distances, and the necessary redox coupling is ensured by long-distance electron transport (Nielsen et al., 2010; Pfeffer et al., 2012). Cable bacteria use oxygen as terminal electron acceptor (Nielsen et al., 2010; Meysman et al., 2015) and recent studies also indicate that nitrate (Marzocchi et al., 2014) and nitrite (Risgaard-Petersen et al., 2014) can be utilized, suggesting that cable bacteria can also play a role in bioavailable nitrogen removal. In addition, marine *Beggiatoa* spp. couple the oxidation of sulfide to nitrate reduction resulting in N_2 and/or NH_4^+ (Mußmann et al., 2003). Previous studies have observed that both denitrification and anammox in anoxic sediments can be supported by intracellular nitrate transport performed by sulfide-oxidizing bacteria like *Thioploca* and *Beggiatoa* (Prokopenko et al., 2013; Mußmann et al., 2007; Jørgensen et al., 2010) down to deeper sediment layers and possibly supplying anammox bacteria with nitrite and/or ammonia produced by DNRA (Teske and Nelson, 2006, Prokopenko et al., 2011).The *nrfA* gene, encoding a nitrite reductase catalyzing the conversion of nitrite to ammonia (Smith et al., 2007), could not be detected in our sediment samples (data not shown) by using general *nrfA* primers (Mohan et al., 2004), however we cannot rule out completely the presence of microorganisms performing DNRA as the primers used could be not the most appropriate ones for this system. Therefore, the presence of sulfide-dependent denitrifiers (such as *Thiobacillus denitrificans*), cable bacteria and *Beggiatoa*-like bacteria present in Lake Grevelingen sediments could potentially support denitrification and anammox processes in March. However, the nitrate and nitrite concentrations in the bottom water in March were reported to be relatively low (28 and 0.7 μM on average, respectively, Fig. S1), which is expected to induce a strong competition for nitrate, nitrite and sulfide with Beggiatoaceae, cable bacteria and other nitrate-reducing bacteria (e.g. *Thiobacillus thioparus*), explaining the limited denitrification potential observed in the Lake Grevelingen sediments.

4.5. Conclusion

Our study has unraveled the coexistence and potential activity of heterotrophic and autotrophic denitrifiers, anammox bacteria as well as SOB/SRB in seasonally hypoxic and sulfidic sediments of Lake Grevelingen. Our starting hypothesis was that the abundance and activity of denitrifiers and anammox bacteria would decrease upon increase of the sulfide concentration found in the sediments during the summer hypoxia. However, *nirS*-type heterotrophic denitrifiers were a stable community regardless of changes in oxygen and sulfide concentrations in different seasons and with a similar abundance to that detected in other sediments not exposed to high sulfide concentrations.

Besides, *nirS*-type denitrifiers outnumbered anammox bacteria leading to the conclusion that anammox does not contribute significantly to the N_2 removal process in Lake Grevelingen sediments. Apart from that, the anammox bacteria population seemed to be affected by the phys-

icochemical changes between seasons. For example, their abundance and activity was higher in lower sulfide concentrations and low carbon input, also supporting a possible inhibition of anammox bacteria by sulfide. The sulfide-dependent denitrifiers in Lake Grevelingen sediments have proven to be abundant and expected to contribute significantly to the N_2-removal in these sediments. Their activity of sulfide oxidation is intuitively expected to reduce the concentration of sulfide, which is in turn toxic for organoheterotrophic denitrifiers and anammox bacteria. However, in this system the detoxification mediated by sulfide-dependent denitrifiers is either not sufficient or other factors are contributing to the low denitrification potential observed in the sediments of Lake Grevelingen.

Recent studies have also reported the presence of cable bacteria and sulfide-oxidizers of the Beggiatoaceae family in the Lake Grevelingen sediments. Both denitrifiers and anammox bacteria activity could be inhibited by the competition with cable bacteria and Beggiatoaceae for electron donors and acceptors (such as sulfide, nitrate and nitrite) before summer hypoxia (March). During summer hypoxia (August), sulfide inhibition and low nitrate and nitrite concentrations seem to limit the activity of heterotrophic and autotrophic (sulfide-dependent) denitrifiers and anammox bacteria. Further studies also involving denitrification rate determinations will be required to further assess the effects of hypoxia and high sulfide concentrations in the sediments of Lake Grevelingen.

Acknowledgements

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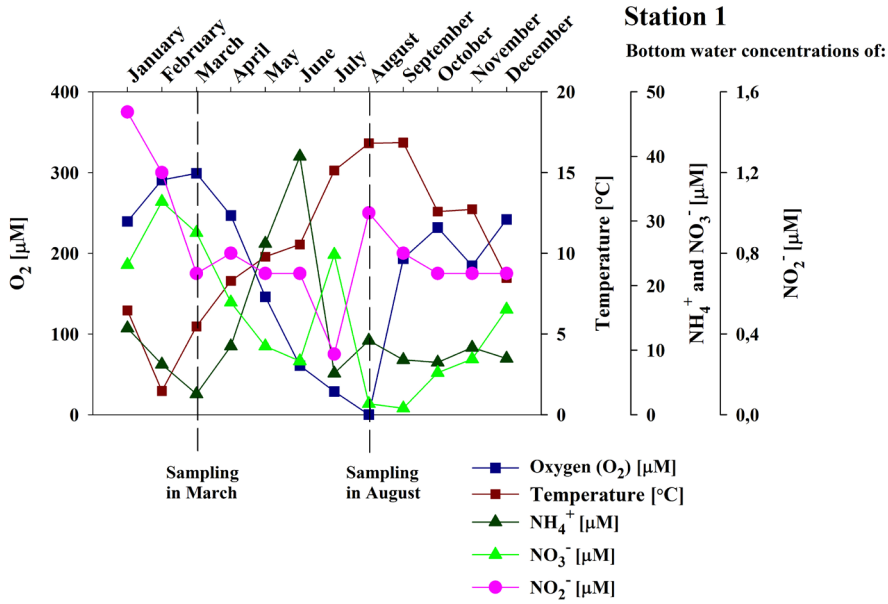

Figure S1: Bottom water temperature [°C] (red square) and concentrations of oxygen [µM] (blue square), ammonium [µM] (dark green triangle), nitrite [µM] (pink triangle) and nitrate [µM] (light green triangle).

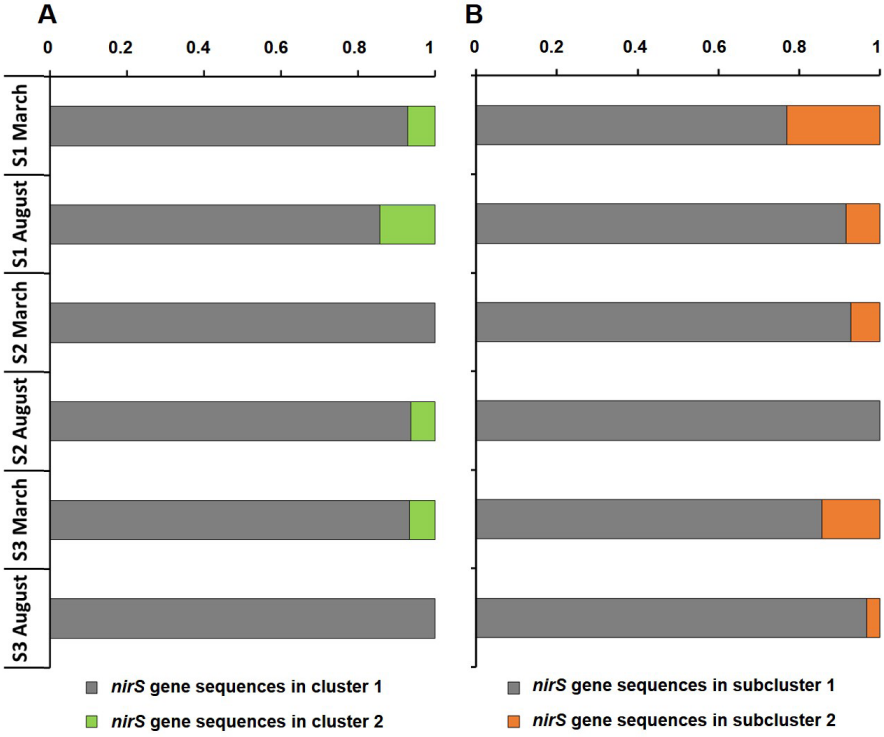

Figure S2: A) Relative percentage of *nirS* sequences of denitrifying bacteria in cluster 1 and 2 and B) in subcluster 1.1 and 1.2 according to the three stations in March and August.

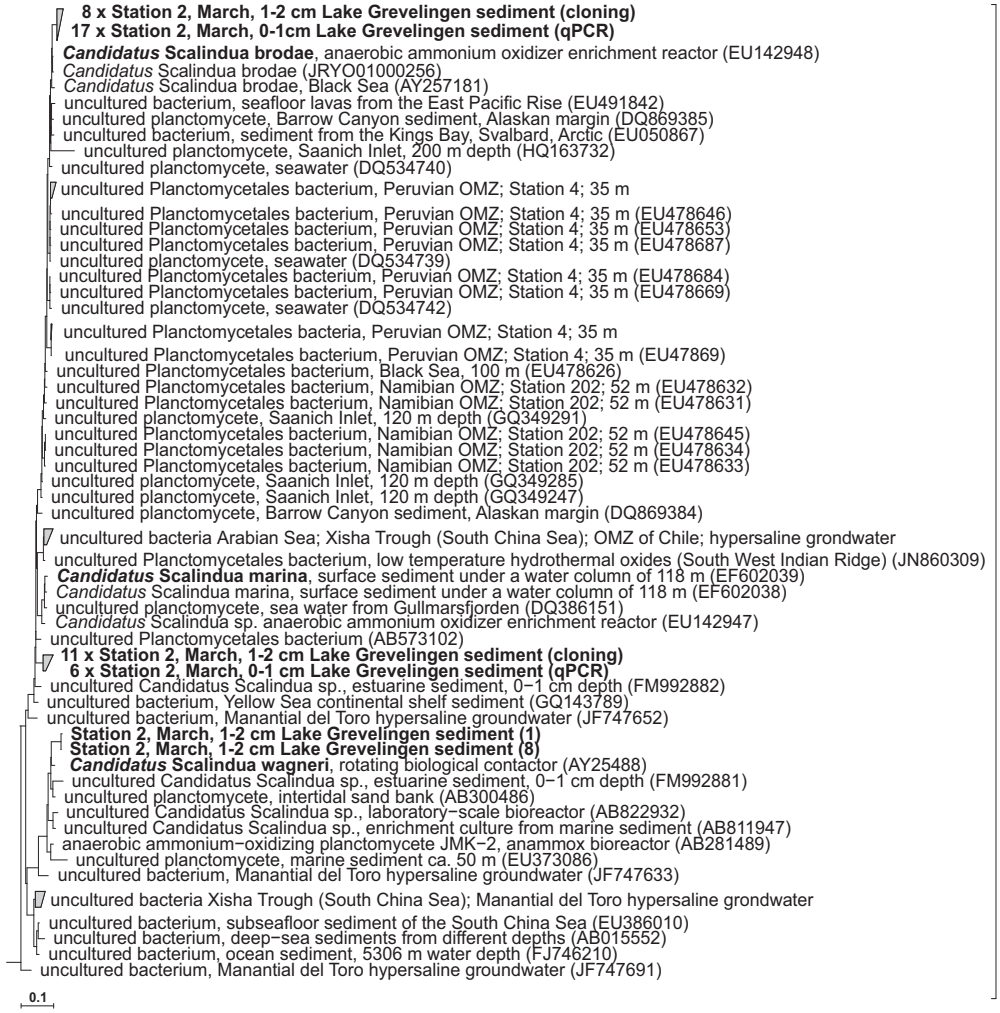

Figure S3: Phylogenetic tree of anammox bacteria partial 16S rRNA gene sequences retrieved in this study (21 DNA sequences recovered by amplification (PCR) and cloning and 21 DNA sequences recovered by amplification (qPCR) and cloning from sediments (0–1 cm) of station S2 in March) and closest relatives (bold: our sequences and closest known relatives); the scale bar indicates 10% sequence divergence.

Table S1: Primer pairs described in the text, PCR conditions and amplicon size used in this study.

Assay	Target	Primer pair	Tm [°C]	Amplicon size [b]	Ref.
qPCR + PCR/ cloning	Anammox bacteria 16S rRNA gene	Brod541F (5'-GAGCACGTAGGTGGGTTTGT-3') Amx820R (5'-AAAACCCCTCTACTTAGTGCCC-3')	qPCR 59/ PCR 58	279	Li et al., 2010
qPCR + PCR/ cloning	*Scalindua* sp. *nirS* gene	Scnir372F (5'-TGTAGCCAGCATTGTAGCGT-3') Scnir845R (5'-TCAAGCCAGACCCATTTGCT-3')	qPCR/ PCR 57	474	Lam et al., 2009
qPCR + PCR/ cloning	Denitrifying bacteria *nirS* gene	nirS1F (5'-CCTAYTGGCCGCCRCART-3') nirS3R (5'-GCCGCCGTCRTGVAGGAA-3')	qPCR/ PCR 60	222	Braker et al., 1998
qPCR+ cloning	Sulfide oxidizing bacteria *aprA* gene	Apr1F (5'TGGCAGATCATGATYMAYGG-3') Apr5R (5'-GCGCCAACYGGRCCRTA-3')	qPCR/ PCR 56	359	Meyer & Kuever, 2007

PCR conditions: 95°C 5 min; 40× [95°C 1 min, Tm 40 s, 72°C 1 min]; 72°C 5 min. qPCR conditions: 95°C 4 min; 40× [95°C 30 s, Tm 40 s, 72°C 30 s]; 80°C 25 s.

Table S2: Total organic carbon (TOC) after acidification of freeze dried sediment.

Station	Sediment depth [cm]	TOC [%]	
		March	August
1	0–1	2.86	1.81
	1–2	3.67	2.47
	2–3	3.83	3.52
	3–4	3.98	3.11
	4–5	4.28	3.66
2	0–1	3.11	2.38
	1–2	3.62	3.29
	2–3	3.77	3.24
	3–4	3.49	3.5
	4–5	3.85	3.52
3	0–1	2.87	3.04
	1–2	4.02	3.9
	2–3	3.89	3.56
	3–4	4.01	4.39
	4–5	3.63	3.73

Table S3: Transcript (cDNA; mRNA copy) abundance detected in sediments of all stations in March and August.

Station	Sediment depth [cm]	*nirS* gene denitrifying bacteria cDNA [copy g^{-1}]		16S rRNA gene anammox bacteria cDNA [copy g^{-1}]		*aprA* gene SOB and SRB cDNA [copy g^{-1}]	
		March	August	March	August	March	August
1	0–1	1.8×10^2	n.d.	1.9×10^5	8.1×10^5	n.d.	3.8×10^3
	1–2	1.1×10^4	n.d.	1.8×10^5	1.3×10^3	n.d.	n.d.
	2–3	n.d.	1.0×10^2	1.0×10^5	2.8×10^5	5.4×10^2	2.5×10^4
	3–4	n.d.	n.d.	6.5×10^4	6.9×10^5	n.d.	1.1×10^4
	4–5	n.d.	n.d.	2.5×10^4	2.3×10^5	n.d.	n.d.
2	0–1	1.9×10^2	n.d.	8.5×10^4	3.0×10^5	n.d.	n.d.
	1–2	1.5×10^3	n.d.	6.0×10^5	1.1×10^6	7.0×10^3	7.4×10^2
	2–3	6.7×10^2	n.d.	9.6×10^5	1.7×10^6	1.4×10^4	3.4×10^3
	3–4	n.d.	n.d.	1.9×10^5	1.3×10^5	1.6×10^3	5.9×10^2
	4–5	n.d.	n.d.	4.8×10^4	5.5×10^4	n.d.	n.d.
3	0–1	1.5×10^2	n.d.	4.9×10^6	2.9×10^5	n.d.	n.d.
	1–2	n.d.	1.1×10^2	3.1×10^6	5.1×10^6	3.5×10^3	1.8×10^4
	2–3	n.d.	n.d.	4.1×10^5	3.1×10^6	5.0×10^2	4.3×10^2
	3–4	n.d.	1.8×10^3	2.2×10^5	1.3×10^5	1.4×10^3	3.7×10^3
	4–5	n.d.	n.d.	8.5×10^4	3.7×10^4	n.d.	n.d.

qPCR conditions: 95°C 4 min; 40 × [95°C 30 s, Tm 40 s, 72°C 30 s]; 80°C 25 s. nd: non detectable, i.e. values were under the detection limit of the qPCR approach.

Table S4: Results of anammox bacteria PC-monoether ladderane lipid analysis at all stations in March and August (0−5 cm sediment depth).

		PC-monoether ladderane lipid [ng g^{-1}]	
Station	**Sediment depth [cm]**	**March**	**August**
1	0–1	20.4	2.4
	1–2	13.3	3.4
	2–3	6.1	2.8
	3–4	3.4	7
	4–5	5	7
2	0–1	10.8	6.2
	1–2	4.7	1.9
	2–3	2	1.9
	3–4	1	1.4
	4–5	1	1.4
3	0–1	8.6	2.4
	1–2	6.1	1.2
	2–3	1.6	1.2
	3–4	1.1	0.9
	4–5	1.4	1.4

Table S5: Results of anammox bacteria FAME analysis of all stations in March and August (0–1 and 4–5 cm sediment depth).

			long chain FAMEs [ng g^{-1}]				sum
Station	**month**	**depth**	**C_{20}-[3]**	**C_{20}-[5]**	**C_{18}-[3]**	**C_{18}-[5]**	**C_{20}-[3]–C_{18}-[5]**
Station 1	March	0.5	3.6	3.8	4.0	5.5	16.8
	March	4.5	3.5	5.4	2.0	5.0	15.8
	August	0.5	2.5	1.5	5.1	3.9	13.1
	August	4.5	1.8	1.8	1.4	2.8	7.8
Station 2	March	0.5	6.1	4.7	6.9	7.8	25.4
	March	4.5	4.8	9.1	4.4	4.4	22.7
	August	0.5	5.3	5.7	19.3	8.4	38.7
	August	4.5	3.1	8.2	4.4	4.1	19.8
Station 3	March	0.5	18.9	16.3	32.1	39.3	106.5
	March	4.5	3.6	7.9	4.0	4.0	19.5
	August	0.5	4.7	4.1	8.9	9.7	27.4
	August	4.5	2.8	3.2	1.6	3.0	10.6

5. Impact of seasonal hypoxia on activity and community structure of chemolithoautotrophic bacteria in a coastal sediment

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Seasonal hypoxia in coastal systems drastically changes the availability of electron acceptors in bottom water, which alters the sedimentary reoxidation of reduced compounds. However, the effect of seasonal hypoxia on the chemolithoautotrophic community that catalyze these reoxidation reactions, is rarely studied. Here we examine the changes in activity and structure of the sedimentary chemolithoautotrophic bacterial community of a seasonally hypoxic saline basin under oxic (spring) and hypoxic (summer) conditions. Combined 16S rRNA gene amplicon sequencing and analysis of phospholipid derived fatty acids indicated a major temporal shift in community structure. Aerobic sulfur-oxidizing Gammaproteobacteria (Thiotrichales) and Epsilonproteobacteria (Campylobacterales) were prevalent during spring, whereas Deltaproteobacteria (Desulfobacterales) related to sulfate reducing bacteria prevailed during summer hypoxia. Chemolithoautotrophy rates in the surface sediment were three times higher in spring compared to summer. The depth distribution of chemolithoautotrophy was linked to the distinct sulfur oxidation mechanisms identified through microsensor profiling, i.e., canonical sulfur oxidation, electrogenic sulfur oxidation by cable bacteria, and sulfide oxidation coupled to nitrate reduction by Beggiatoaceae. The metabolic diversity of the sulfur-oxidizing bacterial community suggests a complex niche partitioning within the sediment probably driven by the availability of reduced sulfur compounds (H_2S, S^0, $S_2O_3^{2-}$) and electron acceptors (O_2, NO_3^-) regulated by seasonal hypoxia.

Chemolithoautotrophic microbes in the seafloor are dependent on electron acceptors like oxygen and nitrate that diffuse from the overlying water. Seasonal hypoxia however drastically changes the availability of these electron acceptors in the bottom water, and hence, one expects a strong impact of seasonal hypoxia on sedimentary chemolithoautotrophy. A multidisciplinary investigation of the sediments in a seasonally hypoxic coastal basin confirms this hypothesis. Our data show that bacterial community structure and the chemolithoautotrophic activity varied with the seasonal depletion of oxygen. Unexpectedly, the dark carbon fixation was also dependent on the dominant microbial pathway of sulfur oxidation occurring in the sediment (i.e., canonical sulfur oxidation, electrogenic sulfur oxidation by cable bacteria, and sulfide oxidation coupled to nitrate reduction by Beggiatoaceae). These results suggest that a complex niche partitioning within the sulfur-oxidizing bacterial community additionally affects the chemolithoautotrophic community of seasonally hypoxic sediments.

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5.1. Introduction

The reoxidation of reduced intermediates formed during anaerobic mineralization of organic matter is a key process in the biogeochemistry of coastal sediments (Soetaert et al., 1996, Jørgensen and Nelson, 2004). Many of the microorganisms involved in the reoxidation of reduced compounds are chemolithoautotrophs, which fix inorganic carbon using the chemical energy derived from reoxidation reactions (dark CO_2 fixation). In coastal sediments, sulfate reduction forms the main respiration pathway, accounting for 50% to 90% of the organic matter mineralization (Soetaert et al., 1996). The reoxidation of the pool of reduced sulfur compounds produced during anaerobic mineralization (dissolved free sulfide, thiosulfate, elemental sulfur, iron monosulfides and pyrite) hence forms the most important pathway sustaining chemolithoautotrophy in coastal sediments (Jørgensen and Nelson, 2004, Howarth, 1984).

Various lineages from the Alpha-, Gamma-, Delta- and Epsilonproteobacteria, including recently identified groups, such as particle-associated Gammaproteobacteria and large sulfur bacteria, couple dark CO_2 fixation to the oxidation of reduced sulfur compounds in oxygen deficient marine waters and sediments, in coastal marine sediments and in lake sediments (Brüchert et al., 2003; Lavik et al., 2009; Sorokin et al., 2011; Grote et al., 2012; Jessen et al., 2016; Ye et al., 2016). Both chemolithoautotrophic sulfur-oxidizing Gammaproteobacteria and sulfur disproportionating Deltaproteobacteria have been identified to play a major role in the sulfur and carbon cycling in diverse intertidal sediments (Lenk et al., 2010; Boschker et al., 2014; Dyksma et al., 2016). Hence, chemolithoautotrophic sulfur-oxidizing communities vary between sediment environments, but it is presently not clear as to which environmental factors are actually determining the chemolithoautotrophic community composition at a given site.

Seasonal hypoxia is a natural phenomenon that occurs in coastal areas around the world (Diaz and Rosenberg, 2008) and provides an opportunity to study the environmental factors controlling sedimentary chemolithoautotrophy. Hypoxia occurs when bottom waters become depleted of oxygen (< 63 μmol O_2 L^{-1}), and has a large impact on the biogeochemical cycling and ecological functioning of the underlying sediments (Diaz and Rosenberg, 2008). The reduced availability or even absence of suitable soluble electron acceptors (O_2, NO_3^-) in the bottom water during part of the year should in principle result in a reduction or complete inhibition of sedimentary sulfur reoxidation, and hence, limit chemolithoautotrophy. At present, the prevalence and temporal variations of chemolithoautotrophy have not been investigated in coastal sediments of seasonal hypoxic basins.

Likely, the availability of soluble electron acceptors (O_2, NO_3^-) in the bottom water is not the only determining factor of chemolithoautotrophy. A recent study conducted in a seasonally hypoxic saline basin (Lake Grevelingen, The Netherlands) indicated that the intrinsic structure and composition of the sulfur oxidizing microbial community also determined the biogeochemistry of the sediment (Seitaj et al., 2015). In this study, three distinct microbial sulfur oxidation mechanisms were observed throughout a seasonal cycle: (1) electrogenic sulfur oxidation by heterotrophic cable bacteria (Desulfobulbaceae); (2) canonical aerobic oxidation of free sulfide at the oxygen-sulfide interface and, (3) sulfide oxidation coupled to nitrate reduction by filamentous members of the Beggiatoaceae family that store nitrate intracellularly. The consequences of these three mechanisms on the chemolithoautotrophic community have however not been studied. The first sulfur oxidizing mechanism has been shown to affect the chemolithoautotrophic community in sediments only under laboratory conditions (Vasquez-Cardenas et al.,

2015) while the third mechanisms may directly involve chemolithoautotrophic Beggiatoaceae as some species are known to grow autotrophically (Nelson and Jannasch, 1983). Accordingly, we hypothesize that the presence of these sulfur oxidation regimes as well as the depletion of O_2 and NO_3^- will result in a strong seasonality in both the chemolithoautotrophic activity and community structure under natural conditions.

To examine the above-mentioned hypothesis, we conducted a multidisciplinary study with intact sediments of Lake Grevelingen, involving both geochemistry and microbiology. Field sampling was conducted during spring (oxygenated bottom waters) and summer (oxygen depleted bottom waters). The dominant sulfur oxidation mechanism was geochemically characterized by sediment microsensor profiling (O_2, H_2S, pH) whereas the abundance of cable bacteria and Beggiatoaceae was performed with fluorescence *in situ* hybridization (FISH). General bacterial diversity was assessed by 16S rRNA gene amplicon sequencing and the analysis of phospholipid derived fatty acids (PLFA). PLFA analysis combined with ^{13}C stable isotope probing (PLFA-SIP) provided the activity and community composition of chemolithoautotrophs in the sediment. This approach was complemented by the analysis of genes involved in dark CO_2 fixation, *i.e.,* characterizing diversity and the abundance of the genes *cbbL* and *aclB* that code for key enzymes in Calvin-Benson Bassham (CBB) and reductive tricarboxylic acid (rTCA) carbon fixation pathways. This multidisciplinary research showed strong temporal and spatial shifts of the chemolithoautotrophic composition and activity in relation to the seasonal hypoxia and the main sulfur oxidation mechanisms present in the sediments of the marine Lake Grevelingen.

5.2. Material and methods

5.2.1. Study site and sediment sampling

Lake Grevelingen is a former estuary located within the Rhine-Meuse-Scheldt delta area of the Netherlands, which became a closed saline reservoir (salinity ~30) by dam construction at both the land side and sea side in the early 1970s. Due to an absence of tides and strong currents, Lake Grevelingen experiences a seasonal stratification of the water column, which in turn, leads to a gradual depletion of the oxygen in the bottom waters (Hagens et al., 2015). Bottom water oxygen at the deepest stations typically starts to decline in April, reaches hypoxic conditions by end of May (O_2 < 63 μM), further decreasing to anoxia in August (O_2 <0.1 μM), while the re-oxygenation of the bottom water takes place in September (Seitaj et al., 2015).

To study the effects of the bottom water oxygenation on the benthic chemolithoautotrophic community we performed a field sampling campaign on March 13th, 2012 (before the start of the annual O_2 depletion) and August 20th, 2012 (at the height of the annual O_2 depletion). Detailed water column, pore water and solid sediment chemistry of Lake Grevelingen over the year 2012 have been previously reported (Seitaj et al., 2015; Hagens et al., 2015; Sulu-Gambari et al., 2016). Sediments were recovered at three stations along a depth gradient within the Den Osse basin, one of the deeper basins in Marine Lake Grevelingen: Station 1 (S1) was located in the deepest point (34 m) of the basin (51.747°N, 3.890°E), Station 2 (S2) at 23 m depth (51.749°N, 3.897°E) and Station 3 (S3) at 17 m depth (51.747°N, 3.898°E). Intact sediment cores were retrieved with a single core gravity corer (UWITEC) using PVC core liners (6 cm inner diameter, 60 cm length). All cores were inspected upon retrieval and only cores with a visually undisturbed surface were used for further analysis.

Thirteen sediment cores for microbial analysis were collected per station and per time point: two cores for phospholipid-derived fatty acid analysis combined with stable isotope probing (PLFA-SIP), two cores for nucleic acid analysis, four cores for ^{13}C-bicarbonate labeling, three cores for microelectrode profiling, one core for quantification of cable bacteria (see supplement), and one core for quantification of Beggiatoaceae (see supplement). Sediment cores for PLFA extractions were sliced manually on board the ship (5 sediment layers; sectioning at 0.5, 1, 2, 4, and 6 cm depth). Sediment slices were collected in Petri dishes, and replicate depths were pooled and thoroughly mixed. Homogenized sediments were immediately transferred to centrifuge tubes (50 mL) and placed in dry ice until further analysis. Surface sediments in August consisted of a highly porous "fluffy" layer that was first left to settle after core retrieval. Afterwards, the top 1 cm thick layer was recovered through suction (rather than slicing). Sediment for nucleic acid analysis was collected by slicing manually at a resolution of 1 cm up to 5 cm depth. Sediment samples were frozen in dry ice, transported to the laboratory within a few hour and placed at −80°C until further analysis.

5.2.2. Microsensor profiling

Oxygen depth profiles were recorded with commercial microelectrodes (Unisense, Denmark; tip size: 50 μm) at 25−50 μm resolution. For H_2S and pH (tip size: 50 and 200 μm), depth profiles were recorded at 200 μm resolution in the oxic zone, and at 400 or 600 μm depth resolution below. Calibrations for O_2, pH and H_2S were performed as previously described (Seitaj et al., 2015; Malkin et al., 2014). ΣH_2S was calculated from H_2S based on pH measured at the same depth using the R package AquaEnv (Hofmann et al., 2010). The oxygen penetration depth (OPD) is operationally defined as the depth below which $[O_2] < 1$ μM, while the sulfide appearance depth (SAD) is operationally defined as the depth below which $[H_2S] > 1$ μM. The diffusive oxygen uptake (DOU) was calculated from the oxygen depth profiles as previously described in detail (Vasquez-Cardenas et al., 2015).

5.2.3. DNA extraction and 16S rRNA gene amplicon sequencing

DNA from 0 cm to 5 cm sediment depth (in 1 cm resolution) was extracted using the DNA PowerSoil® Total Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA). Nucleic acid concentrations were quantified spectrophotometrically (Nanodrop, Thermo Scientific, Wilmington, DE) and checked by agarose gel electrophoresis for integrity. DNA extracts were kept frozen at –80°C.

Sequencing of 16S rRNA gene amplicons was performed on the first cm of the sediment (0–1 cm depth) in all stations in March and August as described before (Moore et al., 2015). Further details are provided in the SI. The 16S rRNA gene amplicon reads (raw data) have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject number PRJNA293286. The phylogenetic affiliation of the 16S rRNA gene sequences was compared to release 119 of the SILVA NR SSU Ref database (http://www. arb-silva.de/; Quast et al., 2013) using the ARB software package (Ludwig et al., 2004). Sequences were added to a reference tree generated from the Silva database using the ARB Parsimony tool.

5.2.4. Sediment incubations and PLFA-SIP analysis

Sediment cores were labeled with ^{13}C-bicarbonate to determine the chemolithoautotrophic activity and associated bacterial community by tracing the incorporation of ^{13}C into bacterial PLFA.

To this end, four intact cores were sub-cored with smaller core liners (4.5 cm inner diameter; 20 cm height). *In situ* bottom water was kept over the sediment and no gas headspace was present. Cores were kept inside a closed cooling box during transport to the laboratory.

Stock solutions of 80 mM of ^{13}C-bicarbonate (99% ^{13}C; Cambridge Isotope Laboratories, Andover, Ma, USA) were prepared as previously described (Boschker et al., 2014). ^{13}C-bicarbonate was added to the sediment from 3 cm above the surface to 8 cm deep in the sediment cores in aliquots of 100 µL through vertically aligned side ports (0.5 cm apart) with the line injection method (Jørgensen, 1978). March sediments were incubated at 17±1°C in the dark for 24 h and continuously aerated with ^{13}C-saturated air to maintain 100% saturated O_2 conditions as those found *in situ*, but avoiding the stripping of labeled CO_2 from the overlying water (Vasquez-Cardenas et al., 2015). In August, sediments were incubated at 17±1°C in the dark for 40 h to ensure sufficient labeling as a lower activity was expected under low oxygen concentrations. In August, oxygen concentrations in the overlying water were maintained near *in situ* O_2 levels measured in the bottom water (S1: 0−4% saturation, S2: 20−26%, S3: 35−80%). A detailed description of aeriation procedures can be found in the SI.

At the end of the incubation period, sediment cores were sliced in five depth intervals (as described for PLFA sediment cores sliced on board) thus obtaining two replicate slices per sediment depth and per station. Sediment layers were collected in centrifuge tubes (50 mL) and wet volume and weight were noted. Pore water was obtained by centrifugation (4500 rpm for 5 min) for dissolved inorganic carbon (DIC) analysis, and sediments were lyophilized for PLFA analysis. Biomarker extractions were performed on freeze dried sediment as described before (Guckert et al., 1985) and ^{13}C-incorporation into PLFA was analyzed as previously reported (Boschker et al., 2014). Nomenclature of PLFA can be found in the SI. Detailed description of the PLFA calculations can be found in the literature (Vasquez-Cardenas et al., 2015; Boschker and Middelburg, 2002). Total dark CO_2 fixation rates (mmol C m^{-2} d^{-1}) are the depth-integrated rates obtained from the 0−6 cm sediment interval.

5.2.5. Quantitative PCR

To determine the abundance of chemolithoautotrophs we quantified the genes coding for two enzymes involved in dark CO_2 fixation pathways: the large subunit of the RubisCO enzyme (ribulose 1,5-bisphosphate carboxylase/oxygenase) *cbbL* gene, and the ATP citrate lyase *aclB* gene. Abundance of the RubisCO *cbbL* gene was estimated by using primers K2f/V2r, specific for the forms IA and IC of the RuBisCO form I large subunit gene *cbbL* which is present in obligately and facultatively lithotrophic bacteria (Nanba et al., 2004; Nigro and King, 2007). The abundance of ATP citrate lyase *aclB* gene was quantified by using the primer set aclB_F/aclB_R, which is based on the primers 892F/1204R (Campbell et al., 2003), specific for the ATP citrate lyase β gene of chemoautotrophic bacteria using the rTCA pathway, with several nucleotide differences introduced after aligning n=100 sequences of *aclB* gene fragments affiliated to Epsilonproteobacteria (See Table S1 for details).

The quantification of *cbbL* and *aclB* genes via quantitative PCR (qPCR) was performed in 1 cm resolution for the sediment interval between 0–5 cm depth in all stations in both March and August. qPCR analyses were performed on a Biorad CFX96™ Real-Time System/C1000 Thermal cycler equipped with CFX Manager™ Software. All qPCR reactions were performed in triplicate with standard curves from 10^0 to 10^7 molecules per microliter. Standard curves and

qPCR reactions were performed as previously described (Chapter 2). Melting temperatures (Tm) are listed in Table S1, qPCR efficiencies (*E*) for *aclB* gene and *cbbL* gene amplifications were 70 and 82%, respectively. Correlation coefficients for standard curves were ≥ 0.994 for *aclB* gene and ≥ 0.988 for *cbbL* gene amplification.

5.2.6. PCR amplification and cloning

Amplifications of the RubisCO *cbbL* gene and the ATP citrate lyase *aclB* gene were performed with the primer pairs specified in Table S1. The PCR reaction mixture was the following (final concentration): Q-solution (PCR additive, Qiagen, Valencia, CA) 1 ×; PCR buffer 1 ×; BSA (200 μg mL^{-1}); dNTPs (20 μM); primers (0.2 pmol μL^{-1}); $MgCl_2$ (1.5 mM); 1.25 U Taq polymerase (Qiagen, Valencia, CA). PCR conditions for these amplifications were the following: 95°C, 5 min; 35 × [95°C, 1 min; Tm, 1 min; 72°C, 1 min]; final extension 72°C, 5 min. PCR products were gel purified (QIAquick gel purification kit, Qiagen, Valencia, CA) and cloned in the TOPO-TA cloning® kit (Life Technologies, Carlsbad, CA) and transformed in *E. coli* TOP10 cells following the manufacturer's recommendations. Recombinant plasmid DNA was sequenced using M13R primer by BASECLEAR (Leiden, The Netherlands).

Sequences were aligned with MEGA5 software (Tamura et al., 2011) by using the alignment method ClustalW. The phylogenetic trees of the *cbbL* and *aclB* genes were computed with the Neighbour-Joining method (Saitou and Nei, 1987). The evolutionary distances were estimated using the Jukes-Cantor method (Jukes and Cantor, 1969) for DNA sequences and with the Poisson correction method for protein sequences (Zuckerkandl and Pauling, 1965) with a bootstrap test of 1,000 replicates. Sequences were deposited in NCBI with the following accession numbers: KT328918–KT328956 for *cbbL* gene sequences and KT328957–KT329097 for *aclB* gene sequences. Further details on experimental procedures and methods are found in the Supplementary Information.

5.3. Results

5.3.1. Geochemical characterization

The seasonal variation of the bottom water oxygen concentration in Lake Grevelingen strongly influenced the porewater concentrations of O_2 and H_2S. In March, bottom waters were fully oxygenated at all stations (299–307 μM), oxygen penetrated 1.8–2.6 mm deep in the sediment, and no free sulfide was recorded in the first few centimeters (Table 1). The width of the suboxic zone, operationally defined as the sediment layer located between the oxygen penetration depth (OPD) and the sulfide appearance depth (SAD), varied between 16–39 mm across the three stations in March 2012. In contrast, in August, oxygen was strongly depleted in the bottom waters at S1 (< 0.1 μM) and S2 (11 μM), and no O_2 could be confidently detected by microsensor profiling in the surface sediment at these two stations. At station S3, the bottom water O_2 remained higher (88 μM), and oxygen penetrated down to 1.1 mm. In August, free sulfide was present near the sediment-water interface at all three stations, and the accumulation of sulfide in the pore water increased with water depth (Fig. 1a). Depth pH profiles showed much larger variation between stations in March compared to August (Fig. 1a). The pH profiles in S1 and S3 in March were similarly characterized by highest values in the oxic zone and low pH values (pH < 6.5) in the suboxic zone. The pH depth profile in S2 showed an inverse pH profile with a pH minimum in the oxic layer and a subsurface maximum below. The pH profiles at S1 and S3

in August 2012 showed a gradual decline of pH with depth, while the pH profile at S2 in August was more or less constant with depth.

5.3.2. Bacterial diversity by 16S rRNA gene amplicon sequencing

The general diversity of bacteria was assessed by 16S rRNA gene amplicon sequencing analysis, which was applied to the surface sediments (0−1 cm) of all stations in both March and August. Approximately 50% of the reads were assigned to three main clades: Gamma, Delta-, and Epsilonproteobacteria (Fig. 2). The remaining reads were distributed amongst the orders Bacteroidetes (14%), Planctomycetes (6%), Alphaproteobacteria (3%), other orders (20%), the candidate phylum WS3 (2%) and unassigned (5%) (given as the average of the three stations and both seasons).

Reads classified within the Gammaproteobacteria were more abundant in March during oxygenated bottom water conditions than in August (Fig. 2). The majority of these reads were assigned to the orders Alteromonadales, Chromatiales and Thiotrichales. The first order includes chemoheterotrophic bacteria that are either strict aerobes or facultative anaerobes (Bowman and McMeekin, 2005). Phylogenetic comparison revealed that the reads assigned to the Chromatiales group were closely related to the Granulosicoccaceae, Ectothiorhodospiraceae, and Chromatiaceae families (Fig. S1). Reads falling in the Thiotrichales group were closely related to sulfur-oxidizing bacteria from the Thiotrichaceae family with 30% of the sequences related to the genera "*Candidatus* Isobeggiatoa", "*Ca.* Parabeggiatoa" and *Thiomargarita* (Fig. S2). It has been recently proposed that the genera *Beggiatoa*, *Thiomargarita* and *Thioploca* should be reclassified into the originally published monophyletic family of Beggiatoaceae (Salman et al., 2011, 2013), and so here, these genera will be further referred to as Beggiatoaceae. Most of the reads assigned to the Beggiatoaceae came from station S2 in spring, whereas the percentage of reads assigned to sulfur oxidizers from the order Thiotrichales decreased in August when oxygen concentrations in the bottom water were low.

Within the Deltaproteobacteria, reads were assigned to the orders Desulfarculales and Desulfobacterales (Fig. S3). Reads within the order of Desulfobacterales were mainly assigned to the families Desulfobacteraceae (between 10−20%) and Desulfobulbaceae (~5%) (Fig. 2). Additional phylogenetic comparison revealed that within the Desulfobulbaceae, 60% of reads, obtained from the three stations in both seasons, clustered within the genus *Desulfobulbus*, of which 30% were related to the electrogenic sulfur-oxidizing cable bacteria (Fig. S4). Overall, the relative abundance of reads assigned to the Desulfobulbaceae family was similar between the two seasons in S2 and S3, whereas in S1 the percentage of reads was approximately 4.5-fold higher in March compared to August (Fig. 2). In contrast, the relative abundance of reads assigned to the Desulfarculales and Desulfobacteraceae increased in August during hypoxia (Fig. 2), and phylogenetic comparison revealed typical sulfate reducer genera *Desulfococcus, Desulfosarcina*, and *Desulfobacterium*, indicative of anaerobic metabolism (Figs. S5a–c).

All Epsilonproteobacteria reads were assigned to the order Campylobacterales, such as the Campylobacteraceae and Helicobacteraceae families. Phylogenetic comparison showed that the reads were closely related to the genera *Sulfurovum*, *Sulfurimonas*, *Sulfurospirillum*, *Arcobacter* (Figs. S6a–d), all capable of sulfur oxidation with oxygen or nitrate (Campbell et al., 2006). The percentage of reads assigned to the Epsilonproteobacteria in August was lower than those in March, with highest numbers present in S2 in March (~6%), (Fig. 2).

Table 1: Geochemical characterization, chemoautotrophy rates, and quantification of cable bacteria and Beggiatoaceae in the three stations in Lake Grevelingen for spring (March) and summer (August).

Month/ Station		°C	Bottom water * conc. [µM] O_2		NO_3^-	DOU [mmol O_2 m^{-2} d^{-1}]	OPD [mm]	SAD [mm]	Suboxic zone** [mm]	pH signature***	Chemolitho-autotrophy [mmol C m^{-2} d^{-1}]	Cable biovolume [mm^3cm^{-2}]	Beggiatoa biovolume [mm^3cm^{-2}]
M	1	5	299	(oxic)	28.2	18.2±1.7	1.8±0.04	17.5±0.7	16	e-SOx	3.1±0.5	2.55	0.02
	2	5	301	(oxic)	27.9	15.8±3.1	2.6±0.65	21.3±2.5	19	Nitrate-storing Beggiatoa	1.9±0.1	ND	0.11
	3	5	307	(oxic)	27.7	17.1±5.7	2.4±0.4	41.8±8.6	39	e-SOx	1.4±0.3	2.08	0.05
A	1	17	0	(anoxic)	1.7	0	0	0.9±1.1	0.9	Sulfate reduction/ canonical sulfur oxidation	0.2±0.1	0.11	0.001
	2	17	12	(hypoxic)	11.6	0	0	0.6±0	0.6		0.8±0.3	ND	3.24
	3	19	88	(hypoxic)	10.6	13.9±2.1	1.1±0.1	4.2±2.7	3		1.1±0.5	0.003	0.004

M - March, A - August, DOU: dissolved O_2 uptake; OPD: O_2 penetration depth; SAD: $\sum H_2S$ appearance depth; e-SOx: electrogenic sulfur oxidation; ND: not determined. *Bottom water is classified as anoxic with O_2 concentration below 1 µM and hypoxic below 63 µM. **The thickness of the suboxic zone is defined as the average SAD minus the average OPD. ***pH signature serves to indicate the sulfur oxidizing mechanism that dominates the porewater chemistry as described by Seitaj et al. (2015). Cable - cable bacteria.

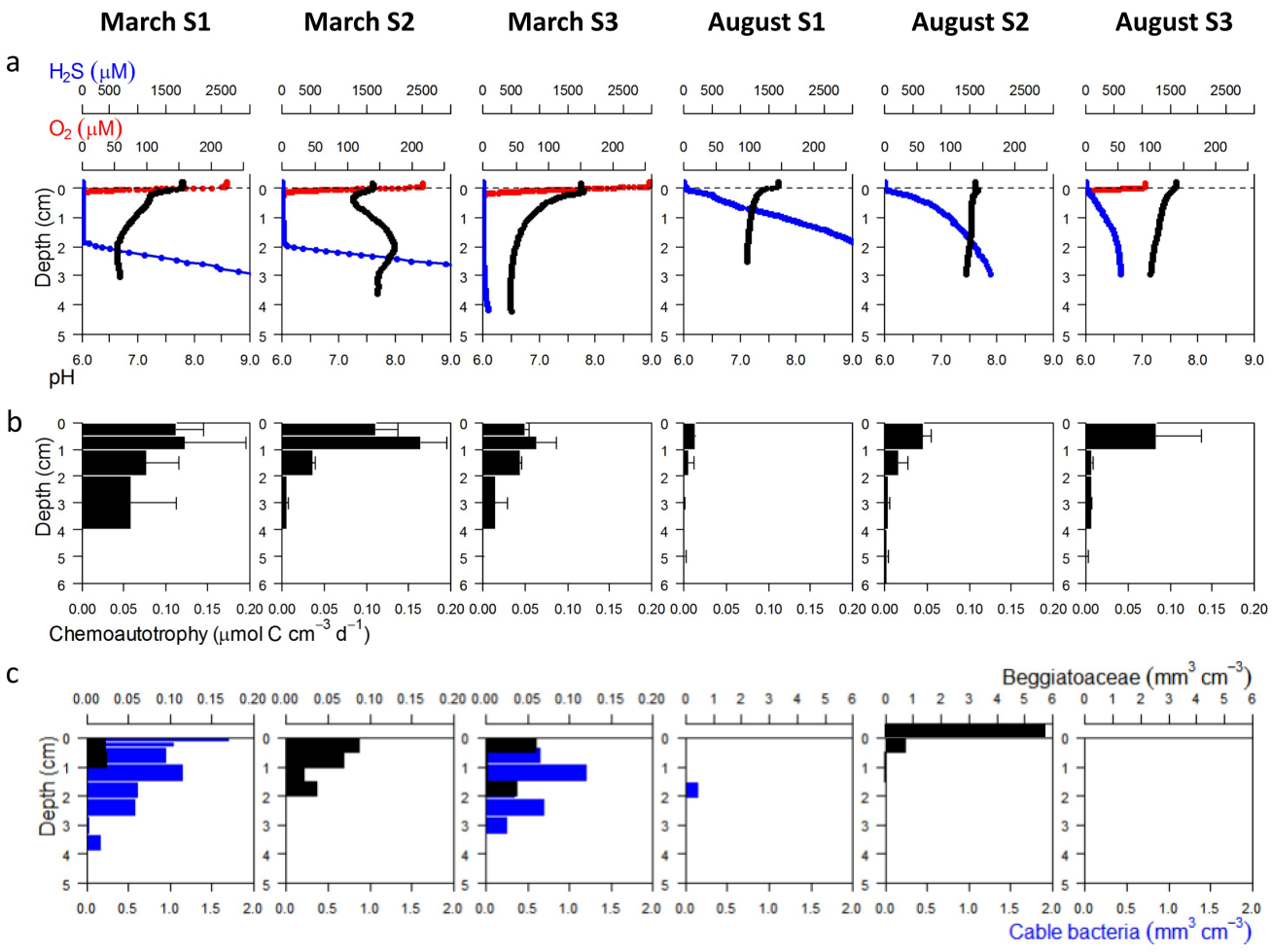

Figure 1: Geochemical fingerprint, (b) chemoautotrophy depth profiles, (c) biovolume of filamentous Beggiatoaceae (black) and cable bacteria (blue) in sediment of Lake Grevelingen for March and August in all three stations (note change in scale for Beggiatoaceae between March and August). S1: station 1 (34 m), S2: station 2 (23 m), S3: station 3 (17 m).

5.3.3. Bacterial community structure by phospholipid derived fatty acid analysis

The relative concentrations of phospholipid derived fatty acid (PLFA) were determined to a depth of 6 cm and were analyzed by principal component analysis (PCA) to determine differences in bacterial community structure (Fig. 3). A total of 22 individual PLFA, each contributing more than 0.1% to the total PLFA biomass, were included in this analysis. Samples with low total PLFA biomass (less than one standard deviation below the mean of all samples) were excluded. The PCA analysis indicated that 73% of the variation within the dataset was explained by the first two principal components (PC). While PC1 correlated with sediment depth (particularly for the March samples), PC2 clearly exposed differences in the bacterial community structure between seasons (Fig. 3a). Surface sediments (0–1 cm) were characterized by high relative concentrations of C_{16} and C_{18} monounsaturated PLFA. In contrast, ai15:0, i17:1ω7c, and 10Me16:0 were more abundant in deeper sediments (Fig. S7a). The surface sediment showed an increased contribution of iso, anteiso and branched PLFAs in August relative to March, and this was more similar to the deeper sediments from March (Fig. 3a). Nonetheless, August sediments also showed a higher abundance of 16:0, 14:0, and 18:1ω9c compared to the deeper sediment layers in March (Fig. S7a).

5.3.4. Chemolithoautotrophic activity and community by ^{13}C-phospholipid fatty acids analysis

Incorporation of ^{13}C-labeled dissolved inorganic carbon was found in bacterial PLFAs after 24 to 40 hours of incubation (Fig. S7b). Depth integrated dark CO_2 fixation rates based on ^{13}C-incorporation (Table 1) showed a significant difference between seasons and stations ($p = 0.0005$). In March, chemolithoautotrophy increased with water depth, while in August, the opposite trend was observed. The dark CO_2 fixation rate in March for S1 was the highest across all seasons and stations, but dropped by one order of magnitude in August. In contrast, at the intermediate station (S2), the dark CO_2 fixation rate was only two times higher in March compared to August, while in the shallowest station (S3) the depth integrated rates were not significantly different between seasons ($p = 0.56$).

The depth distribution of the chemolithoautotrophic activity also differed between seasons ($p = 0.02$; Fig. 1b). In August, the chemolithoautotrophic activity was restricted to the upper cm of the sediment at all stations while in March, chemolithoautotrophic activity was measured deep into the sediment (up to 4 cm). In stations S1 and S3 during March, the activity depth profile showed high activities down to 4 cm, whereas at S2 chemolithoautotrophy only extended down to 2 cm with highest activity in the top 1 cm. This distinction between the depth distributions of chemolithoautotrophy at S2 versus S1/S3 in March correlated with the distinct pH profiles observed for S2 versus S1/S3 (Fig. 1c).

The PLFA ^{13}C-fingerprints were analyzed by PCA to identify differences in chemolithoautotrophic community. Only PLFA that contributed more than 0.1% to the total ^{13}C-incorporation and sediment layers that showed chemolithoautotrophy rates higher than 0.01 μmol C cm^{-3} d^{-1} were taken into account in this analysis. Because chemolithoautotrophy rates were low in August, the PCA analysis mainly analyzed the chemolithoautotrophic communities in March (Fig. 3b). Within the dataset, 64% of the variation was explained by two principal components. PC1 revealed a clear differentiation between station S2, and stations S1 and S3 (Fig. 3b) in agreement with the distinct pH profiles described for March (Fig. 1a). S2 sediment horizons showed a

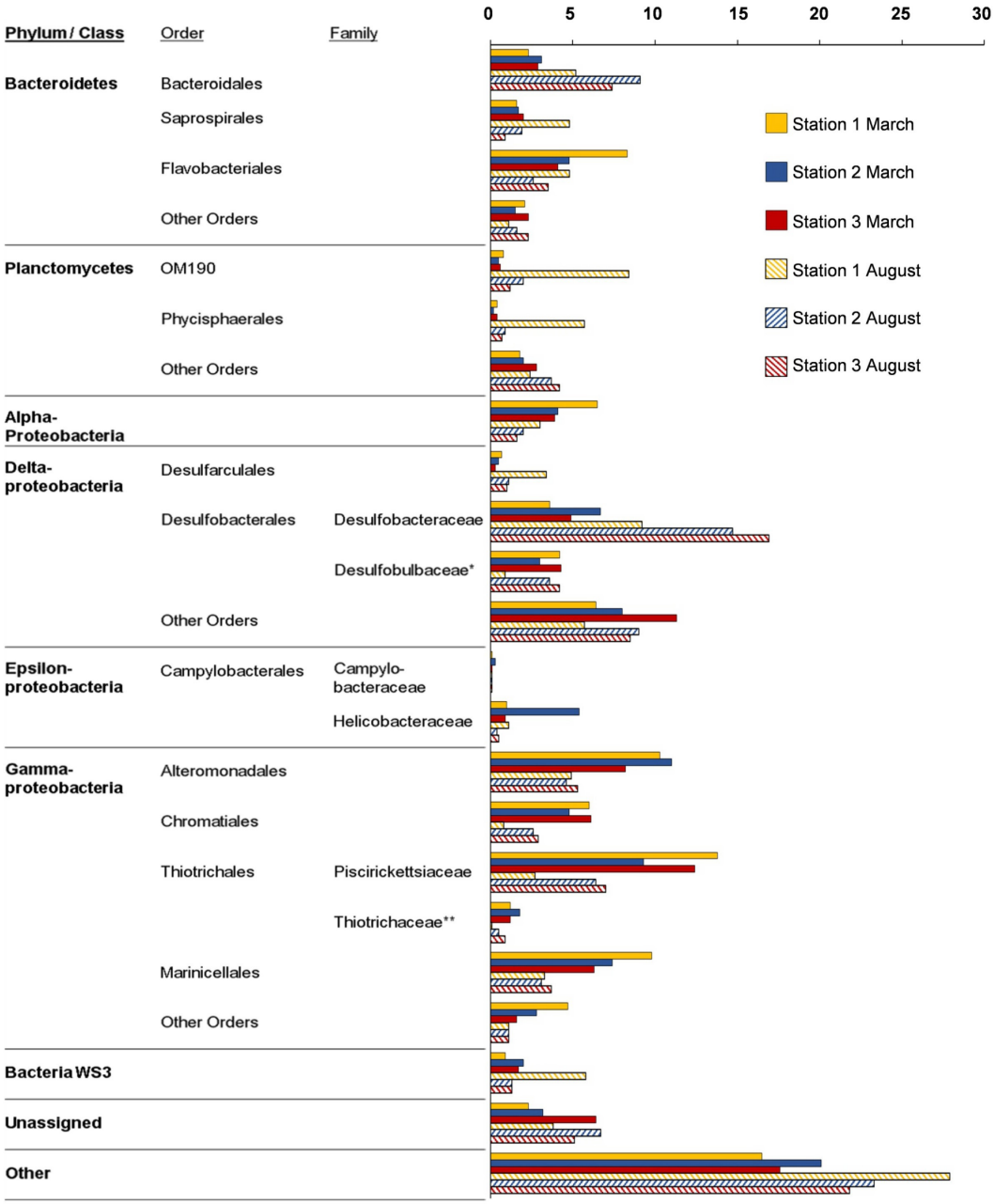

Figure 2: Percentages of total bacterial 16S rRNA gene reads in stations S1 (yellow), S2 (blue) and S3 (red) in March (filled) and August (hatched). Classified bacterial phyla, classes and orders > 3% of the total bacteria reads (in March or August) are reported (exception: family Thiotrichaceae < 3%), *including cable bacteria, **including Beggiatoaceae.

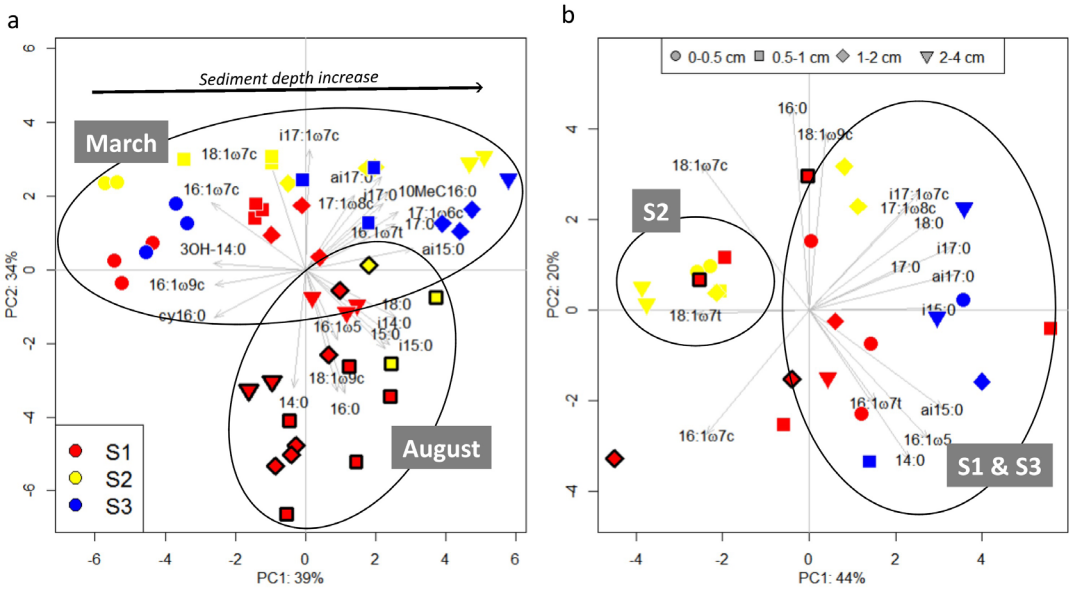

Figure 3: Principal component analysis (PCA) of relative PLFA concentrations (a) and of ^{13}C-incorporation into PLFA (b) in sediments from the three stations in March (no border) and August (black border). The percentage of variability explained by the first two principal components (PC) is indicated on each axis. Red: station S1, Yellow: S2, Blue S3. Symbols indicate sediment depth as seen in plot.

higher contribution of monounsaturated C_{16} and C_{18} fatty acids, whereas sediments from S1 and S3 revealed an increased ^{13}C-incorporation into fatty acids with iso and anteiso C_{15} and saturated C_{14} PLFA (Fig. S7c), together these results indicate distinct chemolithoautotrophic microbial assemblages between S2 and S1/S3. On the contrary, the three sediment samples from August (that had sufficient ^{13}C-incorporation for the analysis) did not cluster in the PCA analysis and exhibited divergent PLFA profiles, thus the chemolithoautotrophic bacterial community for August could not be characterized further based on PLFA analysis.

5.3.5. Chemolithoautotrophic carbon fixation pathways

Various CO_2 fixation pathways are used by autotrophic bacteria (for detailed reviews see Berg, 2011, Hügler and Sievert, 2011). The CBB pathway is utilized by cyanobacteria and many aerobic or facultative aerobic proteobacteria of the alpha, beta and gamma subgroups whereas the rTCA pathway operates in anaerobic or microaerobic members of phyla such as Chlorobi, Aquificae, proteobacteria of the Delta- and Epsilon- subgroups and *Nitrospirae* (Berg, 2011). The spatial and temporal distribution of bacteria possessing these two autotrophic carbon fixation pathways was studied by quantifying the abundance of *cbbL* gene (CBB pathway) (Nigro and King, 2007) and *aclB* gene (rTCA pathway) (Campbell et l., 2006, modified in this study) by quantitative PCR down to 5 cm sediment depth (Fig. 4).The diversity of *cbbL* and *aclB* gene sequences obtained from the surface sediments (0–1 cm) was analyzed (Figs. 5a, b).

Significant differences were found in the abundance of *cbbL* and *aclB* genes ($p = 5.7 \times 10^{-7}$) and between season ($p = 0.002$), but not between stations (Fig. 4). The abundance of the *cbbL* gene copies was at least 2-fold higher than that of the *aclB* gene in March and August in all stations ($p = 5 \times 10^{-5}$). In March, the depth profiles of *cbbL* gene showed a similar trend in stations

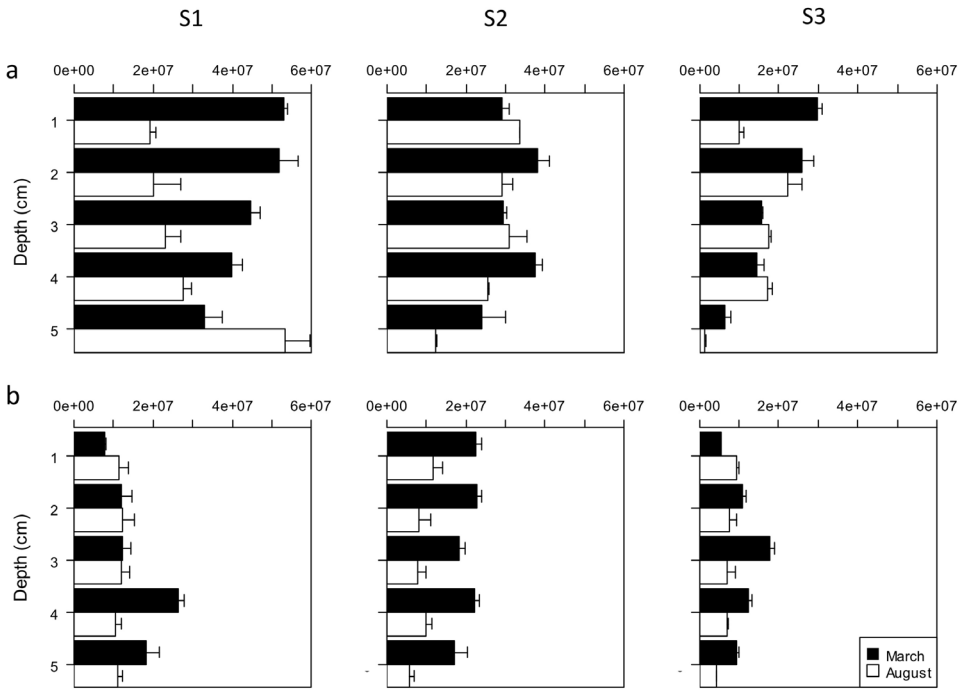

Figure 4: Abundance [copy g^{-1}] of two carbon fixation pathway genes with sediment depth [cm] for S1 (left), S2 (middle) and S3 (right) (a) RuBisCO *cbbL* gene (b) ATP citrate lyase *aclB* gene (black: March, white: August). The error bars represent the standard deviations of qPCR replicates.

S1 and S3 with a decreasing gene abundance from the surface towards deeper layers, but depth integrated abundance of the *cbbL* gene was more than two-fold higher in S1 than in S3 (Fig. 4a). The depth distribution of the *cbbL* gene in the deepest station (S1) differed between seasons ($p = 0.004$), with lower gene copy abundances in the top 4 cm in August (Fig. 4a). In contrast, in the shallow station (S3), which experiences less seasonal fluctuations in bottom water O_2, the *cbbL* gene copy number did not differ significantly between seasons ($p = 0.4$), except for a sharp decrease in the top cm of the sediment. In station S2, abundance and depth distribution of *cbbL* gene copies was similar between the two seasons ($p = 0.3$). All detected *cbbL* gene sequences clustered with uncultured Gammaproteobacteria clones (Fig. 5a), assigned to the orders Chromatiales and Thiotrichales (both *cbbL*1 and *cbbL*2 clusters) reported in intertidal sediment from Lowes Cove, Maine (Nigro and King, 2007).

The abundance of the *aclB* gene significantly differed between months ($p = 0.0004$) and stations ($p = 0.04$). Similar depth profiles of the *aclB* gene were detected in stations S1 and S3, with subsurface maxima in deeper sediments (at 3–4 cm and 2–3 cm deep for S1 and S3 respectively, Fig. 4b). Station S2 showed the highest *aclB* gene abundance in March, which remained constant with sediment depth ($p = 0.86$). In August, *aclB* gene abundance decreased substantially in S2 ($p < 0.001$). All stations showed a uniform distribution of the *aclB* gene abundance with depth in August. Bacterial *aclB* gene sequences in surface sediments (0−1 cm) of all stations were predominantly related to *aclB* sequences of Epsilonproteobacteria (Fig. 5b). Within the Epsilonproteobacteria, sequences clustered in six different subclusters and were mainly affiliated to bacteria in the order of Campylobacterales, *i.e.,* to the genera *Sulfuricurvum, Sulfurimonas, Thiovu-*

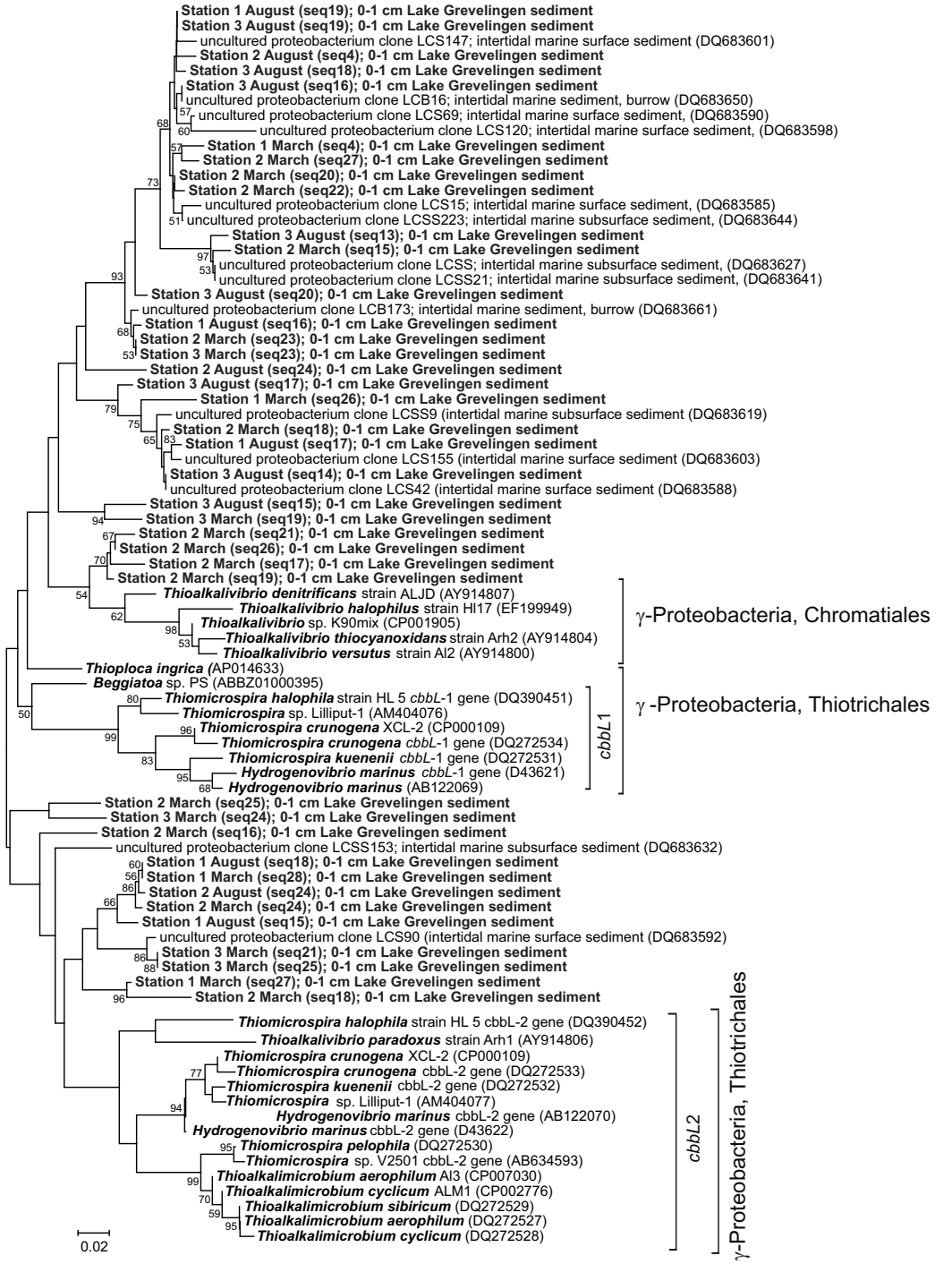

Figure 5a: Phylogenetic tree (amino acid-based) of *cbbL* gene sequences retrieved in this study and closest relatives. The scale bar indicates 2% sequence divergence.

Figure 5b: Phylogenetic tree (amino acid-based) of *aclB* gene sequences retrieved in this study and closest relatives. The scale bar indicates 5% sequence divergence.

lum, Arcobacter, and macrofaunal endosymbionts. As observed for the *cbbL* gene, no clustering of sequences was observed according to station or season (Fig. 5b).

5.3.6. Quantification of cable bacteria and filamentous Beggiatoaceae

We performed a detailed microscopy-based quantification of the biovolume of sulfur oxidizing cable bacteria and filamentous Beggiatoaceae because both groups have been reported to govern the sediment geochemistry and sulfur cycling in sediments of Lake Grevelingen (Seitaj et al., 2015), and thus are likely to influence the chemolithoautotrophic community. Biovolume data of both filamentous bacteria for S1 have been reported before (Seitaj et al., 2015) whereas data for S2 and S3 are novel results from the 2012 campaign. In March, high biovolumes of cable bacteria were detected in S1 and S3 (Table 1) with filaments present throughout the suboxic zone until a maximum depth of 4 cm (Fig. 1c). At the same time, cable bacteria were absent in S2 (Fig. 1c). In August, cable bacteria were only detected between 1 and 2 cm deep at the deepest station (S1), albeit at abundances that were close to the detection limit of the technique. Beggiatoaceae were found in all three stations in March, although the biovolume at S2 was one order of magnitude higher than in the other two stations (Table 1). In station S2, Beggiatoaceae were uniformly distributed up to the sulfide appearance depth (Fig. 1c). In August, Beggiatoaceae were no longer detectable in stations S1 and S3 (Table 1), while at S2, filaments were no longer found in deeper sediment, but formed a thick mat at the sediment-water interface (Fig. 1c).

5.4. Discussion

5.4.1. Temporal shifts of chemolithoautotrophy and the associated bacterial assemblages

Together, our geochemical and microbiological characterization of the sediments of Lake Grevelingen indicates that the availability of electron acceptors (O_2, NO_3^-) constitutes the main environmental factor controlling the activity of the chemolithoautotrophic bacterial community. In the deeper basins of Lake Grevelingen (below water depth of 15 m), the electron acceptor availability changes on a seasonal basis due to the establishment of summer hypoxia (Seitaj et al., 2015; Sulu-Gambari et al., 2016). In March, when high oxygen levels are found in the bottom waters, the chemolithoautotrophy rates were substantially higher than in August when oxygen in the bottom water was depleted to low levels (Fig. 1b; Table 1). Moreover in August, the chemolithoautotrophy rates showed a clear decrease with water depth (from S3 to S1) in line with the decrease of the bottom water oxygen concentration over depth.

The 16S rRNA gene amplicon sequencing analysis in our study reveals that the response of the chemolithoautotrophic bacterial community in the surface sediments of Lake Grevelingen is associated with the seasonal changes in bottom water oxygen (Fig. 2). Generally, Lake Grevelingen surface sediments harbor a distinct microbial community compared to other surface sediments such as coastal marine (North Sea), estuarine and Black Sea sediments (Jessen et al., 2016; Ye et al., 2016; Dyksma et al., 2016) studied by 16S rRNA gene amplicon sequencing analysis.

In March, with oxic bottom waters, the microbial community in the top centimeter of the sediment at all three stations was characterized by high abundances of Epsilon- and Gammaproteobacteria, which are known chemolithoautotrophic sulfur-oxidizers (e.g. capable of oxidizing sulfide, thiosulfate, elemental sulfur and polythionates) using oxygen or nitrate as electron acceptor (Campbell et al., 2006; Sorokin et al., 2001; Takai et al., 2006; Labrenz et al., 2013). In

August, the lack of electron acceptors (O_2, NO_3^-) in the bottom water was accompanied by a decrease in the relative abundance of these Gammaproteobacteria and Epsilonproteobacteria. At the same time, the numbers of reads related to the Desulfobacteraceae family increased in the top centimeter of the sediment, and a shift in the microbial community structure towards sulfate reducing bacteria related to the genera *Desulfococcus* and *Desulfosarcina* was evident. In coastal sediments, these genera are characteristic in deeper sediment layers experiencing anaerobic mineralization (Llobet-Brossa et al., 2002; Miyatake, 2011; Muyzer and Stams, 2008), and thus, they are not unexpected in surface sediments during strongly hypoxic (S2) and anoxic (S1) conditions encountered in August.

Shifts in PLFA patterns are in agreement with the temporal difference in the bacterial community (Fig. 3a), with more PLFAs found in Gammaproteobacteria and Epsilonproteobacteria in March (*i.e.,* 16:1ω7c and 18:1 ω7c; Vestal and White, 1989) as opposed to more PLFAs found in sulfate reducing bacteria in the Deltaproteobacteria in August (*i.e.,* ai15:0, i17:1ω7c, 10Me16:0; Boschker and Middelburg, 2002; Taylor and Parkes, 1983, Edlund et al., 1985). However, sediments below the OPD in March have PLFA patterns that are more similar to those of surface sediments in August, in agreement with the observation that anaerobic metabolism such as sulfate reduction prevail in deeper anoxic sediments also in March.

5.4.2. Spring: Activity and diversity of chemolithoautotrophic bacteria

Although the availability of soluble electron acceptors (O_2, NO_3^-) in the bottom water is important, our data show that it cannot be the only structuring factor of the chemolithoautotrophic communities. In March 2012, all the three stations examined experienced similar bottom water O_2 and NO_3^- concentrations (Table 1), but still substantial differences were observed in the composition of the chemolithoautotrophic communities as determined by PLFA-SIP (Fig. 3b), as well as in the depth distribution of the chemolithoautotrophy rates in the sediment (Fig. 1b). We attribute these differences to the presence of specific sulfur oxidation mechanisms that are active in the sediments of Lake Grevelingen (Seitaj et al., 2015). In March 2012, based on FISH counts and microsensor profiles two separate sulfur oxidation regimes were active at the sites investigated: sites S1 and S3 were impacted by electrogenic sulfur oxidation by cable bacteria, while site S2 was dominated by sulfur oxidation via nitrate-storing, filamentous Beggiatoaceae. Each of these two regimes is characterized by a specific sulfur-oxidizing microbial community and a particular depth distribution of the chemolithoautotrophy. We now discuss these two regimes separately in more detail.

Electrogenic sulfur oxidation

In March, stations S1 and S3 (Fig. 1a) showed the geochemical fingerprint of electrogenic sulfur oxidation (e-SOx) consisting of a centimeter-wide suboxic zone that is characterized by acidic pore waters (pH < 7) (Nielsen et al., 2010; Meysman et al., 2015). Electrogenic sulfur oxidation is attributed to the metabolic activity of cable bacteria (Pfeffer et al., 2012), which are long filamentous bacteria related to the sulfate reducing genus *Desulfobulbus* that extend centimeters deep into the sediment (Schauer et al., 2014). The observed depth-distribution of cable bacteria at S1 and S3 (Fig. 1c) was congruent with the geochemical fingerprint of e-SOx. Cable bacteria couple the oxidation of sulfide in deeper layers to the reduction of oxygen near the sediment-water interface, by channeling electron along their longitudinal axis (long-distance electron transport). Note that stations S1 and S3 also contained some Beggiatoaceae. However, they

attained low biovolumes and were only found at certain depths, suggesting that they did not play a significant role in sulfur oxidation.

When e-SOx was present in the sediment (sites S1 and S3 in March), the chemolithoautotrophic activity penetrated deeply into the sediment and was evenly distributed throughout the suboxic zone (Fig. 1b). These field observations confirm previous laboratory incubations in which cable bacteria were induced under oxic conditions in homogenized sediments, and a highly similar depth pattern of deep chemolithoautotrophy was noted (Vasquez-Cardenas et al., 2015). This deep dark CO_2 fixation is unexpected in two ways. Firstly, cable bacteria are likely not responsible for the deep CO_2 fixation although they do perform sulfur oxidation, as cable bacteria from Lake Grevelingen have been shown to incorporate organic carbon rather than inorganic carbon (Vasquez-Cardenas et al., 2015). Secondly, chemolithoautotrophy is generally dependent on reoxidation reactions, but there is no transport of oxygen or nitrate to centimeters depth in these cohesive sediments, and so the question is: how can chemolithoautotrophic reoxidation occur in the absence of suitable electron acceptors?

To reconcile these observations, it was proposed that in incubated electro-active sediments from Lake Grevelingen, heterotrophic cable bacteria can form a sulfur-oxidizing consortium with chemolithoautotrophic Gamma- and Epsilonproteobacteria throughout the suboxic zone

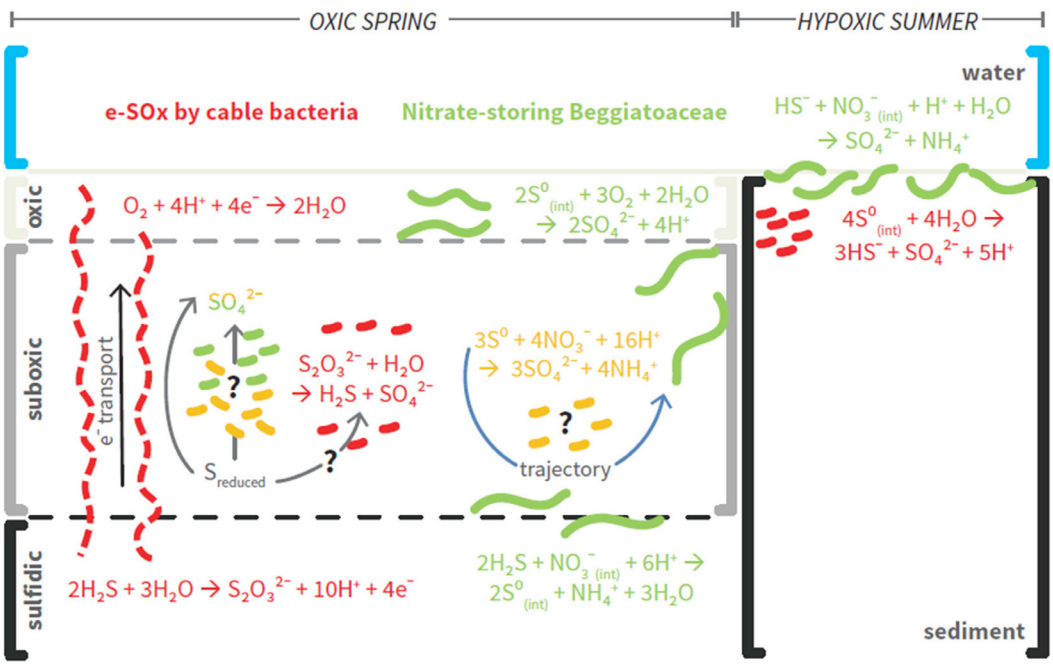

Figure 6: Schematic representation of the main sulfur oxidation mechanisms by chemoautotrophic bacteria under oxic conditions in spring (left) and hypoxic conditions in summer (right) in marine Lake Grevelingen (single-celled and filamentous sulfur-oxidizing bacteria are shown as follows, Epsilonproteobacteria (yellow), Gammaproteobacteria (green), Deltaproteobacteria (red), grey arrows represent possible reoxidation processes by chemoautotrophic bacteria and the color of the reaction indicates the bacterial group involved, blue arrow: trajectory of filamentous Beggiatoaceae (thick green lines) from the surface to the sulfide horizon and back up to the surface, black arrow: long-distance electron transport by cable bacteria (red broken lines).

(Vasquez-Cardenas et al., 2015). The results obtained in our study provide various lines of evidence that support the existence of such a consortium in intact sediment cores. The PLFA-SIP patterns in S1 and S3 (Fig. 3b) showed major ^{13}C-incorporation in PLFA which are present in sulfur-oxidizing Gamma- and Epsilonproteobacteria (Takai et al., 2006; Inagaki et al., 2003; Donachie et al., 2005; Zhang et al., 2005), and this occurred throughout the top 5 cm of the sediment corresponding to the zone of e-SOx activity. In addition, the depth profiles of genes involved in dark CO_2 fixation (Fig. 4) revealed chemolithoautotrophic Gammaproteobacteria using the CBB cycle as well as Epsilonproteobacteria using the rTCA cycle, which confirms the potential dark CO_2 fixation by both bacterial groups deep in the sediment. The higher abundance of *cbbL* genes in S1 compared to S3 indicates a greater chemolithoautotrophic potential of Gammaproteobacteria, which may explain the two-fold higher total chemolithoautotrophy rate encountered in S1. Moreover, the peak in *aclB* gene abundance found in deeper sediment suggests that Epsilonproteobacteria could play an important role in sulfur oxidation in the deeper suboxic zone in both stations. Clearly, a consortium could sustain the high rates of chemolithoautotrophy throughout the suboxic zone. It has been speculated that chemolithoautotrophs use the cable bacteria as an electron sink in the absence of an electron acceptor like O_2 or NO_3^- in centimeter deep sediments (Vasquez-Cardenas et al., 2015). However the question still remains as to how the Gamma- and Epsilonproteobacteria are metabolically linked to the cable bacteria (Fig. 6).

In March, stations S1 and S3 also showed a higher contribution of fatty acids occurring in the sulfate reducing Deltaproteobacteria (Boschker et al., 2014; Taylor and Parkes, 1983; Webster et al., 2006) compared to the PLFA-SIP profiles in station S2 (Fig. 3b). Deltaproteobacteria such as *Desulfobacterium autotrophicum* and *Desulfocapsa* sp., as identified by 16S rRNA gene sequencing, are known to grow as chemolithoautotrophs by performing H_2 oxidation or S^0-disproportionation (Finster et al., 1998, Böttcher et al., 2005; Fig. 6), and are important contributors to the chemolithoautotrophic activity in coastal sediments (Boschker et al., 2014; Thomsen and Kristensen, 1997). However, a further identification of the chemolithoautotrophic Deltaproteobacteria through the functional genes related to carbon fixation pathways was not performed in this study. Although it is known that autotrophic Deltaproteobacteria mainly use the reverse TCA cycle or the reductive acetyl-CoA pathway (Hügler and Sievert, 2011), further development of the functional gene approach, by designing specific primers is necessary to determine and clarify the diversity of Deltaproteobacteria involved in the chemolithoautotrophic activity.

Overall, we hypothesize that such a diverse assemblage of chemolithoautotrophic bacteria in the presence of cable bacteria is indicative of a complex niche partitioning between these sulfur-oxidizers (Gamma-, Epsilon-, and Deltaproteobacteria). In sulfidic marine sediments found in tidal and deep sea habitats, complex S^0 niche partitioning have been proposed where uncultured sulfur-oxidizing Gammaproteobacteria mainly thrive on free sulfide, the epsilonproteobacterial Sulfurimonas/Sulfurovum group oxidizes elemental sulfur, and members of the deltaproteobacterial Desulfobulbaceae family may perform S^0-disproportionation (Pjevac et al., 2014).

Nitrate-storing filamentous Beggiatoaceae

Nitrate-storing filamentous Beggiatoaceae glide through the sediment transporting their electron acceptor (intracellular vacuoles filled with high concentrations of NO_3^-) into deeper sediment

and electron donor (intracellular granules of elemental sulfur) up to the surface, and in doing so, they oxidize free sulfide to sulfate in a two-step process that creates a wide suboxic zone (Seitaj et al., 2015; Mußmann et al., 2003; Sayama et al., 2005; Fig. 6). In March, the microsensor depth profiles at S2 (Fig. 1a) revealed a cm-thick suboxic zone with a subsurface pH minimum at the OPD followed by a pH maximum at SAD, which is the characteristic geochemical fingerprint of sulfur oxidation by nitrate-storing Beggiatoaceae (Seitaj et al., 2015; Sayama et al., 2005). At the same time, microscopy revealed high biovolumes of Beggiatoaceae that were uniformly distributed throughout the suboxic zone (Fig. 1c), and more than half the filaments found were thicker than 15 µm indicating potential nitrate storage. Together these results corroborate the indications of the dominant sulfur-oxidation mechanism suggested by the geochemical fingerprint.

The chemolithoautotrophy depth profile at S2 in March (Fig. 1b) recorded higher activities in the top 1 cm and chemolithoautotrophic activity penetrated down only to 2 cm (at the sulfide appearance depth). The similar depth distribution of Beggiatoaceae and chemolithoautotrophic activity suggests that dark CO_2 fixation was primarily carried out by the nitrate-storing Beggiatoaceae. Beggiatoaceae can indeed grow as obligate or facultative chemolithoautotrophs depending on the strain (Hagen and Nelson DC, 1996). The PLFA-SIP analysis further supported chemolithoautotrophy by Beggiatoaceae as the PLFA patterns obtained at S2 resembled those of *Beggiatoa* mats encountered in sediments associated with gas hydrates (Zhang et al., 2005).

However, the CO_2 fixation by Beggiatoaceae could be complemented by the activity of other chemolithoautotrophs. Beggiatoaceae have previously been reported to co-occur with chemolithoautotrophic nitrate-reducing and sulfur-oxidizing Epsilonproteobacteria (*Sulfurovum* and *Sulfurimonas*) in the deep sea Guyamas basin (Bowles et al., 2012). Interestingly, station S2 in March showed the highest abundance of *aclB* gene copies of all stations and seasons (Fig. 4b), and in addition, had the highest relative percentage of 16S rRNA gene read sequences assigned to the Epsilonproteobacteria (Fig. 2). Therefore, the dark CO_2 fixation at station S2 is likely caused by both the motile, vacuolated Beggiatoaceae as well as the sulfur-oxidizing Epsilonproteobacteria. Yet, as was the case of the cable bacteria above, the metabolic link between Beggiatoaceae and the Epsilonproteobacteria remains currently unknown. However, it seems possible that internally stored NO_3^- is released into the sediment once *Beggiatoa* filaments lyse, allowing sulfur-oxidation with NO_3^- by Epsilonproteobacteria.

5.4.3. Summer: Activity and diversity of chemolithoautotrophic bacteria

In August, when the O_2 levels in the bottom water decreased substantially, a third geochemical regime was observed at all stations, which was different from the regimes associated with cable bacteria or nitrate-storing Beggiatoaceae. The microsensor profiling revealed an upward diffusive transport of free sulfide to the top millimeters of the sediment, which was produced in deeper sediment horizons through sulfate reduction (Fig. 1a). The uniform decrease in pH with depth is consistent with sediments dominated by sulfate reduction (Meysman et al., 2015). As noted above, the chemolithoautotrophy rates strongly decreased compared to March and were restricted to the very top of the sediment. Chemolithoautotrophic activity showed a close relation with the bottom water oxygen concentration. The number of gene copies of the two carbon fixation pathways investigated (CBB and rTCA) also decreased under hypoxic conditions (Fig. 4), suggesting a strong dependence of subsurface chemolithoautotrophy on the availability of oxygen in bottom waters.

The highest chemolithoautotrophy rate was recorded at the shallower station (S3), which was the only station in August where O_2 was found to diffusive into the sediment (Table 1), thus supporting aerobic sulfur oxidation at a shallow oxic-sulfidic interface in the sediment (Meysman et al., 2015). Several studies have shown that chemolithoautotrophy ceases in the absence of oxygen in the overlying water column (Vasquez-Cardenas et al., 2015; Miyatake, 2011; Thomsen and Kristensen, 1997). Such full anoxia occurred in the deepest station (S1), but still limited chemolithoautotrophic activity was recorded in the top layer of the sediment. A possible explanation is that some residual aerobic sulfur oxidizing bacteria were supported by the very low oxygen levels. Alternatively, cable bacteria activity at S1 in spring leads to a buildup of iron (hydr) oxides (FeOOH) in the top of the sediments, which prevents sulfide diffusion to the bottom water during summer anoxia ($3H_2S + 2FeOOH \rightarrow 2FeS + S^0 + 4H_2O$) (Seitaj et al., 2015). The elemental sulfur formed in this reaction may support chemolithoautotrophic sulfur disproportionating bacteria (Finster et al., 1998). At station S2, Beggiatoaceae were found forming a mat at the sediment surface in S2 (Fig. 1b) and chemolithoautotrophy rates were limited to this mat (Fig. 6). Beggiatoaceae can survive hypoxic periods by using stored nitrate as electron acceptor (Schulz and Jørgensen, 2001), which is thought to be a competitive advantage that leads to their proliferation in autumn in S1 (Seitaj et al., 2015). Such survival strategies may be used to produce energy for maintenance rather than growth under hypoxic conditions which would in combination with the low oxygen concentrations explain the low chemolithoautotrophy to biovolume ratio observed in S2 in August compared to March.

5.5. Conclusion

Coastal sediments harbor a great potential for chemolithoautotrophic activity given the high anaerobic mineralization, which produce a large pool of reduced sulfur in these organic rich sediments. In this study, two environmental factors were identified to regulate the chemolithoautotrophic activity in coastal sediments: seasonal hypoxia and the dominant sulfur oxidation mechanism. In sediments where oxygen, the main electron acceptor for chemolithoautotrophs, is depleted because of seasonal hypoxia, chemolithoautotrophy was strongly inhibited. In addition, it is clear that the different sulfur oxidation mechanism (e.g. canonical sulfur oxidation, electrogenic sulfur oxidation, or sulfur oxidation mediated by vacuolated Beggiatoaceae) observed in the sediment also determine the magnitude and depth distribution of the dark CO_2 fixation, as well as the chemolithoautotrophic bacterial community structure. The seasonal variations in electron acceptors and potentially reduced sulfur species suggest complex niche partitioning in the sediment by the sulfur-oxidizing bacterial community. An in depth study on the availability of different sulfur species in the sediment could shed light on the sulfur preferences by the different bacterial groups. Likewise, the potential mechanism used to metabolically link filamentous sulfur-oxidizers and chemolithoautotrophic bacteria remains presently unresolved and requires further study. These tight metabolic relationships may ultimately regulate the cycling of sulfur, carbon and even nitrogen in coastal sediments, including (but not limited to) sediments affected by seasonal hypoxia.

Acknowledgments

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Meer and Sandra Heinzelmann for support onboard and assistance with incubations, Peter van Breugel and Marco Houtekamer for their assistance with stable isotope analysis, Elda Panoto for technical support and Alexandra Vasquez Cardenas for designing Figure 6. This work was financially supported by several grants from Darwin Centre for Biogeosciences to HTSB (grant no: 3061), LV (grant no: 3062) and FJRM (grant no: 3092), FJRM received funding from the European Research Council under the European Union's Seventh Framework Program (FP7/2007–2013) via ERC grant agreement n°[306933], and RS received support from Danish Council for Independent Research-Natural Sciences.

Supplementary information

Experimental procedures

Aeration of ^{13}C- incubations

To maintain the low oxygen levels found in August at S2 and S3, 30 ml of *in situ* overlying water was removed and cores were closed with rubber stoppers. Headspace was then flushed with N_2 through a needle inserted between the core liner and the rubber stopper without disturbing the water surface. After several minutes, N_2 flushing was stopped and 10 and 35% of the headspace was replaced with air using a syringe for S2 and S3, respectively. No air was added to S1 cores. All cores were gently mixed (0.2 rpm) on a shacking plate to homogenize oxygen concentration in headspace with those in overlying water. At the end of the experiment oxygen saturation in overlying water were verified with oxygen optodes (PreSens Fibox 3 LCD) obtaining anoxic conditions for S1 (0−4% air saturation), hypoxic for S2 (20−26%) and S3 (35−80%).

PLFA nomenclature

The shorthand nomenclature used for phospholipid derived fatty acids is as follows. The number before the colon indicates the number of carbon atoms, while the number after represents the number of double carbon bonds in the fatty acids chain. The position of the initial double bond is then indicated by the last number after the number of carbons from the methyl end (ω). The double bond geometry is designated by cis (c) or trans (t). Methyl branching is described as being in the second carbon iso (i), third carbon anteiso (a) or a number followed by Me is used to indicate the position relative to the carboxyl end (e.g. 10Me16:0). Further details can be found in the literature (Vestal and White, 1989).

PCR 16S rRNA gene amplicon library preparation and analysis

PCR reactions were performed with the universal (Bacteria and Archaea) primers S-D-Arch-0519-a-S-15 (5'-CAG CMG CCG CGG TAA-3') and S-D-Bact-0785-a-A-21 (5'-GAC TAC HVG GGT ATC TAA TCC-3') (Klindworth et al., 2013) adapted for pyrosequencing by the addition of sequencing adapters and multiplex identifier (MID) sequences. To minimize bias three independent PCR reactions were performed containing: 16.3 μL H_2O, 6 μL HF Phusion buffer, 2.4 μL dNTP (25 mM), 1.5 μL forward and reverse primer (10 μM; each containing an unique MID tail), 0.5 μL Phusion Taq and 2 μL DNA (6 ng/μL). The PCR conditions were following: 98ºC, 30 s; 25 × [98ºC, 10 s; 53 ºC, 20 s; 72ºC, 30 s]; 72 ºC, 7 min and 4ºC, 5 min.

The PCR products were loaded on a 1% agarose gel and stained with SYBR® Safe (Life technologies, Netherlands). Bands were excised with a sterile scalpel and purified with Qiaquick Gel Extraction Kit (QIAGEN, Valencia, CA) following the manufacturer's instructions. PCR purified products were quantified with Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies, Netherlands). Equimolar concentrations of the barcoded PCR products were pooled and sequenced on GS FLX Titanium platform (454 Life Sciences) by Macrogen Inc. Korea.

Samples were analyzed using the QIIME pipeline (Caporaso et al., 2010). Raw sequences were demultiplexed and then quality-filtered with a minimum quality score of 25, length between 250−350, and allowing maximum two errors in the barcode sequence. Sequences were then clustered into operational taxonomic units (OTUs, 97% similarity) with UCLUST (Edgar, 2010). Reads were aligned to the Greengenes Core reference alignment (DeSantis et al., 2006) using

the PyNAST algorithm (Caporaso et al., 2010). Taxonomy was assigned based on the Greengenes taxonomy and a Greengenes reference database (version 12_10) (McDonald et al., 2012; Werner et al., 2012). Representative OTU sequences assigned to the specific taxonomic groups were extracted through classify.seqs and get.lineage in Mothur (Schloss et al., 2009) by using the greengenes reference and taxonomy files.

In order to determine a more accurate taxonomic classification of the bacterial groups with high percentage of reads and known to contain members with chemolithoautotrophic metabolism, sequence reads of the order Chromatiales and Thiotrichales (Fig. S1, S2), reads of the order Desulfarculales (Fig. S3), the family of Desulfobulbaceae (Fig. S4a) and the genus *Desulfubulbus* (Fig. S4b), reads of the family Desulfobacteraceae (Fig. S5a–c), and additionally reads of the order Campylobacterales (Fig. S6a–d) were extracted from the dataset and added to a phylogenetic tree as described in the supplementary experimental procedures.

Quantification of cable bacteria and Beggiatoaceae

Microscopic identification of cable bacteria was achieved by fluorescence *in situ* hybridization (FISH), using a Desulfobulbaceae-specific oligonucleotide probe (DSB706; 5'-ACC CGT ATT CCT CCC GAT-3'), according to (Schauer et al., 2014). Cable bacteria biovolume per unit of sediment volume ($mm^3\ cm^{-3}$) was calculated based on measured filament length and diameter. The areal biovolume of cable bacteria ($mm^3\ cm^{-2}$) was obtained by depth integration over all sediment layers analyzed.

The minimum limits of quantification via FISH for single cells were 1.5×10^6 cells cm^{-3}, taken as the FISH count with the negative control probe NON338; a minimum of 1000 DAPI (4,6-diamidino-2-phenylindole) stained cells was evaluated for this count. The FISH detection limit for cable bacteria was lower than for single cells, and was calculated to be 10 cm filament cm^{-3} (corresponding to <1 filament in 0.1 mL of sediment).

Filamentous Beggiatoaceae were identified via inverted light microscopy (Olympus IM) within 24 h of sediment retrieval. Intact sediment cores were sectioned at 5 mm intervals over the top 4 cm from which subsamples (20−30 mg) were used to count living Beggiatoaceae (Seitaj et al., 2015). The biovolume was determined by measuring length and width of all filaments found in the subsample, following the counting procedure described in (Jørgensen et al., 2010).

Statistical analysis

All statistical analyses were performed using the CRAN: stats package in the open source software R. A two-way ANOVA (aov) was used to test the effect of station, season, and sediment depth on bacterial biomass, chemolithoautotrophic activity, and gene abundances. PLFA concentrations and ^{13}C-incorporation values were expressed as a fraction of the total bacterial biomass and ^{13}C-incorporation (respectively) per sediment sample. These relative PLFA values were first log-transformed (log (x+1)) and subsequently analyzed with Principal Component Analysis (PCA: prcomp) to distinguish different bacterial and chemolithoautotrophic communities in the sediment.

Table S1: Primer pairs described in the text, PCR conditions and amplicon size used in this study.

Assay	Target	Primer pair	Tm [°C]	Amplicon size [b]	Reference
qPCR + PCR/ cloning	*cbbL* gene	K2f (5'-ACCAYCAAGCCSAAGCTSGG-3') V2r (5'-GCCTTCSAGCTTGCCSACCRC-3')	qPCR 57 PCR 62	492-495	Nigro and King, 2007
qPCR + PCR/ cloning	*aclB* gene	aclB F (5'-TGGACHATGGTDGCHGGKGGT-3') aclB R (5'-ATARTTKGGBCCACCKCKTC-3')	qPCR 54 PCR 53	312	Campbell et al., 2003

PCR conditions: 95°C 5 min; 40 × [95°C 1 min, Tm 40 s, 72°C 1 min]; 72°C 5 min. qPCR conditions: 95°C 4 min; 40 × [95°C 30 s, Tm 40 s, 72°C 30 s]; 80°C 25 s.

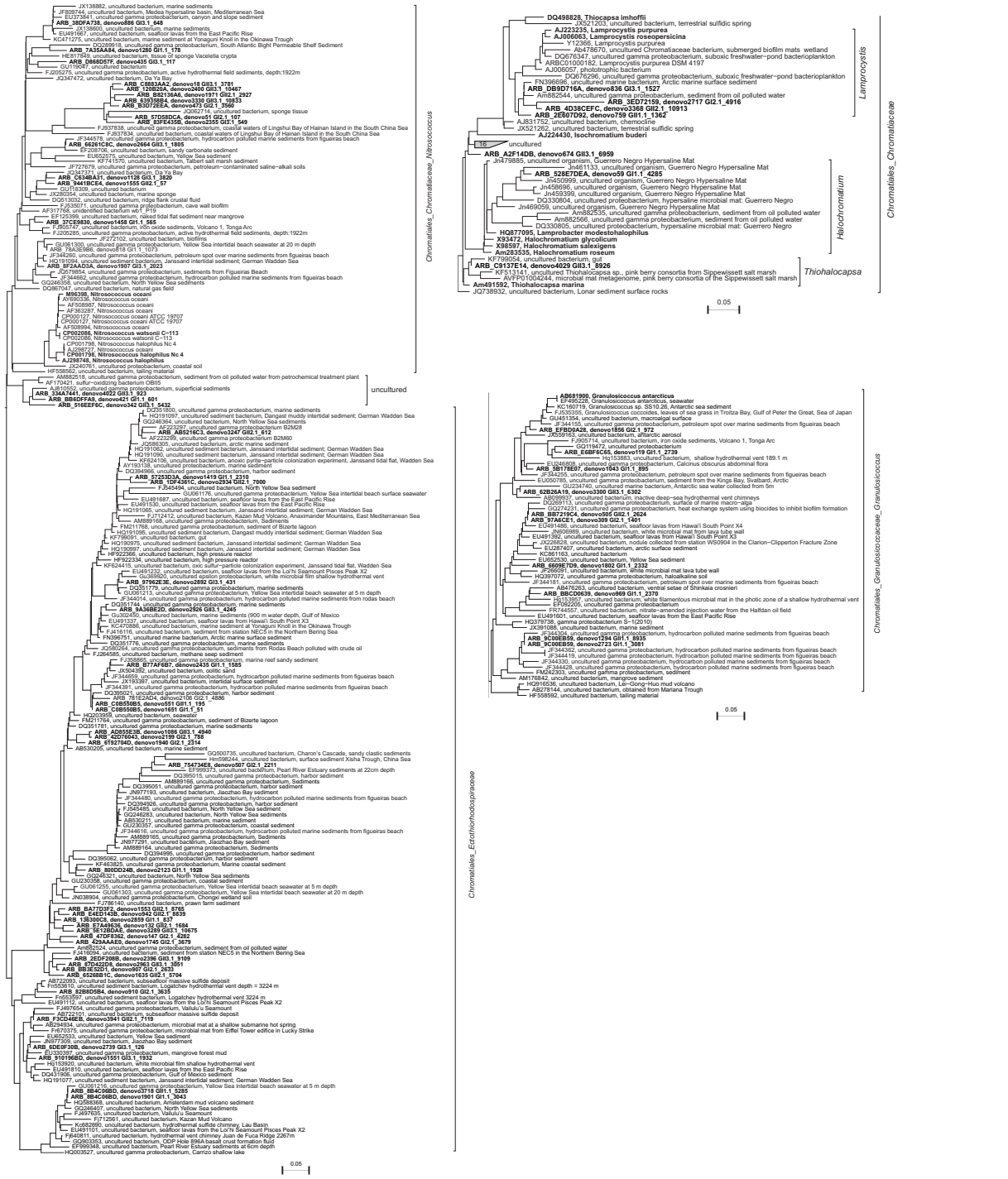

Figure S1: Bacterial 16S rRNA gene amplicon sequences assigned to the class of Gammaproteobacteria, order: Chromatiales (black bold: sequences retrieved in this study and closest relatives).

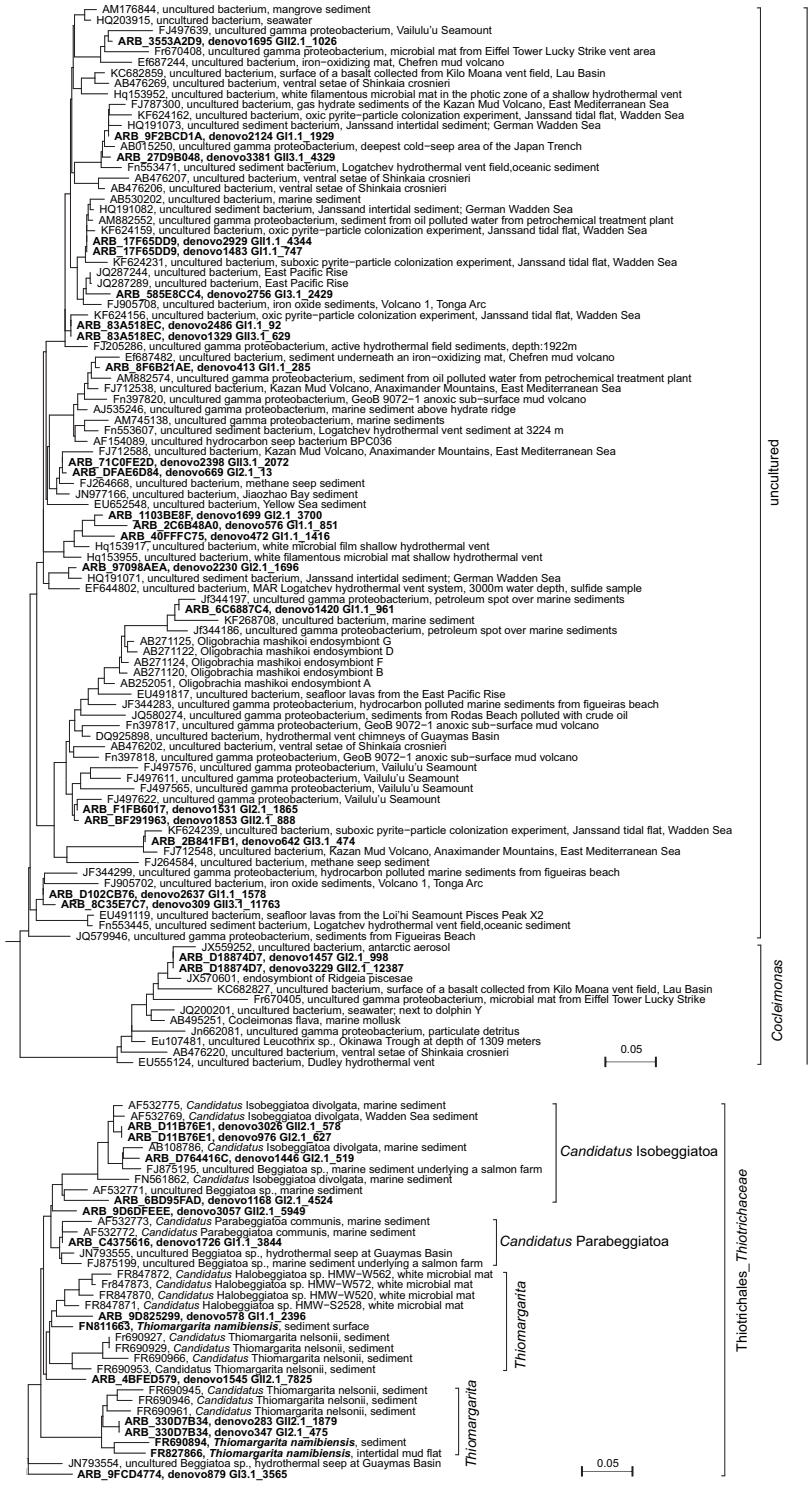

Figure S2: Bacterial 16S rRNA gene amplicon sequences assigned to the class of Gammaproteobacteria, order: Thiotrichales, family: Thiotrichaceae (black bold: sequences retrieved in this study and closest relatives).

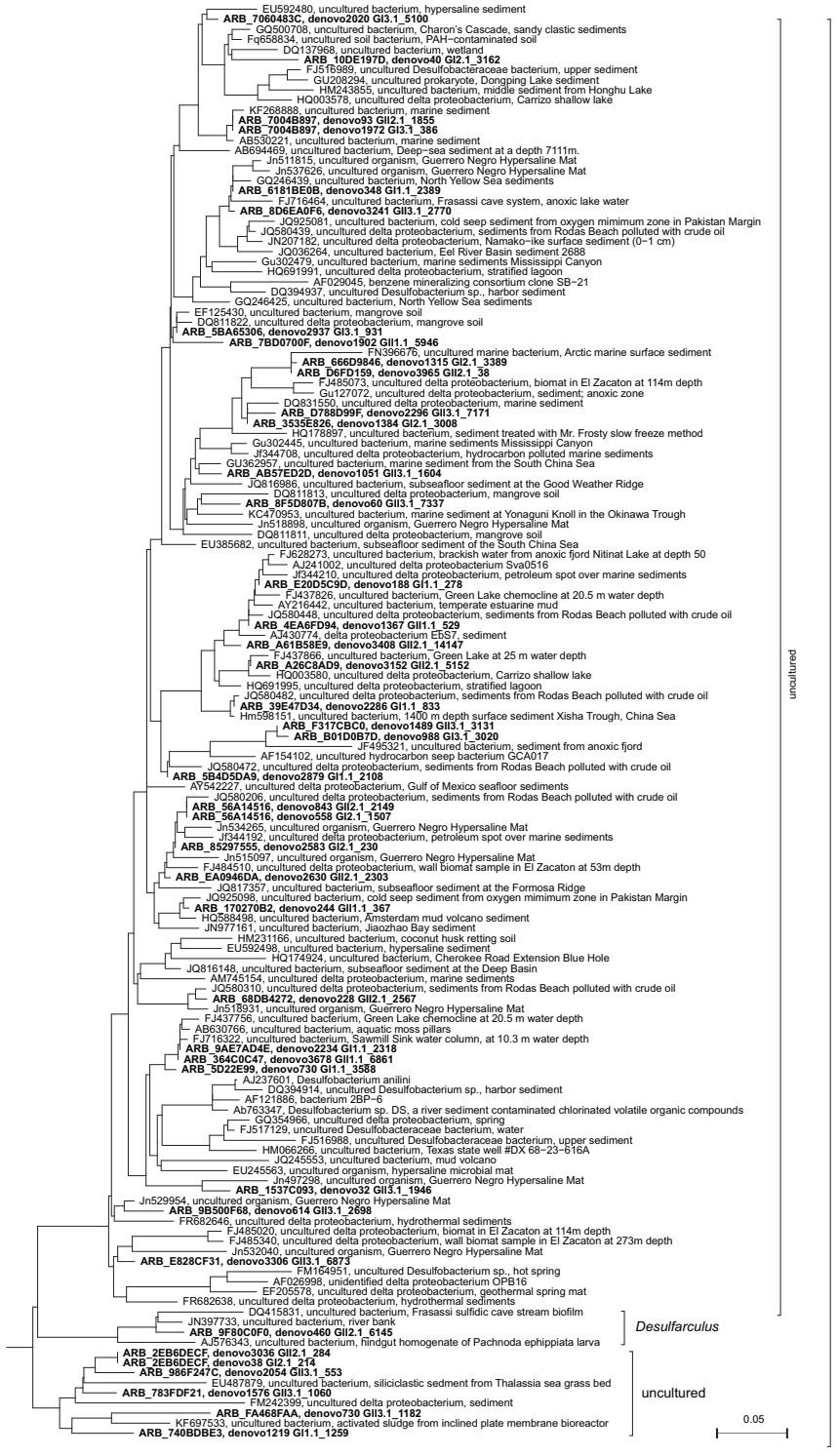

Figure S3: Bacterial 16S rRNA gene amplicon sequences assigned to the class of Deltaproteobacteria, order: Desulfarculales (black bold: sequences retrieved in this study and closest relatives).

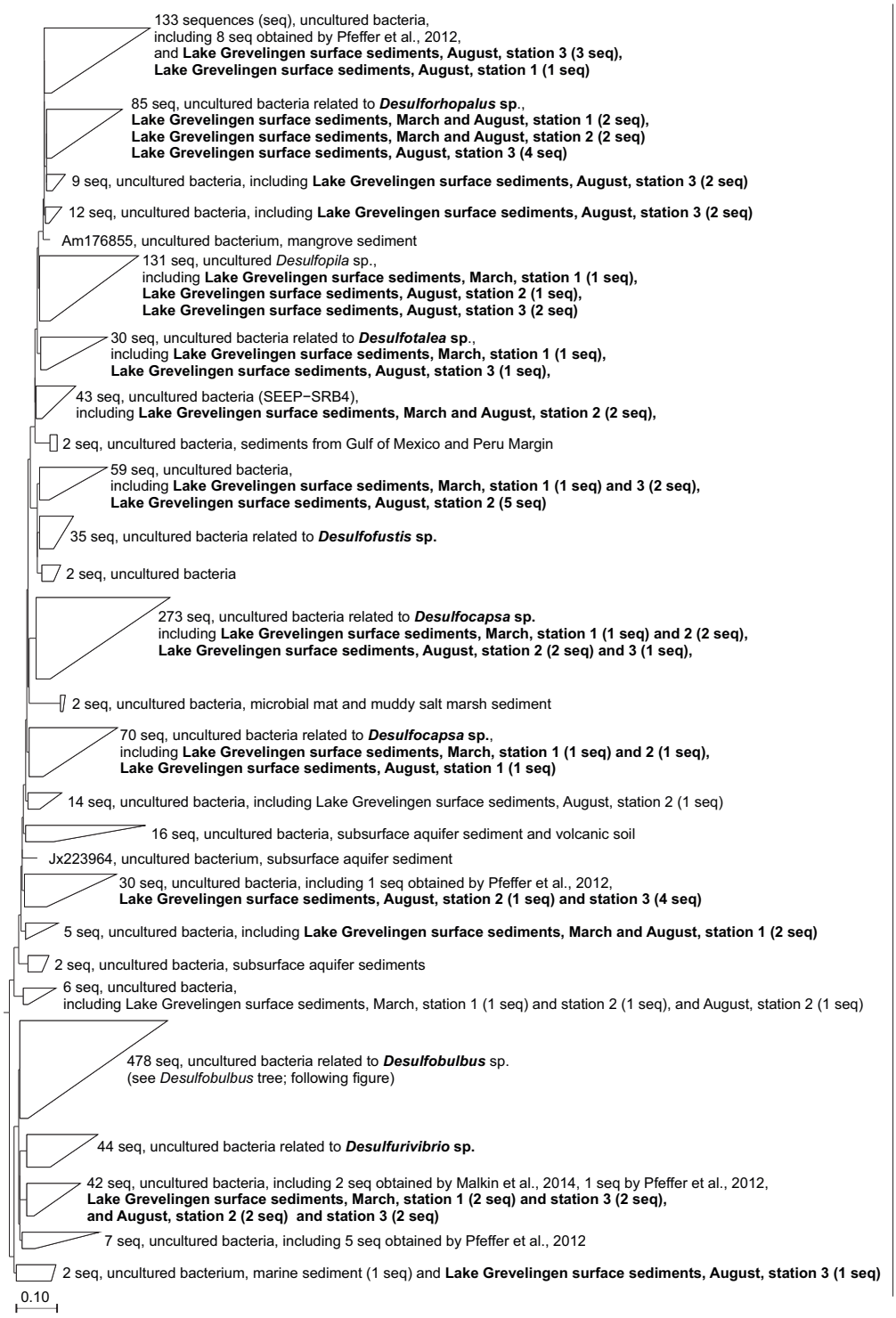

Figure S4a: Bacterial 16S rRNA gene amplicon sequences assigned to the class of Deltaproteobacteria, order: Desulfobacterales, family: Desulfobulbaceae (black bold: sequences retrieved in this study and closest relatives).

Figure S4b: Bacterial 16S rRNA gene amplicon sequences assigned to the class of Deltaproteobacteria, order: Desulfobacterales, family: Desulfubulbaceae, genus: *Desulfubulbus* (black bold: sequences retrieved in this study and closest relatives).

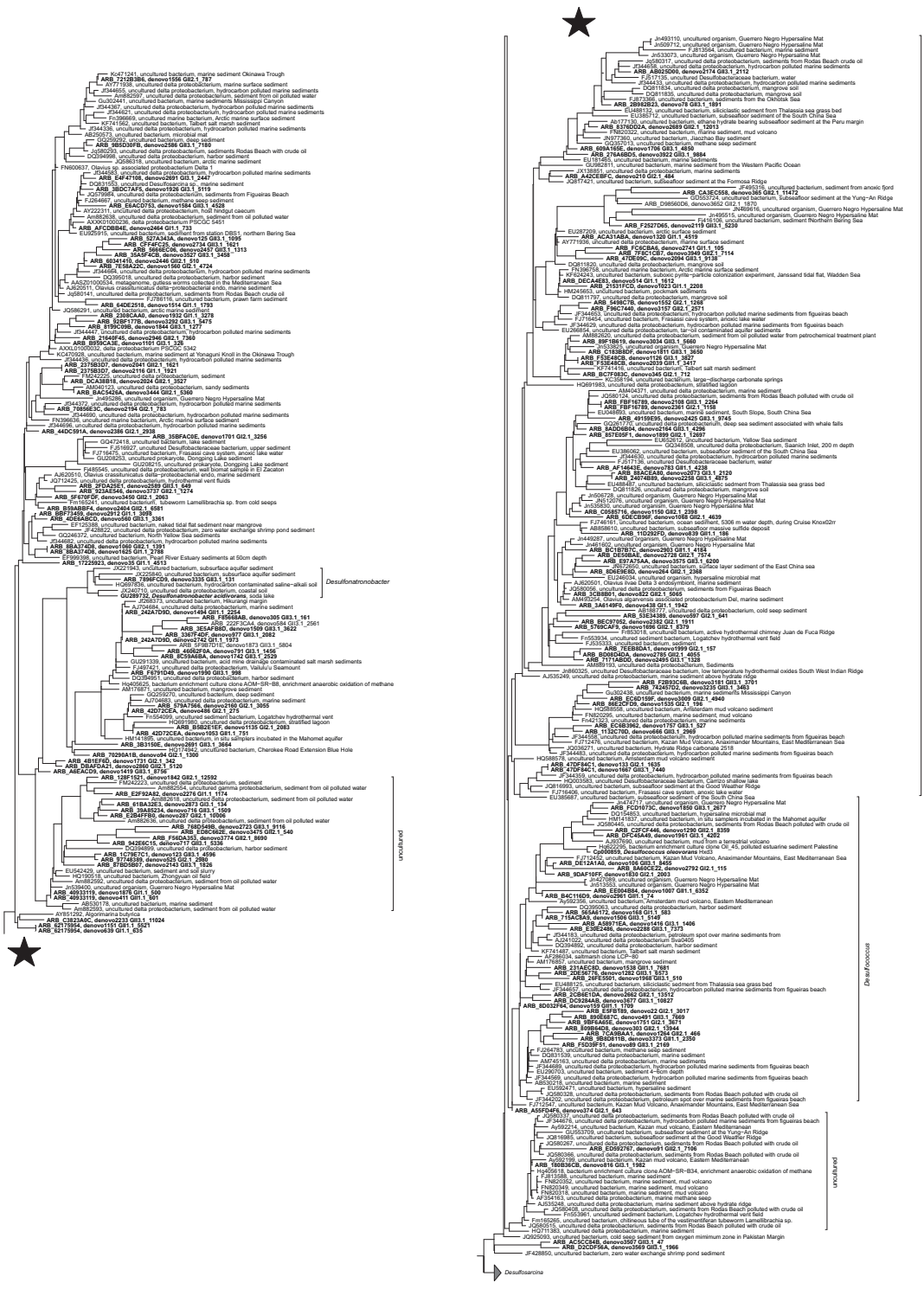

Figure S5a: Bacterial 16S rRNA gene amplicon sequences assigned to in the class of Deltaproteobacteria, order: Desulfobacterales, family: Desulfobacteraceae (black bold: sequences retrieved in this study and closest relatives).

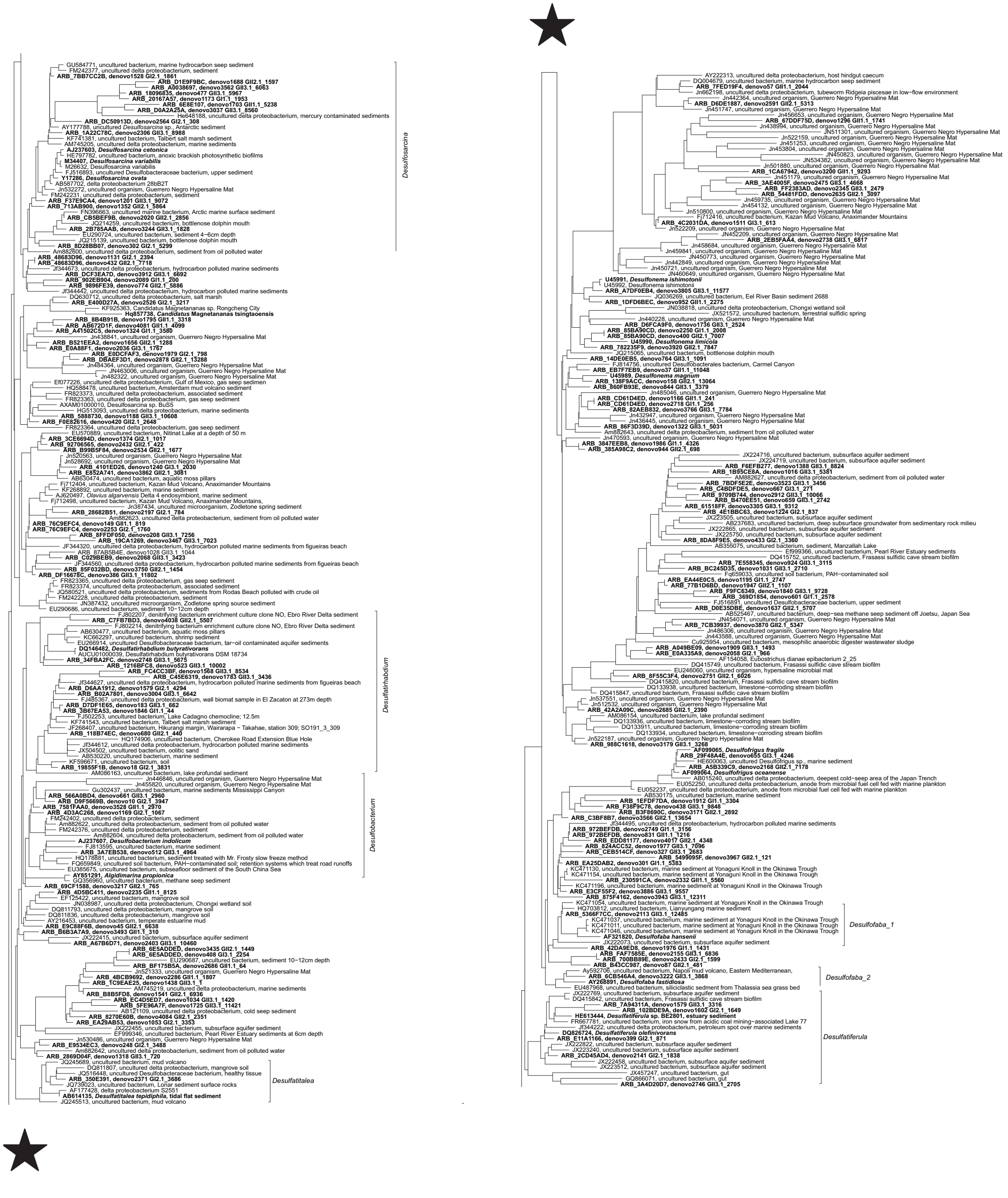

Figure S5b: Bacterial 16S rRNA gene amplicon sequences assigned to in the class of Deltaproteobacteria, order: Desulfobacterales, family: Desulfobacteraceae (black bold: sequences retrieved in this study and closest relatives).

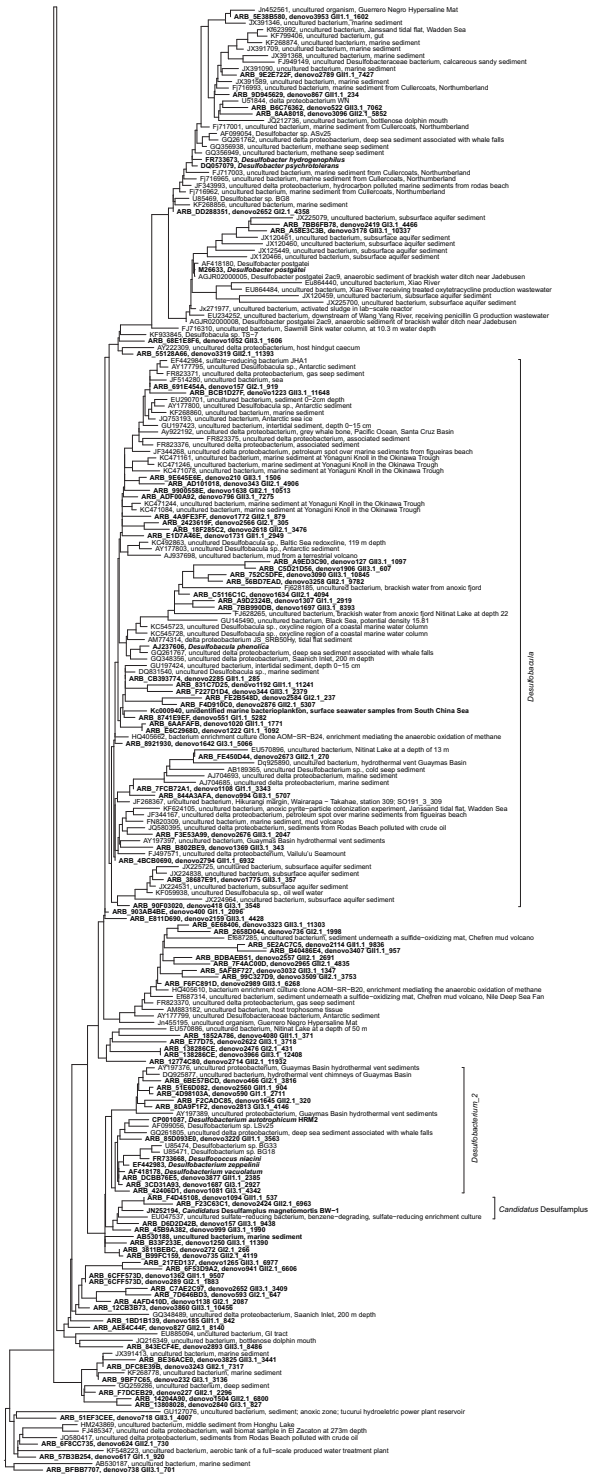

Figure S5c: Bacterial 16S rRNA gene amplicon sequences assigned to in the class of Deltaproteobacteria, order: Desulfobacterales, family: Desulfobacteraceae (black bold: sequences retrieved in this study and closest relatives).

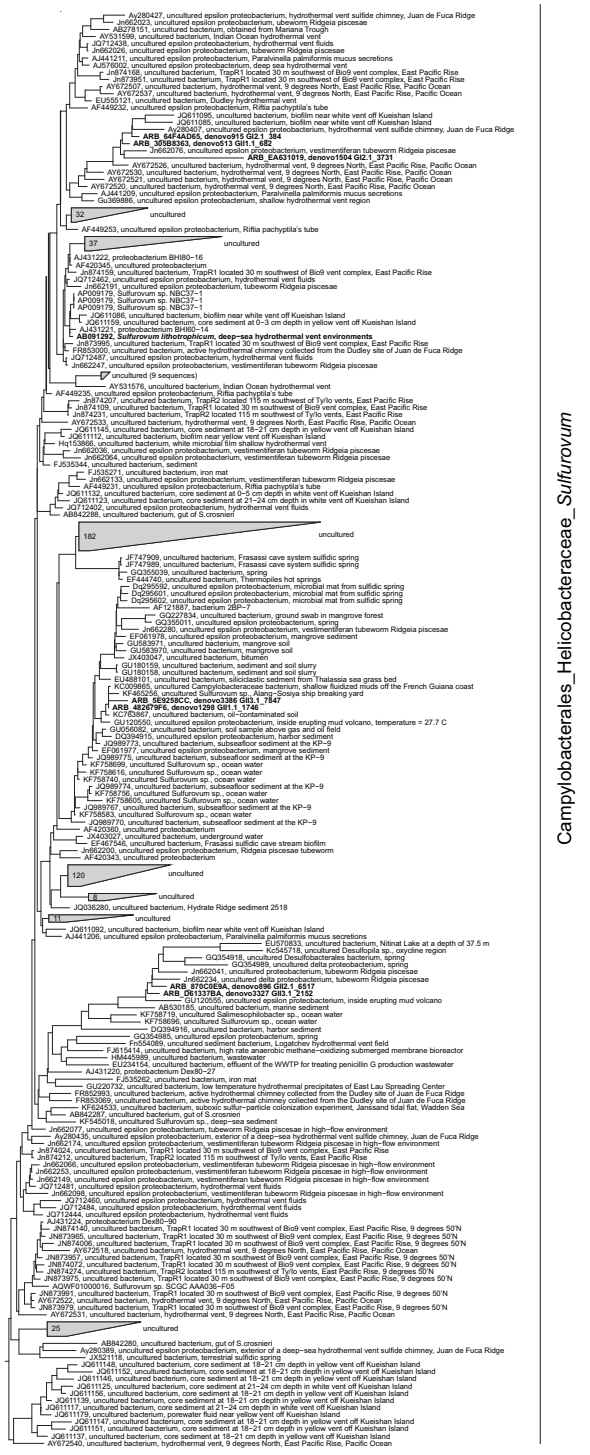

Figure S6a: Bacterial 16S rRNA gene amplicon sequences assigned to the class of Epsilonproteobacteria, order: Campylobacterales, family: Helicobacteraceae, genus: *Sulfurovum* (black bold: sequences retrieved in this study and closest relatives).

Figure S6b: Bacterial 16S rRNA gene amplicon sequences assigned to the class of Epsilonproteobacteria, order: Campylobacterales family: Campylobacteraceae, genus: *Arcobacter* (black bold: sequences retrieved in this study and closest relatives).

Figure S6c: Bacterial 16S rRNA gene amplicon sequences assigned to the class of Epsilonproteobacteria, order: Campylobacterales family: Campylobacteraceae, genus: *Sulfurimonas* (black bold: sequences retrieved in this study and closest relatives).

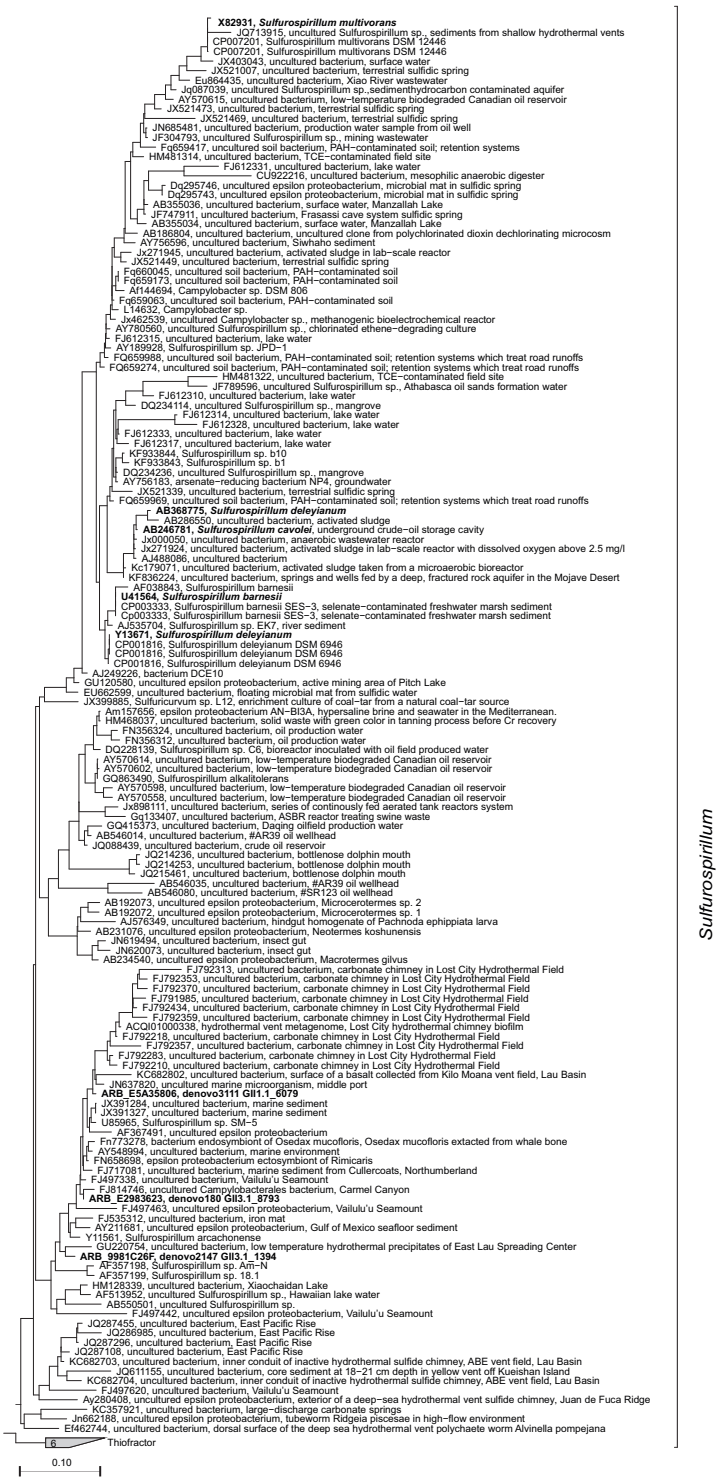

Figure S6d: Bacterial 16S rRNA gene amplicon sequences assigned to the class of Epsilonproteobacteria, order: Campylobacterales family: Campylobacteraceae, genus: *Sulfurospirillum* (black bold: sequences retrieved in this study and closest relatives).

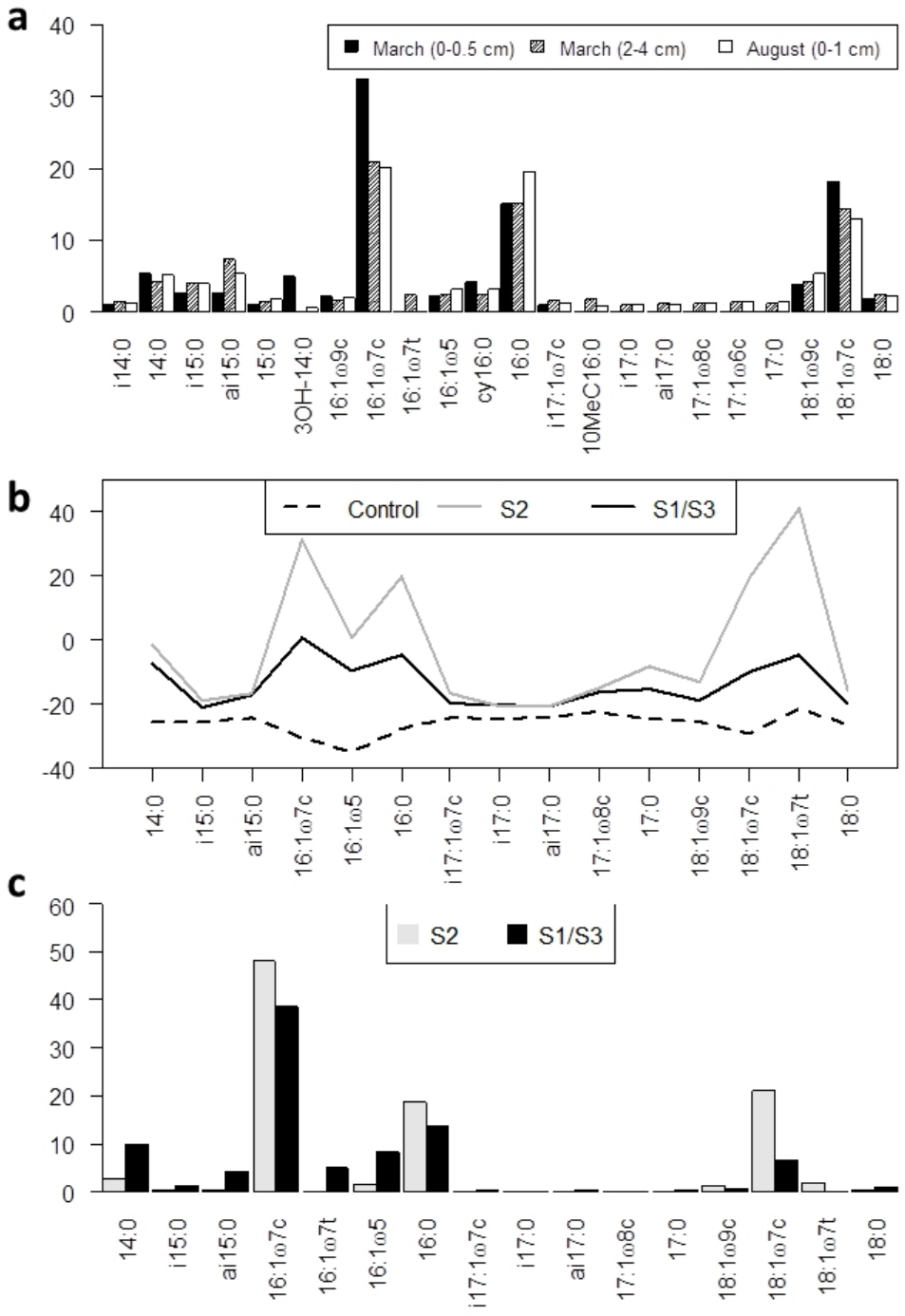

Figure S7: Characteristic PLFA profiles of (a) the average relative concentration of PLFAs for March (0–0.5 cm and 2–4 cm) and August (0–1 cm), (b) the average $\Delta\delta^{13}C$ values and (c) relative ^{13}C incorporation of PLFAs for S2 and S1/S3 March (0–1 cm).

6. Potential recycling of thaumarchaeotal lipids by DPANN archaea in seasonally hypoxic surface marine sediments

Yvonne A. Lipsewers, Ellen C. Hopmans, Jaap S. Sinninghe Damsté, and Laura Villanueva

Thaumarchaeota are known to synthesize specific glycerol dibiphytanyl glycerol tetraethers (GDGTs), the distribution of which is affected by temperature which forms the base of the paleotemperature proxy TEX86. Lipids present in the marine surface sediments are believed to be mostly derived from pelagic Thaumarchaeota; however, some studies have evaluated the possibility that benthic archaea also contribute to the lipid fossil record. Here, we compared the archaeal abundance and composition by DNA-based methods and the archaeal intact polar lipid (IPL) diversity in surface sediments of a seasonally hypoxic marine lake to determine the potential biological sources of the archaeal IPLs detected in surface sediments under changing environmental conditions. The archaeal community changed from March (oxic conditions) to August (euxinic) from a Thaumarchaeota-dominated (up to 82%) to an archaeal community dominated by the DPANN superphylum (up to 95%). This marked change coincided with a one order of magnitude decrease in the total IPL-GDGT abundance. In addition, the polar head groups of the IPL-GDGTs changed from predominantly containing phospho- to glyco- polar head groups. This may indicate a transition to Thaumarchaeota growing in stationary phase or selective preservation of the GDGT pool. However, considering the apparent inability of the DPANN archaea to synthesize their own membrane lipids, we hypothesize that an alternative explanation might be that DPANN archaea use the lipids previously synthesized by the Thaumarchaeota to form their own cell membranes, which would indicate an active recycling of fossil IPLs in the marine surface sediment.

Submitted to Organic Geochemistry

6.1. Introduction

Archaea occur ubiquitously, are abundant in aquatic and terrestrial habitats, and play an important role in global biogeochemical cycles (Jarrell et al., 2011). Marine sediments have been shown to harbor a diverse archaeal community, of which ammonia-oxidizing Thaumarchaeota (marine group I.1a) are the most studied (Pester et al., 2011). Recent genomic studies have revealed the presence of other benthic archaeal groups, such as Miscellaneous Crenarchaeota Group (MCG), Marine Benthic Group-B and D, and archaea of the DPANN superphylum, in marine sediments, which are predicted to be involved in degradation of polymers and proteins under anoxic conditions (Castelle et al., 2015; Lloyd et al., 2013, Meng et al., 2014).

Archaeal lipids occur widespread in marine sediments and are commonly used as biomarkers of Archaea both in present-day systems and in past depositional environments. However, their biological sources are not well constrained, especially in the light of the expanding archaeal diversity. Archaeal lipids in marine surface sediments are thought to be derived mainly from pelagic Thaumarchaeota, which due to grazing and packing in fecal pellets are efficiently transported to the sediment where they get preserved in the sedimentary record (Huguet et al., 2006b). Thaumarchaeota are known to synthesize isoprenoid glycerol dibiphytanyl glycerol tetraether (GDGT) containing 0–4 cyclopentane moieties (GDGT-0 to GDGT-4) as well as the GDGT crenarchaeol (Sinninghe Damsté et al., 2002), containing four cyclopentane moieties and a cyclohexane moiety, which is considered to be characteristic of this phylum (Pearson et al., 2004; Zhang et al., 2006; Pester et al., 2011; Pitcher et al., 2011a; Sinninghe Damsté et al., 2012). The distribution of thaumarchaeotal GDGTs in the marine environment has been shown to be affected by temperature, i.e. with increasing temperature there is an increase in the relative abundance of cyclopentane-containing GDGTs (Schouten et al., 2002; Wuchter et al., 2004, 2005). Based on this relationship, the TEX_{86} paleotemperature proxy was developed and calibrated on sea surface temperature (e.g. Schouten et al., 2002; Kim et al., 2010) and it has been widely applied for more than a decade (Schouten et al., 2013).

Although it is generally thought that GDGTs in marine sediments derive from surface-derived thaumarchaeotal biomass (e.g. Wakeham et al., 2003), some studies have addressed the possibility of a potential contribution of lipids from benthic archaea to the fossil record, which would be relevant for the reliability of TEX_{86} (Shah et al., 2008; Biddle et al., 2006; Lipp et al., 2008; Lipp and Hinrichs, 2009). Archaeal intact polar lipids (IPLs), where the core lipid (CL) GDGTs are attached to polar head groups from the building blocks of membranes of living cells with phospho head groups have been shown to degrade rapidly upon death of the source organism (White et al., 1979; Harvey et al., 1986), while IPLs with glyco polar head groups may be preserved over (much) longer timescales (Lipp et al., 2008; Lipp and Hinrichs, 2009; Bauersachs et al., 2010). IPLs of Archaea have been detected in surface marine sediments and used as biomarkers of the presence of living *in situ* benthic Archaea (Biddle et al., 2006; Lipp et al., 2008; Lipp and Hinrichs, 2009; Lengger et al., 2012). Evidence has been presented that the IPL-GDGTs produced *in situ* in the surface sediments are rapidly recycled upon burial (Lengger et al., 2012). Some studies have suggested that benthic marine archaea recycle fossil CL-GDGTs when producing IPL-GDGTs *de novo* to decrease energy requirements (Liu et al., 2011; Takano et al., 2010).

With the exception of Thaumarchaeota, the membrane lipid composition of other phyla of benthic archaea present in marine sediments has not been yet characterized and, therefore, their potential impact on the archaeal lipid pool preserved in the sedimentary record remains un-

known. In surface sediments where oxygen is still available, Thaumarchaeota are expected to be dominant due to their oxygenic metabolism as nitrifiers (Könneke et al., 2005, Wuchter et al., 2006). However in subsurface sediments, where oxygen is no longer present, archaeal groups such as MBG-D, MCG, Thermoplasmatales and methanogens have been reported to be predominant (Lloyd et al., 2013; Kubo et al., 2012), and may contribute to the total archaeal lipid pool in marine sediments. Recent studies have also observed a high presence of archaea of the superphylum DPANN both in surface and subsurface coastal marine sediments (Choi et al., 2016), as well as in freshwater systems (Ortiz-Alvarez et al., 2016; Ma et al., 2016). However, their contribution to the sedimentary archaeal lipid pool is expected to be negligible as their small genomes (Castelle et al., 2015) lack the genes of the membrane lipid biosynthetic pathway, suggesting that they rely on host cells or cell debris for the synthesis of their lipids (Waters et al., 2003; Jahn et al., 2004).

Here, we determined the archaeal abundance and composition using DNA-based methods in surface sediments of a seasonally hypoxic marine lake with different oxygen and sulfide bottom water concentrations and compared this with the composition of the archaeal IPLs. We aim to identify the potential biological sources of the archaeal IPL lipids detected in surface sediments under changing environmental conditions which could impact the biology of the producer but also the preservation potential of the archaeal lipids.

6.2. Material and Methods

6.2.1. Study site, sediment sampling and physicochemical analyses

Lake Grevelingen is a former estuary located within the Rhine-Meuse-Scheldt delta area of the Netherlands. The delta became a closed saline reservoir (salinity ~30) by dam construction at both the land side and sea side in the early 1970s. As a result of the absence of tides and strong currents, Lake Grevelingen experiences a seasonal stratification of the water column, which in turn, leads to a depletion of oxygen in the bottom waters (Hagens et al., 2015). Bottom water oxygen concentration at the deepest stations starts to decline in April, reaches hypoxic conditions by end of May (O_2 < 63 μM), and further decreases to reach the state of anoxia in August (O_2 < 0.1 μM), while re-oxygenation of the bottom water takes place in September (Seitaj et al., 2015).

Two sampling campaigns were performed on March 13th, 2012 (before the start of the annual O_2 depletion) and on August 20th, 2012 (at the peak of the annual O_2 depletion). Detailed water column, pore water and solid sediment chemistry of Lake Grevelingen over the year 2012 have been previously reported (Seitaj et al., 2015; Sulu-Gambari et al., 2016, Hagens et al., 2015). Intact sediment cores were recovered at three stations along a depth gradient within the Den Osse basin, one of the deeper basins in Marine Lake Grevelingen: Station 1 (S1) was located in the deepest point (34 m) of the basin (51.747°N, 3.890°E), Station 2 (S2) at 23 m depth (51.749°N, 3.897°E) and Station 3 (S3) at 17 m depth (51.747°N, 3.898°E) (Fig. S1). Sediment cores were retrieved with a single core gravity corer (UWITEC) using PVC core liners (6 cm inner diameter, 60 cm length). Further details of sediment sampling have been previously described in detail (Chapter 4). Four sediment cores were sliced with a 1 cm resolution (for the purposes of this study we only focused on the top centimeter) for lipid and DNA/RNA analysis and kept at –80ºC until further processing.

The total organic carbon (TOC) content of the sediment was determined on sub-samples that were freeze-dried, ground to a fine powder and analyzed by an a Thermo Finnigan Delta plus isotope ratio monitoring mass spectrometer (irmMS) connected to a Flash 2000 elemental analyzer (Thermo Fisher Scientific, Milan). Before the analysis, samples were first acidified with 2N hydrogen chloride (HCl) to remove the inorganic carbon (Nieuwenhuize et al., 1994). Concentrations of TOC are expressed as mass % of dry sediment. Oxygen, sulfide, bottom water and pore water ammonium, nitrite and nitrate were determined as previously described (Malkin et al., 2014; Seitaj et al., 2015) and reported in Chapter 5.

6.2.2. DNA/RNA extraction

Sediment was centrifuged and the excess of water was removed by pipetting before proceeding with the extraction of nucleic acids from the sediment. DNA/RNA of surface (0–1 cm) sediments was extracted with the RNA PowerSoil® Total Isolation Kit plus the DNA elution accessory (Mo Bio Laboratories, Carlsbad, CA). Concentration of DNA was quantified by Nanodrop (Thermo Scientific, Waltham, MA) and Fluorometric with Quant-iT™ PicoGreen® dsDNA Assay Kit (Life technologies, Netherlands).

6.2.3. 16S rRNA gene amplicon sequencing and sequencing analyses

PCR reactions were performed with the universal, Bacteria and Archaea, primers S-D-Arch-0159-a-S-15 and S-D-Bact-785-a-A-21 (Klindworth et al., 2013) as previously described in Moore et al. (2015). The archaeal 16S rRNA gene amplicon sequences were analyzed by QIIME v1.9 (Caporaso et al., 2010). Raw sequences were demultiplexed and then quality-filtered with a minimum quality score of 25, length between 250–350, and allowing maximum two errors in the barcode sequence. Taxonomy was assigned based on blast and the SILVA database version 123 (Altschul *et al.*, 1990; Quast *et al.*, 2013). The 16S rRNA gene amplicon reads (raw data) have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject no. PRJNA293286.

6.2.4. PCR amplification, cloning and archaeal 16S rRNA gene quantification

Amplification of the archaeal *amoA* gene was performed as described by Yakimov et al. (2011). PCR reaction mixture was the following (final concentration): Q-solution 1 × (PCR additive, Qiagen); PCR buffer 1 ×; BSA (200 μg ml^{-1}); dNTPs (20 μM); primers (0.2 pmol μl^{-1}); $MgCl_2$ (1.5 mM); 1.25 U Taq polymerase (Qiagen, Valencia, CA, USA). PCR conditions for these amplifications were the following: 95°C, 5 min; 35 × 95°C, 1 min; 55°C, 1 min; 72°C, 1 min]; final extension 72°C, 5 min. PCR products were gel purified (QIAquick gel purification kit, Qiagen) and cloned in the TOPO-TA cloning® kit from Invitrogen (Carlsbad, CA, USA) and transformed in *E. coli* TOP10 cells following the manufacturer's recommendations. Recombinant clones plasmid DNAs were purified by Qiagen Miniprep kit and screening by sequencing ($n \geq 30$) using M13R primer by BaseClear (Leiden, The Netherlands). Obtained archaeal *amoA* protein sequences were aligned with already annotated *amoA* sequences by using the Muscle application (Edgar, 2004). Phylogenetic trees were constructed with the Neighbor-Joining method (Saitou and Nei, 1987) and evolutionary distances computed using the Poisson correction method with a bootstrap test of 1,000 replicates. Quantification of archaeal 16S rRNA gene copies was performed by quantitative PCR (qPCR) by using the primers Parch519F and ARC915R as previously described (Pitcher et al., 2011b).

6.2.5. Lipid extraction and analysis

Total lipids were extracted from surface (upper 0–1 cm) sediments after freeze-drying using a modified Bligh and Dyer method (Bligh and Dyer, 1959) following an experimental protocol previously described by Lengger et al. (2014). The extracts were then dissolved by adding solvent (hexane:isopropanol:H_2O 718:271:10 [v/v/v/v]) and filtered through a 0.45 μm, 4 mm-diameter True Regenerated Cellulose syringe filter (Grace Davison, Columbia, MD, USA).

IPLs were analyzed with Ultra High Pressure Liquid Chromatography- High Resolution Mass spectrometry (UHPLC-HR MS). An Ultimate 3000 RS UHPLC, equipped with thermostated auto-injector and column oven, coupled to a Q Exactive Orbitrap MS with Ion Max source with heated electrospray ionization (HESI) probe (Thermo Fisher Scientific, Waltham, MA), was used. Separation was achieved on a YMC-Triart Diol-HILIC column (250 x 2.0 mm, 1.9 μm particles, pore size 12 nm; YMC Co., Ltd, Kyoto, Japan) maintained at 30°C. The following elution program was used with a flow rate of 0.2 mL min^{-1}: 100% A for 5 min, followed by a linear gradient to 66% A: 34% B in 20 min, maintained for 15 min, followed by a linear gradient to 40% A: 60% B in 15 min, followed by a linear gradient to 30% A: 70% B in 10 min, where A = hexane/2-propanol/formic acid/14.8 M NH_{3aq} (79:20:0.12:0.04 [v/v/v/v]) and B = 2-propanol/water/formic acid/ 14.8 M NH_{3aq} (88:10:0.12:0.04 [v/v/v/v]). Total run time was 70 min with a re-equilibration period of 20 min in between runs. HESI settings were as follows: sheath gas (N_2) pressure 35 (arbitrary units), auxiliary gas (N_2) pressure 10 (arbitrary units), auxiliary gas (N_2) T 50 °C, sweep gas (N_2) pressure 10 (arbitrary units), spray voltage 4.0 kV (positive ion ESI), capillary temperature 275 °C, S-Lens 70 V. IPLs were analyzed with a mass range of m/z 375 to 2000 (resolution 70,000), followed by data-dependent MS^2 (resolution 17,500), in which the ten most abundant masses in the mass spectrum (with the exclusion of isotope peaks) were fragmented successively (stepped normalized collision energy 15, 22.5, 30; isolation window 1.0 m/z). An inclusion list was used with a mass tolerance of 3 ppm to target specific compounds (supplementary File S1). The Q Exactive Orbitrap MS was calibrated within a mass accuracy range of 1 ppm using the Thermo Scientific Pierce LTQ Velos ESI Positive Ion Calibration Solution (containing a mixture of caffeine, MRFA, Ultramark 1621, and *N*-butylamine in an acetonitrile-methanol-acetic solution). IPLs were quantified by integrating the summed mass chromatograms (within 3 ppm mass accuracy) of the dominant adduct formed (in case of MH-, DH- and HPH-IPLs the ammoniated adduct) and the first isotopomer and reported as peak area response per gram of dry sediment extracted, due to the lack of quantitative standards (for details see Pitcher et al., 2011b).

The total lipid extracts from the surface sediments were further analyzed by acid hydrolysis to determine the composition and relative abundance of IPL-derived CL (resulting from the acid hydrolysis of IPLs) and CL-GDGTs using the method described by Lengger et al. (2012) and analyzed by via high-performance liquid chromatography-atmospheric pressure chemical ionization/mass spectrometry (HPLC-APCI/MS) (Schouten et al., 2007) using an internal C_{46} GTGT standard as described previously (Huguet al. 2006a).

6.3. Results

6.3.1. Physicochemical conditions

The seasonal variation of the bottom water oxygen concentration in Lake Grevelingen strongly

influenced the bottom and pore water concentrations of O_2 and sulfide (Table 1). In March, bottom waters were fully oxygenated in all stations (299–307 μM), oxygen penetrated 1.8–2.6 mm deep in the sediment, and no free sulfide was recorded in the first few centimeters (Hagens *et al.*, 2015). The width of the suboxic zone, operationally defined as the sediment layer located between the oxygen penetration depth (OPD) and the sulfide appearance (SAD), varied between 16–39 mm across the three stations in March 2012. In contrast, in August, oxygen was strongly depleted in the bottom waters at S1 (<0.1 μM) and S2 (11 μM), and no O_2 was detected by microsensor profiling in the surface sediment at these two stations. At station S3, the bottom water concentration of O_2 remained higher (88 μM), and oxygen still penetrated into the surface sediment down to 1.1 mm. In August, free sulfide was present near the sediment-water interface at all three stations, and the concentration of sulfide in the pore water increased with water depth.

Bottom water ammonium (NH_4^+) concentrations in station S1 ranged from 3 μM in March to 11.5 μM in August; nitrite (NO_2^-) concentrations were relatively constant (0.7–1 μM) in March and August. Nitrate concentrations ranged from 28 μM in March to <2 μM in August in station S1, whereas in stations S2 and S3 values varied between 28 μM in March to ~10 μM in August (Table 1; for detailed bottom water biogeochemistry see Seitaj et al., 2015; Sulu-Gambari et al., 2016; Hagens et al., 2015; Chapter 4).The TOC content of the sediments varied slightly between stations and seasons, ranging between 1.8 and 4.4% as described previously (Table 1; Chapter 4).

6.3.2. Archaeal lipid diversity and distribution

The archaeal IPL GDGT concentration (quantified as IPL-derived CLs) was almost an order of magnitude higher in March in all stations in comparison with the values in August, although this drop was less for station 3, where slightly lower concentrations of GDGT-0 and crenarchaeol were detected in March in comparison with the other stations (Table 2). In March, total IPL-GDGTs were mostly distributed between IPL-GDGT-0 (approximately 5 μg g^{-1} dry weight of sediment) and IPL-crenarchaeol (average 2.6 μg g^{-1}). In August, the total IPL-GDGT abundance was lower in comparison with March (approximately an order of magnitude less), while the distribution did not change (Table 2). CL-GDGT abundance was also higher in March (26 to 79 μg g^{-1} dry weight of sediment) than in August (5 to 15 μg g^{-1} dry weight of sediment) (Table 2). CL-GDGT abundances were also higher than those of IPL-GDGTs at all stations and seasons (Table 2). In station 1 it was on average three times higher both in March and in August. On the other hand in station 2, CL-GDGTs were in average 8 times higher than IPL-GDGTs in March, and 30 times higher in August, with CL-crenarchaeol as the most abundant CL-GDGT in August (8 μg g^{-1} dry weight of sediment; Table 2), 40 times more of that detected for IPL-crenarchaeol (0.2 μg g^{-1}; Table 2). In station 3, CL-GDGTs were on average 4 times higher in concentration than IPL-GDGTs (Table 2).

The IPL composition was assessed by UHPLC-HRMS analysis and various GDGT-IPLs and archaeol-IPLS were specifically targeted by using an inclusion list as part of the analytical routine (see supplementary File S1). Within the various IPL types detected, the distribution of observed cores was comparable to the distribution obtained after analysis of the IPL-derived CLs. IPLs with GDGT-0 and crenarchaeol as CL are the most abundant IPLs, with the highest concentrations in March (approximately 10^{10} response units g^{-1}), while IPLs with GDGT-1 and -2 as CLs were two orders of magnitude less abundant (Table 3). In August, total IPL-GDGT concentrations decreased by two orders of magnitude as compared with the concentrations in

Table 1: Physicochemical parameters of the bottom water and surface sediment (0–1 cm) in the three stations in Lake Grevelingen in spring (March) and in summer (August). Data included in Seitaj et al., 2015; Sulu-Gambari et al., 2016; Hagens et al., 2015; Chapter 4 and 5.

Bottom water	Station 1		Station 2		Station 3	
	August	March	August	March	August	March
Temperature [°C]	5	17	5	17	5	19
O_2 [µM]	299	0	301	12	307	88
	(oxic)	(anoxic)	(oxic)	(hypoxic)	(oxic)	(hypoxic)
NH_4^+ [µM]	3.2	11.5	3.0	4.3	2.8	2.5
NO_2^- [µM]	0.7	1.0	0.7	0.7	0.7	0.1
NO_3^- [µM]	28.2	1.7	27.9	11.6	27.7	10.6
Surface sediment						
HS^- [µM]	0	810	0	1157	0	211
$NH4^+$ [µM]	279	656	165	550	73	537
TOC [%]	2.86	1.81	3.11	2.38	2.87	3.04
OPD [mm]	1.8±0.04	0	2.6±0.65	0	2.4±0.4	1.1±0.1
SAD [mm]	17.5±0.7	0.9±1.1	21.3±2.5	0.6±0	41.8±8.6	4.2±2.7

Bottom water is classified as anoxic with O_2 concentration below 1 µM and hypoxic below 63 µM; OPD: O_2 penetration depth; SAD: $\sum H_2S$ appearance depth.

Table 2: Abundance and distributiona of IPL-derived (released by acid hydrolysis) and CL GDGTs (μg g^{-1} of dry weight) in the surface sediment (0–1 cm) of the three stations in Lake Grevelingen in spring (March) and in summer (August).

	Station 1		Station 2		Station 3	
	March	**August**	**March**	**August**	**March**	**August**
IPL-derived GDGTs						
GDGT-0	5.1 (56.9)	0.8 (48.2)	5.9 (60.5)	0.2 (45.5)	3.0 (58.6)	1.7 (54.6)
GDGT-1	0.4 (4.6)	0.1 (5.5)	0.4 (4.4)	0.02 (4.9)	0.3 (5)	0.1 (4.6)
GDGT-2	0.2 (2.2)	0.1 (3.7)	0.2 (2.2)	0.01 (2.6)	0.1 (2.7)	0.1 (2.3)
GDGT-3	0.1 (0.9)	0.02 (1.3)	0.1 (0.8)	0.01 (1.1)	0.05 (1)	0.03 (0.9)
Cren[b]	3.1 (34.8)	0.6 (40.8)	3.1 (31.6)	0.2 (44.8)	1.7 (32.5)	1.1 (37.2)
Cren'[b]	0.04 (0.5)	0.01 (0.5)	0.04 (0.4)	0.01 (1.1)	0.02 (0.3)	0.01 (0.4)
Total	**9.0**	**1.6**	**9.7**	**0.5**	**5.1**	**3.0**
CL-GDGTs						
GDGT-0	12.9 (44)	2.1 (43)	36.2 (46)	5.8 (39.3)	11.8 (45.3)	2.9 (40.8)
GDGT-1	1.1 (3.7)	0.2 (3.6)	2.9 (3.7)	0.5 (3.5)	0.9 (3.5)	0.2 (3.5)
GDGT-2	0.5 (1.6)	0.1 (1.6)	1.3 (1.6)	0.2 (1.4)	0.4 (1.5)	0.1 (1.5)
GDGT-3	0.2 (0.8)	0.04 (0.7)	0.6 (0.8)	0.1 (0.7)	0.2 (0.7)	0.05 (0.7)
Cren	14.5 (49.5)	2.5 (50.5)	37.5 (47.6)	8.1 (54.8)	12.7 (48.8)	3.7 (53)
Cren'	0.1 (0.4)	0.03 (0.5)	0.3 (0.4)	0.04 (0.3)	0.1 (0.2)	0.04 (0.6)
Total	**29.2**	**4.9**	**78.8**	**14.8**	**26.0**	**7.1**

[a]Values between parentheses correspond to the fractional (relative) abundance of each individual GDGT as the concentration of the individual GDGT divided by the sum of the concentration of all GDGTs in that fraction. [b] Cren = Crenarchaeol, Cren' = crenarchaeol regioisomer.

Table 3: Absolute abundance as response units per gram of dry weight of detected archaeal GDGT IPLs in surface sediments (0–1cm).

GDGT IPL	Station 1		Station 2		Station 3	
	March	August	March	August	March	August
GDGT-0-MH	9.4×10^{7}	1.3×10^{7}	3.1×10^{8}	4.4×10^{7}	1×10^{8}	4.9×10^{7}
GDGT-0-DH	6.3×10^{7}	n.d.	7.3×10^{7}	n.d.	6.7×10^{7}	n.d.
GDGT-0-DH'	2×10^{8}	n.d.	2.1×10^{8}	n.d.	1.4×10^{8}	2.6×10^{7}
GDGT-0-HPH	1.7×10^{10}	3.3×10^{8}	1.9×10^{10}	8.5×10^{8}	1.4×10^{10}	1.8×10^{9}
GDGT-1-MH	9.4×10^{7}	n.d.	8.9×10^{6}	n.d.	1.2×10^{6}	n.d.
GDGT-1-DH	1.9×10^{8}	n.d.	2.1×10^{8}	n.d.	6.2×10^{7}	n.d.
GDGT-1-DH'	n.d.	n.d.	n.d.	n.d.	8.2×10^{7}	n.d.
GDGT-1-HPH	4.6×10^{8}	n.d.	3.4×10^{8}	n.d.	5.2×10^{8}	n.d.
GDGT-2-DH	1.4×10^{8}	n.d.	2.7×10^{8}	n.d.	1.1×10^{8}	n.d.
GDGT-3-DH	8×10^{6}	n.d.	2.7×10^{7}	n.d.	1×10^{7}	n.d.
GDGT-3-DH'	n.d.	n.d.	1.2×10^{7}	n.d.	n.d.	n.d.
GDGT-4-DH	7.8×10^{7}	n.d.	1.5×10^{8}	n.d.	5×10^{7}	n.d.
GDGT-4-DH'	n.d.	n.d.	n.d.	n.d.	1.3×10^{7}	n.d.
Crenarchaeol-MH	1.5×10^{8}	5.3×10^{7}	3.1×10^{8}	5.7×10^{7}	9.5×10^{7}	7×10^{7}
Crenarchaeol-HPH	1.1×10^{10}	2.4×10^{8}	8.5×10^{9}	6.1×10^{8}	6.6×10^{9}	1.5×10^{9}
Archaeol-DH	3.8×10^{8}	n.d.	n.d.	n.d.	n.d.	n.d.
Total ru g^{-1}	3.0×10^{10}	8.7×10^{8}	3.0×10^{10}	1.6×10^{9}	2.2×10^{10}	3.4×10^{9}

'isomers of specific GDGTs; n.d.: not detected. MH: monohexose, DH: Dihexose, HPH, hexose phosphohexose.

Table 4: Archaeal classes abundances (cells g^{-1} of sediment) calculated by multiplying the percentages of total archaeal 16S rRNA gene reads by the archaeal 16S rRNA gene abundance (copy number g^{-1} of sediment) detected in the surface sediment (0–1cm) of the three stations in March and in August, assuming one 16S rRNA gene copy number per genome.

organism	Station 1		Station 2		Station 3	
	March	August	March	August	March	August
Thermoplasmata, 20a-9	n.d	n.d	n.d	7.8×10^8	n.d	2.0×10^8
Thermoplasmatales, AMOS1A-4113-D04	n.d	n.d	5.5×10^7	6.0×10^7	4.1×10^8	6.0×10^8
Thermoplasmatales, CCA47	n.d	n.d	1.7×10^8	2.4×10^8	2.3×10^8	1.7×10^7
Thermoplasmatales, MBG-D & DHVEG-1	n.d	1.1×10^8	6.1×10^8	7.8×10^8	1.2×10^8	2.0×10^8
Thermoplasmatales, VC2.1 Arc6	n.d	n.d	1.1×10^8	3.0×10^8	5.8×10^7	1.2×10^9
Sum of reads Thermoplasmatales	n.d	1.1×10^8	9.4×10^8	1.4×10^9	8.2×10^8	3.7×10^9
Miscellaneous Crenarchaeota Group, C3	9.9×10^7	1.1×10^8	1.7×10^8	2.1×10^9	n.d	9.0×10^8
Thaumarchaeota, Marine Group I, *Nitrosopumilus*	5.8×10^9	n.d	3.1×10^9	2.9×10^9	2.2×10^{10}	1.2×10^9
Miscellaneous Euryarchaeotic Group (MEG)	n.d	n.d	n.d	1.4×10^9	n.d	n.d
DPANN, Woesearchaeota DHVEG-6	1.1×10^9	4.2×10^9	2.0×10^9	1.9×10^{10}	6.4×10^9	1.2×10^{10}
MBG-B	n.d	n.d	n.d	9.0×10^8	n.d	1.0×10^9
Others	4.9×10^7	n.d	2.2×10^8	6.0×10^7	1.5×10^9	2.3×10^9
Total archaeal abundance [cell g^{-1}]	7.1×10^9	4.4×10^9	6.5×10^9	2.8×10^{10}	3.1×10^{10}	2.1×10^{10}
Total CL-GDGTs and IPL-derived GDGT mg g^{-1}	38.2	6.5	88.5	15.3	31.1	10.1
Calculated femtograms of GDGT per cell	1.3	7.5	3.0	9.8	1.4	2.9

n.d = not detected. MBG-D, Marine Benthic Group D; MBG-B, Marine Benthic Group B.

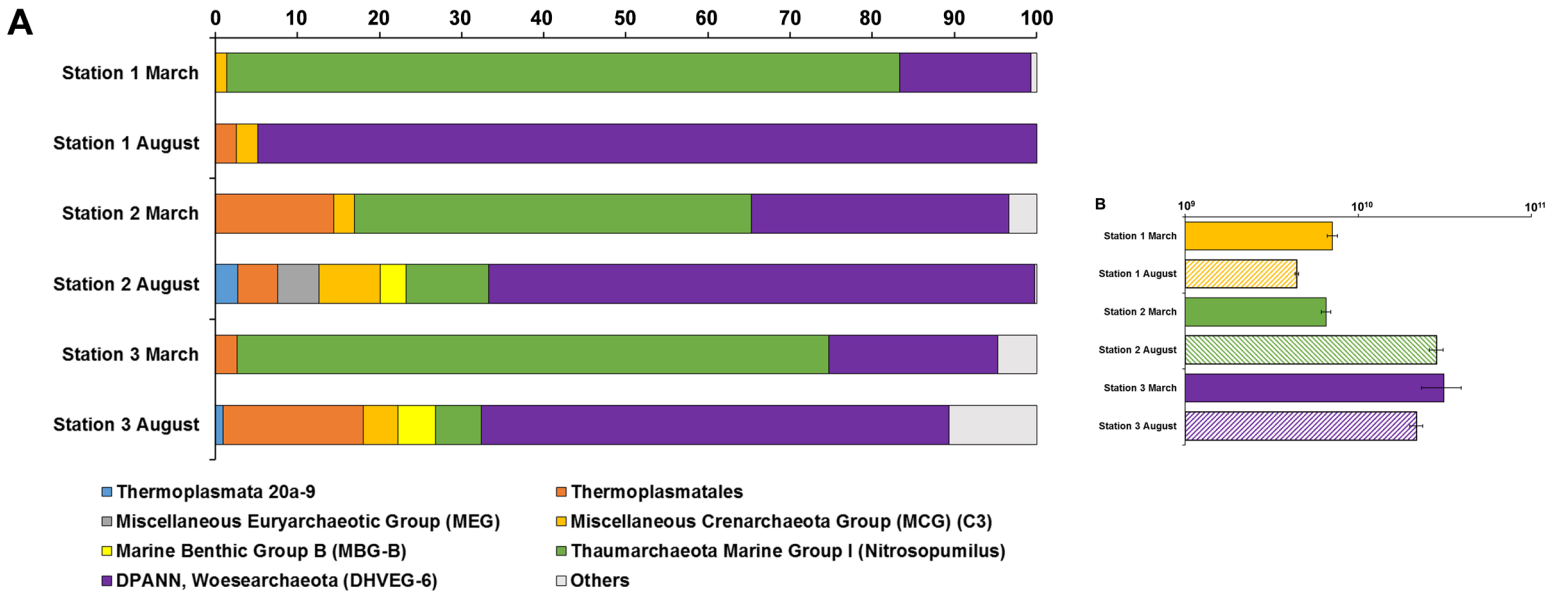

Figure 1: (A) Percentages of total archaeal 16S rRNA gene reads [%] and **(B)** archaeal 16S rRNA gene abundance [copy number g^{-1} of sediment] detected in the surface sediment (0–1cm) of the three stations in March and in August. Only archaeal groups with percentages >3% are reported. qPCR conditions, efficiency = 90%; R_2=0.991.

March. In March both IPLs with GDGT-0 and crenarchaeol cores were mostly found with hexose phosphohexose (HPH) as IPL type, while in August the IPL type MH increased at the expense of HPH (Table 3).

6.3.3. Archaeal 16S rRNA gene diversity and abundance

Archaeal diversity was estimated by 16S rRNA gene amplicon sequencing using universal primers. The archaeal community was dominated by Thaumarchaeota marine group I (MGI) in March both in station 1 and 3 with 72–82% of the total archaeal reads (Fig. 1A, Table S1). In station 2 these archaea were slightly less dominant but still comprised almost half of the total archaeal reads. Other archaeal groups were also present in the surface sediment in March in addition to MGI such as members of the DPANN Woesearchaeota (DHVE-6) (16–31%), Thermoplasmatales (3–14%), and MCG (1.4–2.5%). In August, the percentage of reads attributed to the DPANN Woesearchaeota increased notably from 16 to 95% in station 1, from 31 to 66% in station 2, and from 21 to 57% in station 3 at the expense of the reads assigned to the Thaumarchaeota MGI (Fig. 1A, Table S1). In addition, a slight increase of percentage of reads was observed from March to August for archaea affiliated to the MCG and Thermoplasmatales groups. Total archaeal abundance estimated by qPCR with general archaea 16S rRNA gene primers was comparable between March and August in all stations with slightly lower values in station 1 (average 6×10^9 gene copies g^{-1}) and $1–2 \times 10^{10}$ gene copies g^{-1} for station 2 and 3 (Fig. 1B).

The diversity of Thaumarchaeota was further analyzed by amplification, cloning and sequencing of the *amoA* gene in the surface sediments of the three stations. All *amoA* sequences were closely related to sequences previously detected in marine surface sediments. Although certain variability was detected between the sequences recovered in different stations and seasons, no clear clustering was observed (Figs. S2A, B).

6.4. Discussion

The seasonal changes in the oxygen and sulfide concentration in the bottom water of the marine Lake Grevelingen trigger an important switch in the archaeal community composition in the surface sediment. The archaeal community changed considerably from a Thaumarchaeota MGI-dominated community in March, when oxygen was still present and sulfide was not (Table 1) to an archaeal community dominated by archaea of the DPANN superphylum. Besides, the seasonal change did not induce a diversity change within the Thaumarchaeota as indicated by the *amoA* gene phylogeny (Figs. S2A, B).

By multiplying the percentage of 16S rRNA gene reads of the Thaumarchaeota and the DPANN Woesearchaeota by the total archaea 16S rRNA gene copies per gram of sediments, we can estimate the abundances of these groups (assuming one 16S rRNA gene copy number per genome) (Table 4). For Thaumarchaeota, the abundance decreased dramatically in station 1 (from 6×10^9 cells g^{-1} to undetected) and in station 3 (2×10^{10} to 1.2×10^9 cells g^{-1}), while in station 2 the abundance remained fairly constant (Table 4). On the other hand, members of the DPANN Woesearchaeota were the major component of the archaeal population in August in all stations with absolute numbers increasing in all stations (1×10^9 to 4.2×10^9 cells g^{-1} of sediment in station 1; 2×10^9 to 2×10^{10} cells g^{-1} in station 2, 6.4×10^9 to 1.2×10^{10} cells g^{-1} in station 3; Table 4). This change in the archaeal community is entirely compatible with the metabolism of both Thaumarchaeota and the DPANN archaea, which is aerobic (Könneke

et al., 2005) and anaerobic (Castelle et al., 2015), respectively. Also, nitrification conducted by Thaumarchaeota has been shown to be inhibited by sulfide (Berg et al., 2015), which additionally explains the decrease in the MGI population upon increase of the sulfide concentration in the summer. In the same way, the abundance of the other archaeal benthic groups, which increased in the surface sediments in August, such as the Thermoplasmatales and the MCG, are predicted to have an anaerobic metabolism based on their genome. The environmental control of the change in the archaeal community diversity is also supported by the fact that this change is less evident in the surface sediment of station 3, where oxygen was still present and the sulfide concentration in summer was substantially lower than at stations 1 and 2 (Table 1). Although the seasonality changes in the physicochemical conditions in the bottom and pore water induced a large change in the archaeal community diversity, the total archaeal abundance remained fairly constant between seasons and in the different stations (Fig. 1B). The archaeal community analysis was conducted by primer-based 16S rRNA gene amplicon sequencing. Therefore, we cannot completely rule out a possible primer bias (Sipos et al., 2010; Schloss et al., 2011), although the underrepresentation of DPANN sequences in the databases would imply that if any those primer biases would negatively detect DPANN in our samples, which in that case would induce to a underestimation of this group.

The large change in the archaeal community composition in the surface sediments upon the decrease of oxygen concentration in the summer coincided with an order of magnitude decrease of the total IPL-GDGTs detected. The IPL-GDGT profiles in March were compatible with a Thaumarchaeota-dominated population, due to the relatively high abundance of crenarchaeol, the specific CL of Thaumarchaeota (Sinninghe Damsté et al., 2002). In addition, the IPL type of crenarchaeol (and all other main GDGTs) switched from mostly HPH to MH from March to August (Table 3). The predominance of HPH IPL-crenarchaeol has been previously interpreted as an indication of the presence of an active Thaumarchaeotal population synthesizing membrane lipids *in situ* (Lengger et al., 2012, 2014), due to the labile nature of HPH IPLs (Harvey et al., 1986; Schouten et al., 2010). On the other hand, glycolipid-type IPLs (here mostly MH) have been considered as fossil signal (Lengger et al., 2012, 2014) or, alternatively, as the preferred IPL type in conditions where the Thaumarchaeota are in a stationary phase of growth (Elling et al., 2014). This latter explanation is not likely in the case where the surface sediment became fully sulfidic.

Recent studies suggest that members of the DPANN have a reduced genome with limited metabolic capabilities (Rinke et al., 2013; Castelle et al., 2015), suggesting that these archaea may have a symbiotic or parasitic lifestyle. In addition, they also lack most if not all the genes coding for the enzymes of the archaeal lipid biosynthetic pathway (Jahn et al., 2004; Villanueva et al., 2017). They are, therefore, not expected to contribute to the total IPL-GDGT pool by actively synthesizing lipids but may recycle the membrane lipid of other archaea. The decline of the total archaeal IPL-GDGTs in August coinciding with the switch to a DPANN-dominant population with similar or even higher cell abundances to that reported in March would therefore imply that the DPANN Woesearchaeota make use of the preserved ('fossil') pool of IPL-GDGTs to make their membranes. A question with respect to this membrane lipid acquisition mechanism is whether the pool of 'fossil' IPL-GDGTs is able to fulfill the membrane requirements of the large archaeal DPANN population detected in the surface sediments sampled in August in view of the much reduced concentration of IPL-GDGTs, which is an order of magnitude lower. This

can only work when the DPANN archaea have a much smaller cell size than the Thaumarchaeota that thrive in spring. The size of the DPANN Woesearchaeota present in Lake Grevelingen surface sediment is unknown. The only characterized archaeon from the DPANN superphylum is *Nanoarchaeum equitans,* which is considered to be a symbiont of the archaeon *Ignicoccus sp.* by growing on its surface. The lipids of *N. equitans* have been shown to be derived from *Ignicoccus sp.* (Jahn et al., 2004), but the uptake mechanisms remain unknown. *N. equitans* is 5 times smaller than its host, the archaeon *Ignicoccus sp.*. Assuming a similar size difference between the DPANN Woesearchaeota found in the Lake Grevelingen surface sediments and its potential host, sufficient IPLs would be available to sustain the DPANN population detected in the sediments in August.

Alternatively, the DPANN archaea could recycle the CL-GDGTs present in the sediment as previously suggested (Liu et al., 2011; Takano et al., 2010), which is presumed to involve hydrolysis and reformation of the ether bond to the glycerol backbone as suggested by the ^{13}C-glucose labeling studies of Takano et al. (2010). However, since the members of the DPANN generally lack the genes coding for the enzymes involved in producing the ether bonds to the glycerol backbone (Villanueva et al., 2017), this is unlikely. Alternatively, they could also use the CL-GDGTs and add the polar head groups *de novo*. Waters et al. (2003) observed the presence of genes for lipid modification, such as glycosylation, in the genome of *N. equitans*. We performed a search for the gene encoding cytidine diphosphate (CDP)-archaeol synthase (CarS, E.C. 2.7.7.67) that catalyzes the activation of 2,3-bis-*O*-geranylgeranylglyceryl diphosphate (DGGGP) by cytidine triphosphate (CTP) to form the intermediate for polar head group attachment (i.e. CDP-archaeol), and we found homologs in most of the DPANN Micrarchaeota genomes and in some of the DPANN Parvarchaeota and Diapherotrites (perfomed by find function in JGI Integrated Microbial Genomes, IMG with genomes available in July 2017). However, the genes coding for the enzymes that catalyze the subsequent replacement of cytidine monophosphate of the cytidine diphosphate (CDP)-archaeol of CDP-diacylglycerol with a polar head group in DPANN genomes were not detected, in contrast to *N. equitans* (Waters et al., 2003). This may indicate that the members of the DPANN either do not have the capacity of adding the polar head groups to the CL-GDGT or that the polar head groups of their IPLs are different and added by enzymes different to those already characterized.

6.5. Conclusion

We observed a dramatic change in the archaeal community composition and lipid abundance in surface sediments of a seasonally hypoxic marine lake which corresponded to a switch from a Thaumarchaeota-dominated to a DPANN-dominated archaeal community, while the total IPLs detected were significantly reduced. Considering the reduced genome of the members of the superphylum DPANN and their apparent inability to synthesize their own membrane lipids, we hypothesize that they use the CLs previously synthesized by the Thaumarchaeota to form their membrane.

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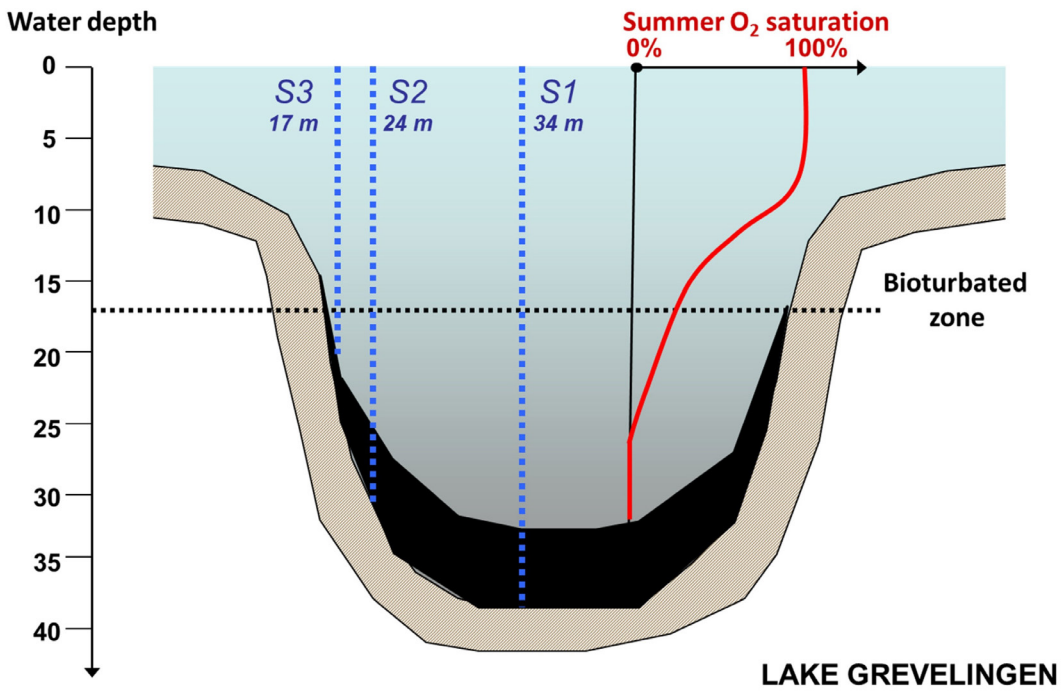

Figure S1: Scheme of the sampling locations in Lake Grevelingen

Table S1: Percentages of total archaeal 16S rRNA gene reads [%] detected in the surface sediment (0–1cm) of the three stations in March and in August. Only archaeal groups with percentages >3% are reported.

organism	Station 1		Station 2		Station 3	
	March	August	March	August	March	August
Thermoplasmata, 20a-9	n.d.	n.d.	n.d.	2.7	n.d.	0.9
Thermoplasmatales, AMOS1A-4113-D04	n.d.	n.d.	0.8	0.2	1.3	2.8
Thermoplasmatales, CCA47	n.d.	n.d.	2.5	0.8	0.7	7.9
Thermoplasmatales, MBG-D & DHVEG-1	n.d.	2.6	9.3	2.7	0.4	0.9
Thermoplasmatales, VC2.1 Arc6	n.d.	n.d.	1.7	1.1	0.2	5.6
Sum of reads Thermoplasmatales	n.d.	2.6	14.4	4.9	2.6	17.1
Miscellaneous Crenarchaeota Group, C3	1.4	2.6	2.5	7.4	n.d.	4.2
Thaumarchaeota, Marine Group I, *Nitrosopumilus*	81.9	n.d.	48.3	10.1	72.1	5.6
Miscellaneous Euryarchaeotic Group (MEG)	n.d.	n.d.	n.d.	5.1	n.d.	n.d.
DPANN, Woesearchaeota DHVEG-6	16.0	94.9	31.4	66.5	20.6	56.9
MBG-B	n.d.	n.d.	n.d.	3.2	n.d.	4.6
Others	0.7	n.d.	3.4	0.2	4.7	10.6

n.d = not detected. MBG-D, Marine Benthic Group D; MBG-B, Marine Benthic Group B.

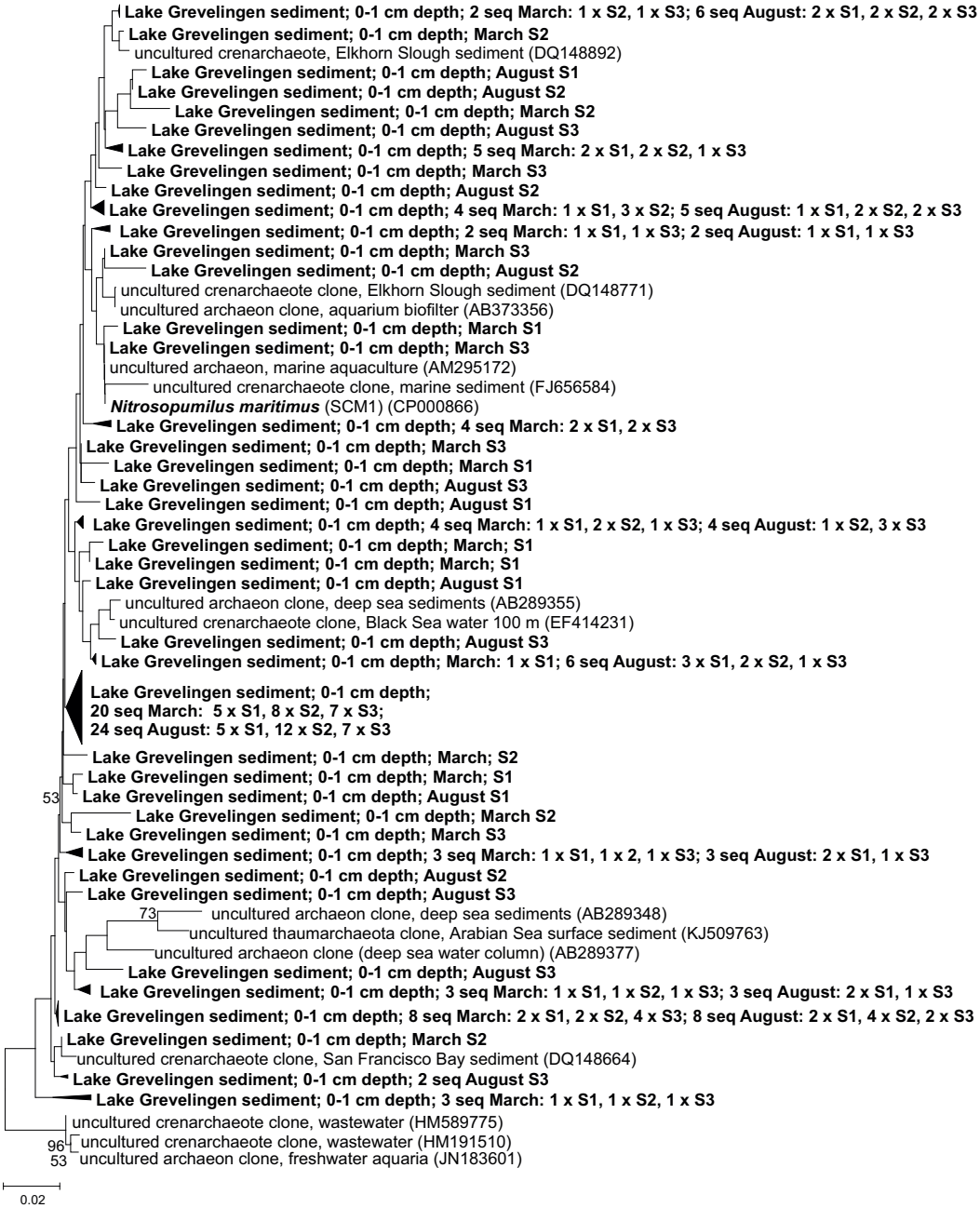

Figure S2A: Phylogenetic tree of *amoA* protein sequences recovered from the surface sediment (0–1 cm) of Stations 1, 2 and 3 in Lake Grevelingen during March and August (black bold: sequences retrieved in this study and closest relatives) constructed with the Neighbor-Joining method (Saitou and Nei, 1987). Scale bar indicates 2% sequence dissimilarity. The evolutionary distances were computed using the Poisson correction method with a bootstrap test of 1,000 replicates (values higher than 50% are shown on the branches).

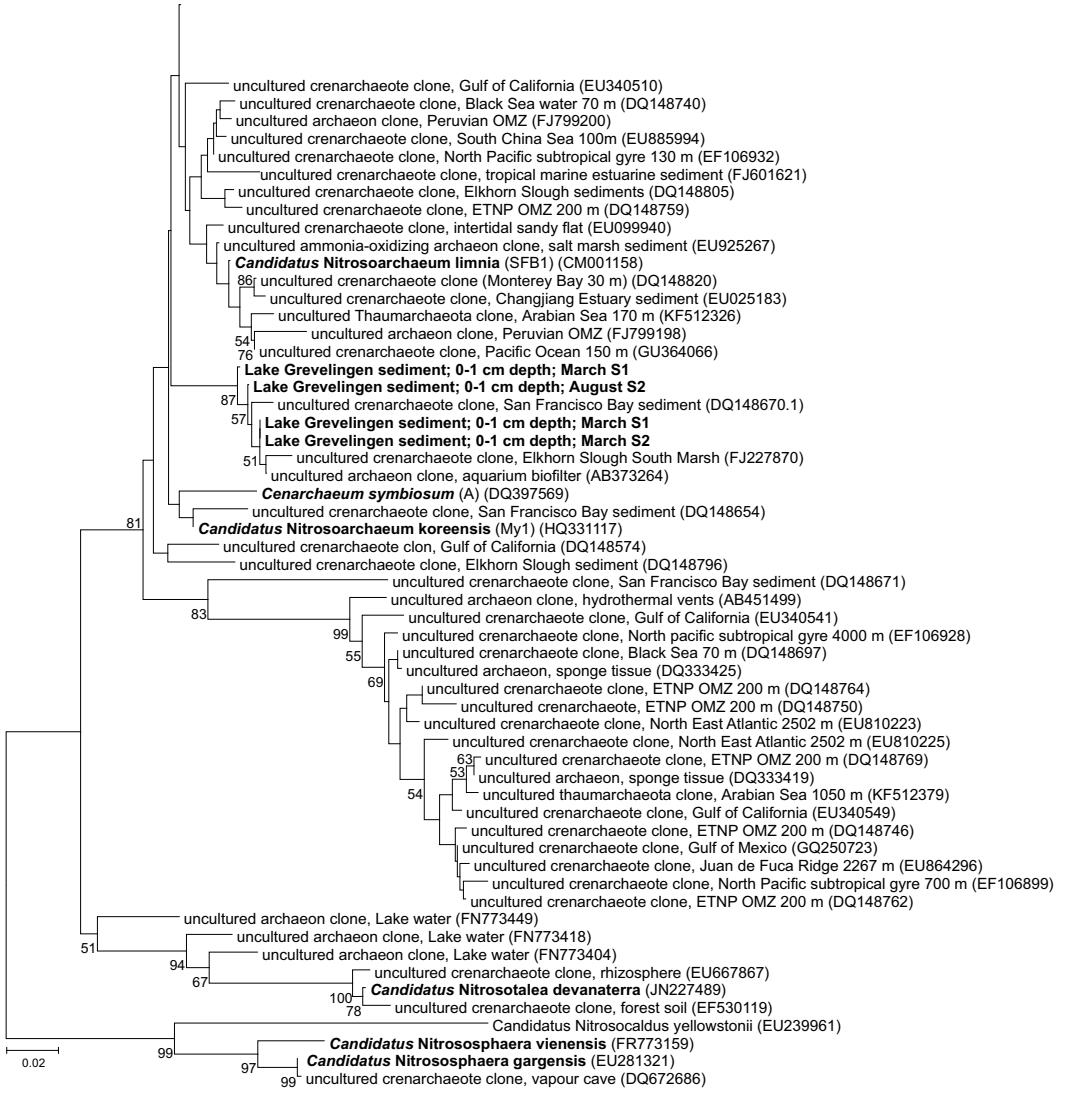

Figure S2B: Phylogenetic tree of *amoA* protein sequences recovered from the surface sediment (0–1 cm) of Stations 1, 2 and 3 in Lake Grevelingen during March and August (black bold: sequences retrieved in this study and closest relatives) constructed with the Neighbor-Joining method (Saitou and Nei, 1987). Scale bar indicates 2% sequence dissimilarity. The evolutionary distances were computed using the Poisson correction method with a bootstrap test of 1,000 replicates (values higher than 50% are shown on the branches).

7. Synthesis

The role of chemolithoautotrophic microorganisms has been considered to be of minor importance in coastal marine sediments although it has not been investigated in depth. Additionally, the impact of seasonal hypoxic/anoxic conditions on microbial chemolithoautotrophy in coastal marine sediments has not been examined. Therefore, in this thesis the diversity, abundance and activity of specific groups of chemolithoautotrophic microorganisms involved in the nitrogen and sulfur cycling, such as ammonia oxidizing archaea and bacteria, denitrifying and anammox bacteria, sulfate reducing and sulfur oxidizing bacteria, as well as the total microbial community, has been determined in coastal sediments by using DNA/RNA- and lipid-based biomarker approaches. Results show the presence and potential importance of chemolithoautotrophs in the biogeochemical cycling of carbon, nitrogen and sulfur. Additionally, temporal changes of the diversity, abundance and activity of chemolithoautotrophic microorganisms coinciding with seasonal hypoxia in coastal sediments have been determined.

7.1. Physicochemical factors of the sediments harboring chemolithoautotrophs studied in the framework of this thesis

In this thesis, coastal sediments with different sediment properties and oxygen conditions were examined. In this section, I focus on the most important environmental factors affecting chemolithoautotrophic microorganisms targeted in this thesis, i.e. aerobic (ammonia oxidizing archaea and bacteria, AOA and AOB) and anaerobic ammonia oxidizing (anammox) bacteria, denitrifying bacteria, as well as sulfate reducing and sulfur oxidizing bacteria including sulfide-dependent denitrifiers. Effects on the seasonal and spatial distribution of the diversity, abundance and activity of the target organisms, as well as applied methods are discussed in detail in the following sections.

Sediments of the Oyster Grounds (Chapter 2), a deep depression in the central North Sea and a temporary deposition center for sediment, play an important role in the carbon and nitrogen cycle in the region (van Raaphorst et al., 1998; Weston et al., 2008). High levels of benthic fauna has been reported for this area (Duineveld et al., 1991; van Raaphorst et al., 1992) even during summer stratification. Oxygen concentrations in the water column commonly decrease from winter to summer (as seen in our case between the sampling performed in February and August), as well as the oxygen penetration depth (OPD) into the sediment. In this thesis, sediments sampled in February, May and August were investigated.

Also Lake Grevelingen sediments (Chapters 4, 5, and 6) were exposed to varying oxygen concntrations, i.e. bottom water hypoxia due to summer stratification of the water column, and sulfide accumulation in the sediment pore water due to intense sulfate reduction. Sediments, located at different water depths of a deep basin within Lake Grevelingen were investigated (S1 at 34 m, S2 at 23 m, and S3 at 17m). Bottom waters were fully oxygenated in March (S1-S3), whereas in August oxygen was strongly depleted in the bottom waters and no oxygen has been detected in the surface sediments (S1, S2). Bottom water oxygen concentration at S3 remained higher in August (~88 μM), and oxygen penetrated down to 1.1 mm into the sediment. In August, free sulfide was present near the sediment-water interface at all three stations, and the accumulation of sulfide in the sediment pore water increased with water depth (S3 to S1).

In general, my results support that the oxygen concentration of the bottom water and the OPD into the sediment is one of the major factors shaping the temporal and spatial distribution of the diversity, abundance and activity of chemolithoautotrophic microorganisms, due to different oxygen requirements of their respective metabolism. Next to the availability of electron donors and acceptors, I have concluded that other factors such as bioturbation (Kristensen, 2000; Meyer et al., 2005; Beman et al., 2012) and the interactions between different microbial groups are important factors allowing for the activity of aerobic and anaerobic microorganisms together in the same niche. The abundance and activity of Thaumarchaeota and anammox bacteria was detected in Oyster Grounds sediments (Fig. 1) independent of the season and decreasing oxygen concentrations in the bottom water, whereas in Lake Grevelingen sediments, the abundance and activity of Thaumarchaeota was restricted to March, when the bottom water was still oxygenated (Fig. 2). These results suggest that the presence of bioturbation, oxygenating deeper layers in Oyster Grounds sediments, favors the presence and activity of aerobic ammonia oxidizing microorganisms, which could in turn fuel the activity of anammox bacteria by consuming oxygen and providing nitrite (Meyer et al., 2005; Laverock et al., 2013), compared to Lake Grevelingen sediments, where bioturbation was absent and sulfide accumulates in the sediment pore water during summer hypoxia.

In comparison to Oyster Grounds sediments, temporal and spatial differences of the community structure, as well as of the abundance and activity of studied microorganisms were more pronounced in Lake Grevelingen sediments (Fig. 2), most likely due to the full development of hypoxia and rising sulfide concentrations as it has been shown recently for Black Sea sediments (Jessen et al., 2017). High sulfide concentrations are not only toxic for benthic bioturbating animals as sulfide has also been shown to inhibit ammonia oxidation (Joye and Hollibaugh, 1995; Berg et al., 2015) and the last steps of denitrification (Sørensen, 1980; Porubsky et al., 2009). My results showed that decreasing oxygen and rising sulfide concentrations induce drastic changes in the archaeal and bacterial community structure, and limit the abundance and/or the activity as well of specific groups involved in the sedimentary nitrogen and sulfur cycle, i.e. Thaumarchaeota (AOA), AOB, anammox (Chapters 2, 3, 4) and denitrifying bacteria, sulfate and sulfur oxidizing bacteria including sulfide-dependent denitrifiers in Lake Grevelingen sediments (Chapters 4 and 6), as well as of chemolithoautotrophic bacteria (Chapter 5). Additionally, the potential activity of anammox bacteria was relatively low in Lake Grevelingen sediments compared to Oyster Grounds sediments, suggesting that this group is strongly affected by increasing sulfide concentrations. The activity of other groups, i.e. denitrifying bacteria, sulfate reducing and sulfur oxidizing bacteria including sulfide-dependent denitrifiers, was relatively low in seasonally hypoxic Lake Grevelingen sediments independent of the season, suggesting that the activity of these groups is as well affected by decreasing oxygen and rising sulfide concentrations between spring and summer.

Both anammox and denitrification pathways have been suggested to be more important in sediments with low sulfide concentrations, but also with low carbon input (Burgin and Hamilton, 2007). The TOC content in Lake Grevelingen sediments is high (2.5−4.5%) and fresh organic rich sediment rapidly accumulates (> 2 cm y^{-1}), which might additionally inhibit the activity of anammox and denitrifying bacteria. The TOC content in Oyster Grounds sediments was low (0.15−0.38%), which might explain the higher activity of anammox activity compared to Lake Grevelingen sediments. However, sediment incubation experiments (using labeled ni-

trite) have previously shown that the ratio of anammox to denitrification depends on the carbon to nitrogen (C/N) ratio, i.e. the activity of anammox bacteria increased with higher nitrogen relative to carbon content (lower C/N) ratio of the source substrate (Babbin et al., 2014). However, in my studies, I have observed a difference in the potential activity of anammox bacteria with higher expression levels in Oyster Ground compared to Lake Grevelingen sediments. The C/N ratio is relatively high in sediments of the Oyster Ground (7−8) and Lake Grevelingen (9.2 ± 0.2; Rao et al., 2016), indicating that this does potentially not explain the differences in the potential activity of anammox bacteria in the sediments studied here. Generally, the activity of chemolithoautotrophic bacteria in Lake Grevelingen sediments was restricted to March under oxygenated bottom water concentrations, indicating that chemolithoautotrophic bacteria in general are affected by bottom water hypoxia/anoxia, the lack of bioturbation and rising sulfide concentrations (Chapter 5).

Additionally, the abundance and metabolic capacities of the chemolithoautotrophic aerobic ammonia oxidizing archaea (AOA), i.e. Thaumarchaeota, were also evaluated in other marine sediment systems, i.e. in sandy to muddy sediments of the Iceland shelf. Thaumarchaeota were detected at all stations but their metabolic activity was low based on lack of incorporation of ^{13}C-labeled substrate in archaeal GDGT lipids. The low activity of Thaumarchaeota is in agreement with the low oxygen penetration into the sediment, supporting that the absence of oxygen is limiting the activity of Thaumarchaeota. Ammonia and nitrite concentrations showed no changes during incubations, indicating that other factors than the availability of ammonia might inhibit the activity of Thaumarchaeota.

7.2. Diversity of chemolithoautotrophic microorganisms in coastal sediments

The general diversity of the microbial community was studied in Lake Grevelingen surface sediments by applying 16S rRNA amplicon sequencing analysis (Chapters 5, 6), before and during summer hypoxia. In general, these sediments harbor a distinct bacterial community comparable to other surface sediments, such as coastal marine (North Sea), estuarine and Black Sea sediments (Jessen, et al., 2016; Ye et al., 2016; Dyksma et al., 2016). Approximately, 50% of the bacterial reads were assigned to the clades of Gammaproteobacteria, Deltaproteobacteria and Epsilonbacteria, whereas the remaining reads were distributed among the orders of Bacteroidetes (14%), Planctomycetes (6%), Alphaproteobacteria (2%) and other orders (22%). The majority of reads assigned to the Gammaproteobacteria were closely related to the orders Alteromomodales, Chromatiales and Thiotrichales, whereas most of the reads of Epsilonproteobacteria were assigned to the order of Campylobacterales. Within the Deltaproteobacteria, most reads were assigned to the orders of Desulfarculales and Desulfobacterales.

In March, under oxygenated bottom water conditions, the surface sediment was characterized by high relative abundances (percentage of 16S rRNA gene reads) of Gammaproteobacteria and Epsilonproteobacteria, which are known chemolithoautotrophic sulfur oxidizers, e.g. capable of oxidizing sulfide, thiosulfate, elemental sulfur and polythionates, using oxygen and nitrate as electron acceptor. In August, the lack of electron acceptors in the bottom water, i.e. oxygen and nitrate, coincided with a decrease in the relative abundance of Gammaproteobacteria and Epsilonproteobacteria. At the same time, the number of reads assigned to the order of Desulfobacterales increased in the top centimeter of the sediment and a shift in the bacterial

community toward chemoorganoheterotrophic sulfate-reducing bacteria related to the genera *Desulfococcus* and *Desulfosarcina* was evident. In coastal sediments, these genera are characteristic in deeper sediment layers experiencing anaerobic mineralization (Llobet-Brossa et al., 2002; Miyatake, 2011; Muyzer and Stams, 2008). The DNA-based 16S rRNA gene amplicon sequencing was complemented with the analysis of bacterial phospholipid derived fatty acids (PLFA) analysis in Lake Grevelingen sediments down to 6 cm sediment depth in all seasons and stations. In March, surface sediments were characterized by relative high concentrations of C_{16} and C_{18} monosaturated PLFAs found in Gammaproteobacteria and Epsilonproteobacteria (Vestal and White 1989), whereas in deeper sediment layers, ai15:0, i17:1ω7c, and Me16:0, indicating that sulfate-reducing Deltaproteobacteria were more abundant (Boschker et al., 2002; Taylor and Parks, 1983; Edlund et al., 1985). In August, surface sediment showed an increased contribution of iso, anteiso, and branched PLFAs, more similar to the deeper sediments from March, but showed higher abundances of 16:0, 14:0, and 18:1ω9c compared to March. These shifts in the PLFA patterns are in agreement with the temporal difference in the bacterial community shown by 16S rRNA gene amplicon sequencing. However, the results of the PLFA analysis have to be interpreted with caution because there is now evidence that the method used here (Guckert et al., 1985), i.e. the separation of phospholipids from glycolipids and neutral lipids using silica column chromatography, does not result in a fraction only containing phospholipids but also other lipid classes (Heinzelmann et al., 2014). Thus, commonly reported PLFA composition might be not derived purely from phospholipids but from of other lipid classes.

Additionally, the archaeal diversity was estimated by 16S rRNA gene amplicon sequencing in surface sediments of Lake Grevelingen (Chapter 6). The archaeal community was dominated by chemolithoautotrophic Thaumarchaeota marine group I (MGI) in March (50−82%), when oxygen was still present and sulfide was absent, next to the presence of other archaeal groups, such as members of the DPANN Woesearchaeota, Thermoplasmatales and members of the Miscellaneous Crenarchaeotic group (MCG). In August, the percentage of reads, attributed to the DPANN Woesearchaeota increased notably (57−95%) at the expense of reads assigned to the Thaumarchaeota. This change in the archaeal community is compatible with the aerobic metabolism of Thaumarchaeota (Könneke et al., 2005) and the anaerobic metabolism of DPANN archaea (Castelle et al., 2015). Thaumarchaeota have been shown to be inhibited by sulfide (Berg et al., 2015), potentially explaining the decrease in the MGI population upon the increase of sulfide concentrations in the summer.

In summary, results of the community analysis revealed a diverse microbial community in Lake Grevelingen surface sediments consisting of chemolithoautotrophic and chemoorgano-heterotrophic microorganisms, responding to changes in the availability of reduced nitrogen and sulfur compounds and electron acceptors (oxygen and nitrogen) induced by hypoxia. Nevertheless, the bacterial and archaeal community analysis was performed by 16S rRNA gene amplicon sequencing, therefore I cannot completely exclude a possible primer bias (Sipos et al., 2010; Schloss et al., 2011). Additionally, the diversity analysis of the 16S rRNA gene does not give information about absolute abundances, the metabolism and more importantly, about the activity of sedimentary microorganisms and their role in biogeochemical cycling of carbon, nitrogen and sulfur.

To further estimate the diversity of microorganisms fixing CO_2 via the Calvin-Benson-Bassham (CBB) and the reductive tricarboxylic acid (rTCA) pathways, surface sediments of Lake Grevelingen sediments (Chapter 5) were investigated by cloning and sequencing the *cbbL* and

aclB gene, encoding for the key enzymes ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) and ATP citrate lyase, respectively. All detected *cbbL* sequences clustered with uncultered gammaproteobacterial clones, assigned to the orders Chromatiales and Thiotrichales, reported in intertidal sediment from Lowes Cove, ME (Nigro and King, 2007). The *aclB* gene sequences in surface sediments were predominantly related to *aclB* sequences of Epsilonproteobacteria of the order Campylobacterales, macrofaunal symbionts and to sequences of Euryarchaeota. No clustering of *cbbL* and *aclB* gene sequences according to one of the stations or one of the seasons was observed. Results revealed a stable community of bacteria using the CBB cycle, as well as bacteria and archaea using the rTCA pathway in Lake Grevelingen surface sediments. Until today, there is no PCR-based approach available to identify microorganisms using other CO_2 fixation pathways, such as the reductive acetyl-CoA, or Wood-Ljungdahl (WL) pathway utilized by Firmicutes, Planctomycetes, Deltaproteobacteria, Spirochaeta and Euryarchaeota, or the 3-hydroxypropionate/4-hydroxybutyrate (3-HP/4-HB) cycle and the dicarboxylate/4- hydroxybutyrate (DC/4-HB) cycle operating in Crenarchaeota (Hügler and Sievert, 2011), mainly due to the diversity of key genes of these pathways which impairs the development of a PCR-based detection method. Thus, an underestimation of the diversity of chemolithoautotrophic microorganisms in the coastal marine sediments under study in this thesis is possible.

Another goal of this thesis was to further estimate the diversity of chemolithoautotrophic microorganisms involved in the sedimentary nitrogen and sulfur cycle by using group specific 16S rRNA genes and functional genes involved in reoxidation pathways. This approach was applied in sediments of the Oyster Grounds (North Sea) and Lake Grevelingen surface sediments. In Oyster Grounds surface (0–1 cm) and deeper (9–10 cm) sediments, the diversity of ammonia oxidizing archaea (Thaumarchaeota, AOA) and ammonia oxidizing bacteria (AOB), was determined by targeting the functional *amoA* gene of AOA and AOB. Results showed that *amoA* gene sequences of AOA amplified from Oyster Grounds surface sediments fell into distinct subclusters of *Nitrosopumilus* sp., whereas most of the archaeal *amoA* sequences obtained from Lake Grevelingen sediments were closely related to *Nitrosopumilus maritimus* (SCM1) and in minor proportion to "*Candidatus* Nitrosoarchaeum limnia" found in low-salinity habitats (Mosier et al., 2012), showing a different community composition of AOA in Lake Grevelingen surface sediments, which is most likely explained by the lower salinity (~30 psu) compared to Oyster Grounds sediments (~34.5 psu). AOB *amoA* gene sequences retrieved from Oyster Grounds sediments were closely related to *Nitrospira* sp. and *Nitrosolobus multiformis*.

Another important chemolithoautotrophic microbial group present in anoxic sediment and involved in the nitrogen cycle is anammox bacteria. One issue much in debate is the relevance of anammox bacteria vs. heterotrophic denitrifiers in the N_2 removal process in the environment. In this thesis I tackled this issue by determining the diversity and role of anammox bacteria, heterotrophic denitrifiers and other microbial groups involved in nitrogen removal (e.g. sulfur oxidizers coupled to nitrate reduction) in Lake Grevelingen sediments (Chapter 4). The diversity of the anammox 16S rRNA gene, whereas the *nirS* gene of denitrifying and anammox bacteria, as well as the *aprA* gene of sulfur oxidizing and sulfate reducing bacteria, were determined in Lake Grevelingen surface sediments (chapter 4). The diversity of *nirS*-type denitrifiers in surface sediments (0−1 cm) revealed a temporally stable community of denitrifying bacteria involved in autotrophic denitrification coupled to sulfide or iron oxidation (e.g. *Thiobacillus denitrificans, Sideroxidans lithotrophicus*), and of diverse heterotrophic denitrifiers. Most of the anammox bacterial

nirS gene sequences obtained from Lake Grevelingen surface sediments were closely related to "*Candidatus* Scalindua profunda", whereas anammox bacterial 16S rRNA gene sequences were closely related to "*Candidatus* Scalindua marina" and "*Candidatus* Scalindua brodae" as found in Oyster Grounds sediments (0–1 cm and 9–10 cm sediment depth).

The analysis of the *aprA* gene also revealed a diverse sulfur oxidizing community; including affiliations to autotrophic sulfur-dependent denitrifying Beta- and Gammaproteobacteria (*Thiobacillus denitrificans* and *Sulfuricella denitrificans*), chemolithoautotrophic sulfur-dependent nitrate reducing Betaproteobacteria (*Thiobacillus thioparus*), heterotrophic sulfate reducing Deltaproteobacteria (e.g. *Desulfosarcina* sp., *Desulfobulbus propionicus*) or phototrophic sulfur oxidizing bacteria (*Lamprocystis purpurea*). In addition, the diversity of genes involved in carbon fixation and reoxidation pathways were also estimated showing no clustering of sequences according to the depth, station or season. These results were interpreted as an indication of the presence of a stable population of ammonia oxidizing archaea and bacteria, anammox (Chapter 2) and denitrifying bacteria, as well as sulfate reducing and sulfur oxidizing bacteria including sulfide-dependent denitrifiers (Chapter 4) in coastal sediments experiencing decreasing bottom water oxygen concentrations due to summer stratification.

In general, the microbial community in Lake Grevelingen surface sediments consisted of phototrophic, heterotrophic and autotrophic microorganisms involved in carbon-, nitrogen and sulfur cycling. Thaumarchaeota have been detected in Oyster Ground sediments and Lake Grevelingen sediments, where they seem to play an important role in the reoxidation of reduced inorganic compounds in order to sustain biogeochemical cycling of carbon and nitrogen compounds especially under oxygenated bottom water conditions and/or in bioturbated sediments (Fig. 1). The diversity of anammox bacteria in Oyster Grounds sediment is comparable to the anammox bacterial diversity in Lake Grevelingen sediments by the 16S rRNA gene diversity, indicating that the diversity of anammox bacteria is less affected by seasonal hypoxia and rising sulfide concentrations compared to the archaeal population as shown in chapter 2 and 4.

7.3. Abundance and distribution of chemolithoautotrophic microorganisms in coastal sediments

Microbial processes such as nitrification, denitrification and anammox are important for nitrogen and carbon cycling, and are tightly coupled in marine sediments (Zhang et al., 2013). The abundance and seasonal variations of archaeal and bacterial ammonia oxidizers, anammox and denitrifying, and sulfide dependent denitrifiers, as well as environmental factors affecting these groups, are not well studied in coastal marine sediments. In this thesis, the seasonal and depth distribution of the abundance of these groups was estimated in marine sediments of the Oyster Grounds (North Sea), in sediments of the Iceland shelf, as well as in Lake Grevelingen sediments to get insights of the importance of these groups and their impact in the carbon and nitrogen cycles in selected marine sediments.

In Oyster Grounds sediments (Chapter 2) this was achieved by quantifying specific intact polar lipids attributed to ammonia oxidizing Thaumarchaeota (AOA) (i.e. hexose phosphohexose (HPH) crenarchaeol) and anammox bacteria (i.e. phosphatidylcholine (PC)-monoether ladderane), as well as the abundance of the 16S rRNA genes, the ammonia monooxygenase subunit A (*amoA*) gene of AOA and AOB, and the hydrazine synthase (*hzsA*) of anammox bacteria, in February, May, and in August during decreasing bottom water oxygen concentrations. One of

the highlights of the study in the Oyster Grounds sediments is the detection of aerobic ammonia oxidizing microorganisms at depths where the oxygen concentration was expected to be undetectable, which is counterintuitive with their aerobic metabolism. This was attributed to the intense bioturbation reported in these sediments which could provide enough oxygen to support the presence and activity of these microbial groups. This is further supported by other studies like the one of Beman et al. (2012), where AOA and AOB *amoA* genes were detected up to 10 cm sediment depth in bioturbated sediments of Catalina Island (CA, USA). My results, and those of Beman et al. (2012), suggest that aerobic ammonia oxidizers are indeed living and actively involved in the marine sedimentary nitrogen cycle in deeper layers of coastal marine sediments than previously thought, which is relevant as it extends the area of active dark carbon fixation and nitrification in marine sediments.

Another conclusion of the study performed in the Oyster Grounds sediments is that seasonal variation in the abundance of Thaumarchaeota (AOA) within the sediment given by quantification of 16S rRNA and *amoA* gene were not reflected in the specific Thaumarchaeota lipid biomarker HPH-crenarchaeol quantification, which was stable with depth in the first 12 cm. HPH-crenarchaeol has been shown to be more labile compared to crenarchaeol with a glycosidic head group, and its concentration corresponds well to the DNA-based abundance of Thaumarchaeota (Pitcher et al., 2011a; Schouten et al., 2012; Lengger et al., 2012a). These results might suggest a possible preservation of this biomarker within the sediment vertical profile which would invalidate the use of it as indicator of abundance of living Thaumarchaeota in deep sediments.

The presence and potential activity of Thaumarchaeota were also assessed in Iceland shelf sediment incubations with ^{13}C-labeled substrates (chapter 3). This study also included a direct analysis of IPL-crenarchaeol as specific lipid of Thaumarchaeota. The IPL-crenarchaeol with (HPH)- head group was between 80−90% in surface sediments of all stations and it decreased up to 10−25% in the deepest sediment layer. However, the presence of HPH-crenarchaeol in anoxic parts of the sediment, as seen in the Oyster Grounds sediments (Chapter 2), can again be due to preservation of this compound and cannot be then taken as a biomarker of a living Thaumarchaeota population.

In order to rule out a possible preservation effect of lipid biomarkers in deep sediments, the analysis or archaeal lipids was later restricted to surface sediments in Lake Grevelingen in order to determine the abundance and distribution of chemolithoautotrophic ammonia oxidizing Thaumarchaeota as well as other archaeal groups present. The methodological improvement of this study with respect to the one included in Chapter 2 and chapter 3 is that a new method was applied for the detection of the polar head groups of different IPL-GDGTs and was not only restricted to the specific GDGT crenarchaeol of Thaumarchaeota. With this, it was possible to extend the analysis of IPL-GDGT to the entire archaeal population present in the sediments. The detection of HPH-GDGTs in March in Lake Grevelingen sediment, coinciding with a higher presence of Thaumarchaeota detected by 16S rRNA gene amplicon sequence, indicated that the Thaumarchaeota population was actively living in those sediments. On the other hand, the switch to an archaea DPANN-dominated population in August with a similar IPL-GDGT profile, and the fact that DPANN archaea are not genetically capable of synthesizing their own lipids, suggest that this archaea group might be recycling the IPL-GDGTs already present in the sediment. This factor complicates the interpretations of the lipid sources in sedimentary settings

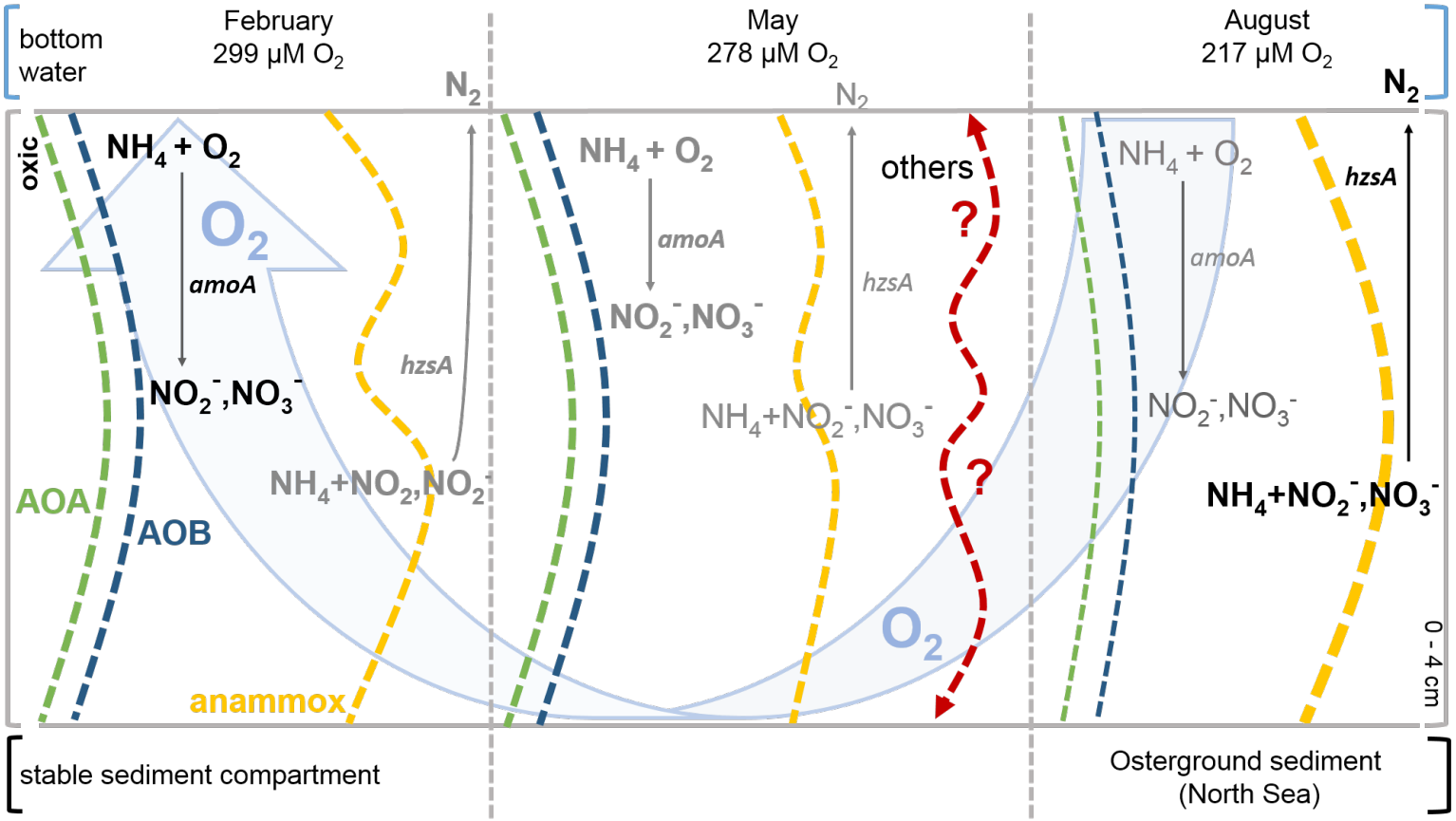

Figure 1: The abundance and activity of AOA (green) and AOB (dark blue), as well as of anammox bacteria (yellow) in bioturbated sediments of the Oyster Grounds (North Sea) (0–4 cm sediment depth). Big blue arrow indicates oxygen supply by bioturbation. Red arrow indicates potential abundance and activity of other microbial groups. Please note that the dashed lines do not reflect the actual depth profiles of biomarkers (find in chapter 2), but represent the profile distribution (taken together the abundance and potential activity); (thin dashed line) lower abundance and activity, (thicker dashed lines) higher abundance and activity.

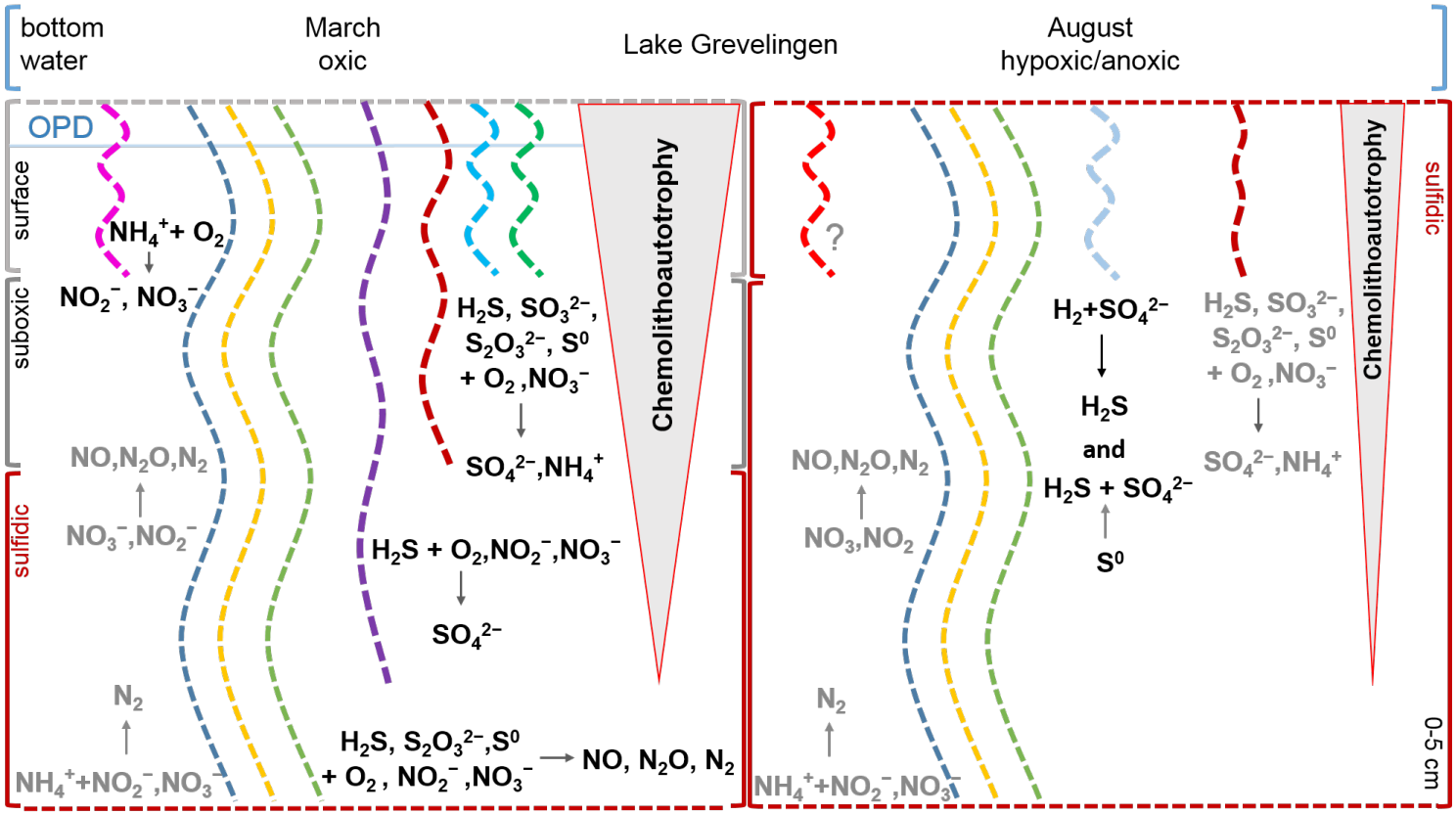

Figure 2: Representation of the profile distribution (taken together the abundance and potential activity) (dashed lines) and chemolithoautotrophic activity (^{13}C PLFA-SIP) (triangle) in Lake Grevelingen sediments during oxygenated bottom water conditions (left) and during hypoxia (right) (0–5 cm sediment depth) (Chapter 4, 5 and 6). [color code: (pink) Thaumarchaeota, (dark blue) heterotrophic and autotrophic denitrifying bacteria, (green) sulfate reducing and sulfur oxidizing microorganisms including sulfide-dependent denitrifiers, (purple) cable bacteria, (red) Beggiatoaceae, (light blue) sulfur oxidizing Gammaproteobacteria, (dark green) sulfur oxidizing Epsilonproteobacteria, (orange) DPANN Woesearchaeota, (light blue) sulfate reducing and sulfur disproportioning Deltaproteobacteria]. (black characters) abundant and potentially active, (grey characters) abundant and low potential activity. Only reduced species and electron acceptors are shown.

and highlight the need of combining different methodological approaches (i.e. lipid biomarkers, gene-based detection, activity measurements) to determine the chemolithoautotrophic potential and biological sources. Also, this study highlights the dramatic change in the archaea population in Lake Grevelingen surface sediments upon summer hypoxia and increase of sulfide concentration in the pore water. This can be explained by a toxic effect of sulfide on the Thaumarchaeota population, together with decrease in oxygen available for their aerobic metabolism. In fact, a similar toxic effect of sulfide has been observed in Thaumarchaeota enrichment cultures from Baltic Sea water (Berg et al., 2015).

In this thesis, I have also determined the abundance and distribution of anammox bacteria in different marine sediment systems both by using specific lipid biomarkers and quantification of specific 16S rRNA and metabolic genes. For example, anammox bacteria 16S rRNA and the hydrazine synthase (*hzsA*) gene abundance were detected throughout the Oyster Grounds sediment with more pronounced depth and seasonal differences than those observed for AOA and AOB. Both, anammox bacteria 16S rRNA and *hzsA* gene abundances were higher in August compared to February and May, which was not reflected in the abundance of the anammox specific lipid biomarker PC-monoether ladderane. This can be explained by partial preservation of this biomarker lipid in the sediment due to a relatively low turnover rate (Brandsma et al., 2011; Bale et al., 2014). I also observed a ten-fold difference between abundances of the anammox 16S rRNA gene and the *hzsA* gene. This difference between the 16S rRNA gene and functional genes of anammox bacteria has been previously observed and is possibly explained by primer biases of the 16S rRNA gene primers, suggesting the amplification of 16S rRNA gene fragments of bacteria other than anammox bacteria (Harhangi et al., 2012; Bale et al., 2014).

The presence of anammox bacteria biomarkers in the upper layers of Oyster Grounds sediments (0−4 cm) is still remarkable as the metabolism of anammox bacteria is expected to be incompatible with the presence of oxygen. Some studies have reported that anammox bacteria can be present at high oxygen levels, by being dormant or using anaerobic niches (Kuypers et al., 2005; Woebken et al., 2007). It is also possible that the metabolic activity of AOA/AOB and heterotrophic bacteria support the presence of anammox bacteria by oxygen consumption allowing the presence of anammox bacteria even in the oxygenated sediments. My results suggest that anammox bacteria can tolerate the presence of oxygen (Kalvelage et al., 2011; Yan et al., 2012) and co-exist with aerobic ammonia oxidizers and potentially have an important role in the nitrogen cycle in marine sediments.

Similarly, anammox bacteria detected in Lake Grevelingen sediments showed a clear seasonal contrast in their nitrite reductase (*nirS*) gene abundance with higher copy numbers in August during summer hypoxia compared to March, similar to the 16S rRNA and *hzsA* gene abundance of anammox bacteria detected in Oyster Ground sediments (Chapter 2). The seasonal trend was as well not reflected in the abundance of the anammox bacteria lipid biomarker PC-monoether ladderane lipid, as it has been reported for Oyster Grounds sediments (Brandsma et al., 2011; Bale et al., 2014). In contrast, the concentration of ladderane lipid fatty acids correlated with the abundance of anammox bacteria determined by the 16S rRNA gene abundance, suggesting that the abundance of ladderane lipid fatty acids could be interpreted as a proxy for the abundance of anammox bacteria, together with specific gene quantification. A difference of three to four orders of magnitude between abundances of the anammox 16S rRNA gene and the "*Candidatus* Scalindua" specific *nirS* gene was detected. This discordance between the 16S rRNA gene and

functional genes of anammox bacteria has been previously observed and has been discussed above in detail, thus the possibility of amplifying other bacteria has been ruled out by cloning and sequencing of the product generated during the quantitative PCR reaction for Lake Grevelingen sediments.

Overall, the *nirS* gene abundance of denitrifiers was three to four orders higher compared to the anammox *nirS* gene abundance, suggesting that *nirS*-type denitrifiers have a more prominent role in the nitrogen removal in Lake Grevelingen sediments compared to anammox bacteria. The abundance of denitrifying bacteria did not vary substantially despite of differences in the oxygen and sulfide availability, whereas anammox bacteria were more abundant in the summer hypoxia, but in those sediments with lower sulfide concentrations, indicating that the abundance of anammox bacteria is impaired by high sulfide concentrations as previously suggested by others (Dalsgaard et al., 2003; Jensen et al., 2008). Anammox abundance was also highest in Oyster Grounds sediments (0.5 cm) in August under low bottom water oxygen conditions and decreasing oxygen penetration. These results are most likely explained by of the fact that anammox bacteria are favored by hypoxic conditions (i.e. Neubacher et al., 2013). Besides, the optimum temperature of anammox bacteria of 15°C has been reported in temperate shelf sediments (Dalsgaard et al., 2005) which is close to the summer temperature in Oyster Grounds and Lake Grevelingen sediments (~17°C). Additionally, bioturbation has been shown to extend the area of aerobic processes such as nitrate reduction and thus the availability of reduced compounds such as nitrite, which would fuel the anammox process (Meyer et al., 2005). In Lake Grevelingen sediments, anammox abundance was highest in bioturbated compared to non bioturbated sediments (S3), indicating that bioturbation might additionally favor the abundance of anammox bacteria. Bioturbation has also been reported in Oyster Grounds (Duineveld et al., 1991), which together with the reported high activity of AOA, AOB and/or other aerobic heterotrophs removing the available oxygen, could contribute to the proliferation of anammox bacteria.

In order to determine the abundance of other microorganisms involved in nitrogen removal in Lake Grevelingen sediments (Chapter 4), I also quantified the nitrite reductase (*nirS*) gene of chemoorganoheterotrophic denitrifying bacteria and archaea, performing the stepwise conversion of nitrate/nitrite to dinitrogen gas (Zumft, 1997), as well as the *aprA* gene of sulfate reducing and sulfur oxidizing bacteria including autotrophic sulfide-dependent denitrifying members of the Alpha-, Beta-, Gamma-, and Epsilonproteobacteria (Shao et al., 2010).The *nirS* gene abundance has previously been show to correlate positively with potential denitrification rates (Mosier and Francis, 2010). Here, the *nirS* gene abundance of denitrifying bacteria was relatively constant with sediment depth, suggesting a stable community of heterotrophic and autotrophic denitrifying bacteria actively involved in the denitrification process in Lake Grevelingen sediments. However, assumptions have to be made with caution, because foraminiferal denitrification might be as important as heterotrophic denitrification in marine sediments, as well as metal-dependent denitrification (Devol, 2015). Besides, and some denitrifying microorganisms use the copper-containing nitrite reductase encoded by the *nirK* gene which could have led to the underestimation of the diversity and abundance of total denitrifiers by exclusively targeting the *nirS* gene. However, *nirS* denitrifiers are more likely to have a complete denitrification pathway (including *nor* and *nos* genes), suggesting that *nirS*-containing denitrifiers may be more likely to reduce nitrite to N_2 (Graf et al. 2014). In addition, the *nirS* gene has been shown to be more abundant and more diverse than *nirK* in estuarine sediments, although this pattern sometimes

shifts in different estuarine regions (Nogales et al. 2002; Abell et al. 2010; Mosier and Francis 2010; Beman, 2014; Smith et al. 2015b; Lee and Francis, 2017). In general, the *nirS* gene abundance was slightly higher in March in all three stations in Lake Grevelingen sediments (Chapter 4), but the difference was more pronounced in station 1, which can be most likely explained by lower sulfide concentrations in March compared to August. The lower sulfide concentrations in March could favor the proliferation of heterotrophic denitrifiers, as sulfide has been shown to impair denitrification by inhibition of NO and N_2O reductases (Sørensen et al., 1980).

The abundance of chemolithoautotrophic microorganisms utilizing the CBB and rTCA pathways for carbon fixation was also estimated in Lake Grevelingen sediments (Chapter 5) by quantifying the *cbbL* and *aclB* gene, encoding for the key-enzymes ribulose 1,5 bisphosphate carboxylase/oxygenase (CBB) and ATP citrate lyase (rTCA). Significant differences were determined in the abundance of *cbbL* and *aclB* genes between the seasons but not between the stations, suggesting a stable community of community of autotrophic microorganisms using the CBB and rTCA pathway. The abundance of the *cbbL* gene was at least 2-fold higher than *aclB* gene abundance independent of the season or station, indicating a more prominent role of microorganisms using the CBB cycle compared to those using the rTCA pathway. The results indicated a more important role of autotrophic aerobic or facultative aerobic Gammaproteobacteria of the orders Chromatiales and Thiotrichales compared to Epsilonproteobacteria of the order Campylobacterales and Euryarchaeota in Lake Grevelingen sediments. However, these assumptions have to be made with caution, as the primers used to amplify *aclB* genes are mainly based on sequences of Epsilonproteobacteria (Campbell et al., 2006), suggesting that potentially there is an underestimation of the abundance of microorganisms using the rTCA pathway in these sediments, as well as the abundance of microorganisms using other carbon fixation pathways than the CBB or rTCA pathway (Hügler and Sievert, 2011; Berg et al., 2010, 2011).

Taking all the considerations mentioned above, the determination of the abundance of microorganisms based on DNA sequences and lipid biomarker molecules in marine coastal and estuarine sediments has to be interpreted with caution. DNA-based methods employed in this thesis depend on PCR amplification with primers, designed based on the sequences available in the databases, potentially causing an underestimation of the diversity as well as of the abundance of target organisms. As the abundance of functional and 16S rRNA genes was estimated by qPCR and 16S rRNA gene amplicon sequencing, I cannot completely rule out primer induced PCR biases (Schloss et al., 2011; Sipos et al., 2010). Additionally, DNA and lipid biomarkers can be partly preserved in anoxic sediments, which possibly leads to an overestimation of the abundance of the studied microorganisms (Torti et al., 2015; Lengger et al., 2012; Rush et al., 2012).

7.4. Activity of chemolithoautotrophic microorganisms in coastal sediments

In my thesis, I have estimated activity by determining incorporation of ^{13}C-labeled substrates in membrane lipids as well as the gene expression of 16S rRNA and functional genes as proxy of transcriptional activity. The abundance and gene expression of functional genes has been frequently interpreted as an indication of the process rate in which the protein-coding gene is involved (Nogales et al., 2002; Smith et al., 2007; Abell et al., 2010, 2011; Bowen et al., 2014). However, this is not always the case and it is in general dependent on the regulation of the specific gene under study. As an example, the transcript levels of the coding gene for the dissimilatory sulfite reductase (*dsrA*), catalyzing the final step in sulfate reduction, has been seen to positively

correlate with sulfate reduction rate (SRR) when growing under limited electron acceptor (sulfate) while no correlation between SRR and *dsr*A gene expression was observed when grown under electron donor limitation (Villanueva et al., 2008). Similarly, a recent study by Carini et al. (2017) observed that ammonia limitation in the thaumarchaeon "*Candidatus* Nitrosopelagicus brevis" induced a decrease in the expression of the ammonia oxidation core proteins, which could be interpreted as a confirmation that lower ammonia oxidation activity is correlated to lower *amoA* gene expression levels. However, this study also concluded that the genes involved in ammonia oxidation constitute a large part of the total transcript pool irrespective of growth and environmental conditions, which makes it difficult to connect changes in gene expression to differences in the metabolic process.

Examples like this illustrate the need of studying, in pure cultures and under controlled conditions, the regulation of the expression of metabolic genes and its potential correlation to the process rates before making definite conclusions based on environmental samples. In this thesis, several metabolic genes involved in key re-oxidation pathways were analyzed for their gene expression patterns in environmental samples. For some of these genes, previous information on the regulation of their gene expression was available (e.g. AOA *amoA* gene; Abell et al., 2011), which allowed us to interpret the results more confidently. For some other genes, e.g. anammox bacterial *hzsA* gene (Harhangi et al., 2011), the determination of their gene expression and comparison with their gene abundance and process rate in environmental samples in this thesis (Chapters 2, 4), has allowed to get a better understanding of the environmental conditions that can affect the activity of the metabolic process.

To estimate the activity of aerobic and anaerobic ammonia oxidizers and anammox bacteria in coastal sediments, the gene expression of their 16S rRNA gene, the *amoA* gene of AOA and AOB, and the expression of the anammox bacterial *hzsA* gene, were analyzed here by quantifying the transcript abundance by reverse transcription and subsequent qPCR, in sediments of the Oyster Grounds (North Sea) (Chapter 2) and in Lake Grevelingen (chapter 4). The detection of the transcriptional activity of the AOA 16S rRNA gene up to 12 cm depth in Oyster Grounds sediment supports that AOA are indeed active in subsurface sediments. The potential transcriptional activity, by the 16S rRNA gene RNA:DNA ratio, was higher during winter, whereas the abundance was slightly higher during the summer months. The AOA *amoA* gene RNA:DNA ratio has been detected in August throughout the core but only in the upper 3 cm in February, because the quantity of transcripts in other seasons and depths was under the detection limit of the qPCR assay. However, the relationship of *amoA* gene expression and *in situ* ammonia oxidation is still unclear (Lam et al., 2007; Nicol et al., 2008; Abell et al., 2011; Vissers et al., 2013). Recent studies have observed a good correlation between nitrification rates and the abundance of *amoA* genes and transcripts in coastal waters (Smith et al., 2014; Urakawa et al., 2014), indicating that the detection of AOA *amoA* transcripts suggest AOA were active in Oyster Grounds sediments in the summer throughout the sediment in comparison with the winter in which AOA would be only active in the upper part of the sediment (upper 2 cm).

The abundance of AOB *amoA* transcripts was higher than for AOA, and was detected up to 12 cm sediment depth in all seasons, which would suggest a higher AOB activity compared to AOA. However, a study by Urakawa et al. (2014) in estuarine waters did not observe a correlation between nitrification rates and AOB *amoA* transcripts. Additionally, previous studies have detected the presence of AOB *amoA* transcripts in non-active starved cells (Bollmann et al., 2005;

Geets et al., 2006; Sayaveda-Soto and Arp, 2011) indicating that the AOB *amoA* transcriptional activity is not a good indicator of AOB nitrification activity due to a longer half-life of AOB *amoA* transcripts than expected for AOA *amoA* transcripts.

The metabolic capacities of sedimentary Thaumarchaeota are still under debate. To shed further light on this important chemolithoautotrophic metabolism in marine sediments, we also performed labeling experiments using ^{13}C-labeled substrates, such as bicarbonate, pyruvate, amino acids and glucose in sediment cores of the Iceland Shelf (Chapter 3) and determined the degree of ^{13}C-incorporation into the most abundant GDGT lipids, i.e. crenarchaeol and GDGT-0. These compounds have been suggested to be taken up by Thaumarchaeota in the environment (Ouverney and Fuhrman, 2000; Wuchter et al., 2003; Herndl et al., 2005; Takano et al., 2010; Pitcher et al., 2011c), or in enrichment cultures (Park et al., 2010; Jung et al., 2011; Tourna et al., 2011; Kim et al., 2012), and are possibly the most suitable substrates resulting in uptake by sedimentary Archaea. However, the incubation with labeled ^{13}C substrates did not result in any substantial incorporation of the ^{13}C into the biphytanyl chains of these lipids. No incorporation of ^{13}C in IPL-GDGT was detected in clay rich and organic rich sediments, whereas some enrichment in δ^{13}C (1−4‰) of IPL-GDGTs recovered from sandy sediment was detected after incubation with bicarbonate and pyruvate. This apparent lack of uptake could be due to several reasons. It is possible that the Thaumarchaeota were active, but the substrates were not taken up. Secondly, it is possible that most of the IPLs measured are fossil, and thus only a small amount of these IPLs are part of living and active sedimentary Thaumarchaeota, as also discussed above. Another possibility is that the metabolism and growth rates are so slow that no incorporation could be detected over the timescales investigated. Thus, the incubation period of 96−144 h, may have been too short. These results suggested that sedimentary Thaumarchaeota grow slowly in contrast to other sedimentary organisms, and/or a slow turn-over of IPL-GDGTs compared to bacterial/eukaryotic PLFAs. Interestingly, enrichment culture experiments with a thaumarchaeal strain closely related (99%) to *Nitrosopumilus maritimus* (SCM1), discovered that α-keto acids, such as pyruvate and oxaloacetate, were not directly incorporated into AOA cells, but functioned as chemical scavengers that detoxified intracellularly produced H_2O_2 during ammonia oxidation (Kim et al., 2016), indicating that previous findings of pyruvate incorporation could have been misinterpreted as evidence for mixotrophic growth of AOA.

In order to determine the potential activity of anammox bacteria in Oyster Grounds and Lake Grevelingen sediments, the potential transcriptional activity of the 16S rRNA gene, as well as of the functional genes *hzsA* and *nirS* encoding for the key-enzymes of the anammox reaction, hydrazine synthase and nitrite reductase (Chapter 2 and 4, respectively). A previous study by Bale et al. (2014) observed a good correlation between anammox rate activity and anammox bacterial 16S rRNA transcript abundance in North Sea sediments, suggesting that the transcriptional activity of anammox bacteria 16S rRNA gene is a good proxy for anammox bacteria activity. In Oyster Grounds sediments, the transcriptional activity of anammox bacteria 16S rRNA and *hzsA* gene was detected throughout the sediment in all seasons, and the 16S rRNA RNA:DNA ratio was higher during summer as observed for the anammox bacteria abundance. In Lake Grevelingen sediments the anammox 16S rRNA gene transcript abundance was detected throughout the sediment. However, the anammox bacteria 16S rRNA RNA:DNA ratio was low (0.01−1) compared to the values reported in sediments of the Oyster Grounds (1.6−34.5), further supporting a low anammox activity in Lake Grevelingen sediments.

Nevertheless, quantification results of anammox bacteria 16S rRNA gene have to be interpreted with caution because there is evidence that the ribosome content does not decrease during the period of starvation (Schmid et al., 2005; Li and Gu, 2011). Besides, the increase of anammox bacteria 16S rRNA gene transcriptional activity during summer in Oyster Grounds sediments was not observed for the *hzsA* gene, which remained stable with sediment depth and also for the different seasons. The low and constitutive transcriptional activity of the *hzsA* gene indicates that the *hzsA* gene transcript abundance does not reflect variations of anammox bacteria activity in environmental sediment samples, thus the *hzsA* transcript abundance does not seem to be an adequate biomarker for the estimation of the activity of anammox bacteria. Further experiments, preferably with pure cultures and under controlled physiological conditions, should be performed to clarify the regulation of the *hzsA* gene expression.

Environmental conditions favoring the abundance and activity of anammox bacteria have been discussed above in detail. Bioturbation activity could explain the differences in the activity of anammox bacteria in sediments of the Oyster Grounds compared to the Lake Grevelingen sediments, where only in station S3 bioturbators have been recognized in March, coinciding with the highest anammox abundance compared to the other stations. Additionally, the activity of anammox bacteria might be inhibited by high carbon and sulfide concentrations, as discussed above, probably explaining the higher activity of anammox bacteria in Oyster Grounds (low TOC), compared to Lake Grevelingen sediments (high TOC).

To further estimate the potential activity of microbial groups involved in denitrification other than anammox bacteria, i.e. denitrifying bacteria, sulfate reducing/sulfur oxidizing bacteria including sulfide-dependent denitrifying microorganisms, in seasonal hypoxic and sulfidic sediments of Lake Grevelingen (Chapter 4), I also analyzed the potential transcriptional activity of these groups. This was achieved by quantifying the *nirS* transcript abundance of denitrifying bacteria, as well as the *aprA* transcript abundance of sulfate reducing and sulfur oxidizing microorganisms. Unfortunately, there is a lack of studies correlating the *nirS* gene expression of denitrifying bacteria with denitrification rates, and some studies have shown a lack of direct relationship between *nirS* gene expression and modeled rates of denitrification in marine surface sediments (Bowen et al., 2014), whereas other studies have observed a correlation between a decrease of *nirS* transcript abundance and the reduction of denitrification rates coinciding with declining concentrations of nitrate in estuarine sediments (Dong et al., 2000, 2002; Smith et al., 2007). These studies would justify the use of *nirS* gene transcript abundance as a proxy for denitrification performed by *nirS*-type denitrifiers. In my study, the *nirS* transcript abundance of *nirS*-type denitrifiers was two or three orders lower compared to values previously reported for estuarine sediments, suggesting a low denitrification rate in surface sediments of all stations in March and in deeper sediment layers in August. Gene expression of the *nirS* gene was detected in surface sediments in March (all stations) and in deeper layers in August (stations S1 and S3), which is most likely explained by the availability of nitrate/nitrite in the bottom water. The potential transcriptional activity of sulfate reducing and sulfur oxidizing including chemolithoautotrophic sulfide-dependent denitrifiers, determined by the *aprA* gene RNA:DNA ratio was relatively low (≤ 0.00088), which also indicated a low denitrification potential of these groups.

Although denitrification was more relevant than the anammox process as suggested by the gene abundance and potential transcriptional activity, and measured denitrification rates (between 36 and 96 $\mu M\ m^{-2}\ d^{-1}$; Seitaj, D. personal communication) in Lake Grevelingen sediments

which were low compared to other marine sediments. Denitrification rates vary between 30 and 270 μM m^{-2} d^{-1} in marine arctic sediments (Rysgaard et al., 2004), whereas in estuarine sediments of the lower St. Lawrence estuary, denitrification rates of 270 ± 30 μM m^{-2} d^{-1} were measured (Crowe et al., 2012). The lower denitrification and anammox potential in Lake Grevelingen sediments could be explained by the effect of high concentrations of sulfide, potentially inhibiting both heterotrophic denitrification and anammox. Another explanation of the low denitrification and anammox potential of Lake Grevelingen sediments can be found in the interactions of anammox and heterotrophic denitrifiers with sulfur-oxidizers also involved in the nitrogen cycle. Large filamentous sulfur oxidizing bacteria have been detected in Lake Grevelingen sediments, i.e. cable bacteria and Beggiatoaceae including the genera *Beggiatoa*, *Thiomargarita* and *Thioploca* (Salman et al., 2011, 2013). Cable bacteria perform electrogenic sulfur oxidation (e-SOx), in which the oxidation of the electron donor and the reduction of the electron acceptor are separated over cm-scale distances and the redox coupling is ensured by long distance electron transport (Nielsen et al., 2010; Pfeffer et al., 2012). Cable bacteria can play a role in bioavailable nitrogen removal, as they have been shown to use oxygen but might as well use nitrite and nitrate (Meysman et al., 2015; Marzocchi et al., 2014; Risgaard-Petersen et al., 2014). Additionally, Beggiatoaceae couple the oxidation of sulfide to nitrate reduction resulting in N_2 and/or NH_4^+ (Mußmann et al., 2003). Previous studies have observed that in anoxic sediments both denitrification and anammox can be supported by intracellular nitrate transport, performed by sulfide-oxidizing *Thioploca* and *Beggiatoa* (Mußmann et al., 2007; Jorgensen, 2010; Prokopenko et al., 2013) down to deeper sediment layers and possibly supplying anammox and denitrifying bacteria with nitrite and/or ammonia produced by DNRA (Teske and Nelson, 2006; Prokopenko et al., 2011). Cable bacteria have been detected in S1 and S3 in March, whereas station S2 was dominated by Beggiatoaceae down to ~3 cm, but both groups were hardly detected in subsurface sediments (0.5 cm downwards) during summer hypoxia. The presence of sulfide-dependent denitrifiers, as shown by the *aprA* gene abundance and diversity, as well as the presence of cable bacteria and Beggiatoa-like bacteria could potentially support the denitrification and anammox processes in March. However, the nitrate and nitrite concentrations in the bottom water in March were reported to be relatively low (28 and 0.7 μM on average, respectively). This limitation could induce a strong competition for nitrate, nitrite and sulfide with Beggiatoaceae, cable bacteria and other nitrate-reducing bacteria, which may explain the limited denitrification potential observed in March. During summer hypoxia (August), sulfide inhibition and low nitrate and nitrite concentrations seem to limit the activity of heterotrophic and autotrophic denitrifiers and anammox bacteria. Further studies, involving denitrification rate measurements will be required to further assess the effects of hypoxia and high sulfide concentrations in the sediments of Lake Grevelingen.

The chemolithoautotrophic activity of the bacterial community has also been determined by PLFA analysis combined with ^{13}C-stable isotope probing in Grevelingen sediments (Chapter 5). In March, under oxygenated bottom water conditions, the chemolithoautotrophic rates were substantially higher than those in August, when oxygen in the bottom water was depleted to low levels. Moreover, in August, the chemolithoautotrophic rates showed a clear decrease in with water depth (S3 to S1), which is in line with the bottom water oxygen concentration over water depth. At the same time, the 16S rRNA gene amplicon sequencing revealed that the response of the chemolithoautotrophic bacterial community in surface sediments is associated

with the seasonal changes in the bottom water oxygen concentrations. As described above, in March the microbial community in surface sediments of all stations was characterized by high relative abundances of chemolithoautotrophic sulfur oxidizing Gammaproteobacteria and Epsilonproteobacteria using oxygen or nitrate as electron acceptor. In August, the lack of oxygen and nitrate in the bottom water was accompanied by a decrease in the relative abundances of Gamma- proteobacteria and Epsilonproteobacteria, and an increase of the relative abundance of heterotrophic sulfate-reducing Deltaproteobacteria using sulfate as electron acceptor. These results support the chemolithoautotrophic rates measured in March and August, indicating that the lack of electron acceptors such as oxygen and nitrate induced by hypoxia limiting the activity of chemolithoautotrophic bacteria in August. Recently, a study by Jessen et al. (2017) addressed the effects of hypoxia on the microbial community composition in Black Sea sediments, and showed that variations in oxygen supply to the seafloor influenced the composition of the bacterial community in surface sediments. Similar to my results, they reported an increase of Deltaproteobacteria under variable to anoxic conditions, whereas Gammaproteobacteria reached highest abundances under variable hypoxic conditions while decreasing toward stable oxic and anoxic conditions (Jessen et al., 2017). Together, these results show the important impact of bottom water hypoxia on the microbial community composition and especially on chemolithoautotrophs in sediments.

7.5. Outlook

In the last five years, new and improved methods have been developed and their cost reduced, which would now make them compatible with studies with a massive amount of samples. In the last two chapters of this thesis, I have already applied both 16S rRNA gene amplicon sequencing and lipid analysis with high resolution accurate mass/mass spectrometry that showed a quantitative and qualitative increase in the amount of data and the estimation of the diversity of not only chemolithoautotrophic microorganisms.

The studies included in this thesis regarding the gene expression and abundance of key metabolic genes of chemolithoautotrophs under different environmental and physiological conditions have shed further light on the role of this group in the carbon and nitrogen cycle in coastal sediments. In this thesis, the diversity of chemolithoautotrophic microorganisms was mostly determined with gene-targeted approaches, either by amplifying and sequencing specific 16S rRNA gene or functional genes involved in the carbon fixation of re-oxidation pathways. In this synthesis, I have listed the disadvantages of this approach mainly due to the biases introduced by the primer design, lack of sequences in databases, and inherent PCR amplification biases. Currently, these limitations could have been overcome by metagenomic and metatranscriptomic approaches, which are now much more affordable, the data analysis is more feasible, and most importantly they are not PCR-biased. With this approaches, I could have determined both diversity and the gene expression of chemolithoautotrophic key genes. However, the connection between gene expression and the activity of the actual process is still problematic. This information can only be validated by laboratory experiments in which the regulation of the gene of interest is thoroughly studied under different physiological and environmental conditions. Another recommendation is the combination of activity proxy measurements included in this thesis with direct rate measurements when possible to directly assess if the gene expression can be correlated to the activity measurement. Apart from using metatranscriptomics, the activity

of specific microorganisms could now also detected by using more sensitive approaches than the lipid-SIP approaches used in this thesis. For example, nano-scale secondary ion mass spectrometry (NanoSIMS) in combination with catalyzed reporter deposition (CARD)- fluorescence *in situ* hybridization (FISH) has proven to be very effective for the determination of metabolic capacity linked to phylogenetic identity at the single cell level (Behrens et al., 2008; Musat et al., 2008; Morono et al., 2011). However, a problem that remains is that this method still relies on incubation with labeled substrates with the inherent limitations of labeling-based incubations including labeling incubation time and cross-feeding of ^{13}C metabolites produced by the primary consumers of the ^{13}C substrate.

Finally, in this thesis both specific lipid biomarkers for targeted groups (i.e. crenarchaeol for Thaumarchaeota, ladderane lipids for anammox) and bacterial and archaeal lipid characterization were performed in sediment samples with the aim of determining the abundance and activity (i.e. Lipid-SIP) of chemolithoautotrophs. However, I have seen that some of this biomarkers (i.e. HPH-crenarchaeol, PC-ladderane) are potentially preserved in deeper sediments invalidating their use as proxies of living biomass. In this regard, a recommendation would be to continue in the search of lipid biomarkers that can be more representative of living biomass and less prone to preservation. Laboratory cultures together with characterization of the microbial lipidome with methods similar to the ones applied in Chapter 6 (i.e. high resolution accurate mass spectrometry) to detect intact polar lipids would be key in improving this issue. Also in chapter 6, I have seen the potential recycling of IPLs by archaeal groups like the DPANN. Further studies need to carefully assess the effect of such lipid recycling in sediment and the interpretations derived from those results.

Summary

The role of chemolithoautotrophic microorganisms has been considered to be of minor importance in coastal marine sediments although it has not been investigated in depth. Additionally, the impact of seasonal hypoxic/anoxic conditions on microbial chemolithoautotrophy in coastal marine sediments has not been examined. Therefore, in this thesis the diversity, abundance and activity of specific groups of chemolithoautotrophic microorganisms involved in the nitrogen and sulfur cycling, as well as the total microbial community, has been determined in coastal sediments by using DNA/RNA- and lipid-based biomarker approaches. Results show the presence and potential importance of chemolithoautotrophs in the biogeochemical cycling of carbon, nitrogen and sulfur. Temporal changes of the diversity, abundance and activity of chemolithoautotrophic microorganisms coinciding with seasonal hypoxia in coastal sediments have been determined.

The diversity, abundance and activity of chemolithoautotrophic microorganisms, such as ammonia oxidizing archaea (AOA) and bacteria (AOB), anammox and denitrifying bacteria, as well as sulfate reducing and sulfur oxidizing microorganisms, including sulfide dependent denitrifiers, have been determined. Additionally, the diversity and activity of chemolithoautotrophic microorganisms involved in the CBB and rTCA cycle as well as the metabolism of Thaumarchaeota and the abundance of Archaea have been addressed. In the following studies, we focused on coastal marine sediments, exposed to summer hypoxia, i.e. (1) the Oyster Grounds in the central North Sea, exposed to decreasing bottom water oxygen concentrations, (2) different sediment types (sand, clay, and mud) of the Iceland Shelf, and (3) seasonally hypoxic and sulfidic sediments of saline Lake Grevelingen (The Netherlands).

In Oyster Grounds sediments (chapter 2), seasonal variations of archaeal and bacterial ammonia oxidizers (AOA and AOB) and anammox bacteria, as well as the environmental factors affecting these groups, were addressed. We examined the seasonal and depth distribution of the abundance and potential activity of these microbial groups in coastal marine sediments of the southern North Sea. We quantified specific intact polar lipids (IPLs) as well as the abundance and gene expression of their 16S rRNA gene, the ammonia monooxygenase subunit A (*amoA*) gene of AOA and AOB, and the hydrazine synthase (*hzsA*) gene of anammox bacteria. AOA, AOB and anammox bacteria were detected and transcriptionally active down to 12 cm sediment depth. This study unraveled the coexistence and metabolic activity of AOA, AOB, and anammox bacteria in bioturbated marine sediments of the North Sea, leading to the conclusion that the metabolism of these microbial groups is spatially coupled based on the rapid consumption of oxygen that allows the coexistence of aerobic and anaerobic ammonia oxidizers. AOA outnumbered AOB throughout the year which may be caused by the higher oxygen affinity of AOA compared to AOB. Anammox bacterial abundance and activity were higher during summer, indicating that their growth and activity are favored by higher temperatures and lower oxygen available in the sediments due to summer stratifying conditions in the water column. During the summer, anammox bacteria are probably not in competition with AOA and AOB for ammonia as the concentrations were relatively high in the sediment pore water most likely as a result of ammonification processes and the activity of denitrifiers and DNRA.

In Iceland Shelf sediments (chapter 3), we investigated the activity of Thaumarchaeota in sediments by supplying different ^{13}C-labeled substrates which have previously been shown to be

incorporated into archaeal cells in water incubations and/or enrichment cultures. We determined the incorporation of ^{13}C-label from bicarbonate, pyruvate, glucose and amino acids into thaumarchaeal intact polar lipid-glycerol dibiphytanyl glycerol tetraethers (IPL-GDGTs) during 4–6 day incubations of marine sediment cores from three sites on the Iceland Shelf. Thaumarchaeal IPLs were present in sediment cores recovered from the Iceland Shelf and the presence of labile IPL-biomarkers for Thaumarchaeota, such as hexose phosphohexose (HPH)-crenarchaeol, suggested the presence of living Thaumarchaeota. However, incubations with ^{13}C-labeled substrates bicarbonate, pyruvate, glucose and amino acids did not result in any substantial incorporation of the ^{13}C into the biphytanyl chains of these lipids. Turnover times and generation times of thaumarchaeal lipids were estimated to be at least several years, suggesting slow growth of sedimentary Thaumarchaeota in contrast to other sedimentary organisms, and/or a slow degradation of IPL-GDGTs, in contrast to bacterial or eukaryotic PLFAs.

In Lake Grevelingen sediments (chapter 4), we evaluated the abundance, diversity and potential activity of denitrifying, anammox, and sulfide-dependent denitrifying bacteria in the sediments of the seasonally hypoxic saline Lake Grevelingen, known to harbor an active microbial community involved in sulfur oxidation pathways. Depth distributions of 16S rRNA gene, *nirS* gene of denitrifying and anammox bacteria, *aprA* gene of sulfur-oxidizing and sulfate-reducing bacteria, and ladderane lipids of anammox bacteria were studied in sediments impacted by seasonally hypoxic bottom waters. This study unraveled the coexistence and potential activity of heterotrophic and autotrophic denitrifiers, anammox bacteria as well as SOB/SRB in seasonally hypoxic and sulfidic sediments of Lake Grevelingen. The *nirS*-type heterotrophic denitrifiers were a stable community regardless of changes in oxygen and sulfide concentrations in different seasons and with a similar abundance to that detected in other sediments not exposed to high sulfide concentrations. Besides, *nirS*-type denitrifiers outnumbered anammox bacteria leading to the conclusion that anammox does not contribute significantly to the N_2 removal process in Lake Grevelingen sediments. The anammox bacteria population seemed to be affected by the physicochemical conditions between seasons and their abundance and activity was higher in lower sulfide concentrations and low carbon input, also supporting a possible inhibition of anammox bacteria by sulfide. The sulfide-dependent denitrifiers in Lake Grevelingen sediments have proven to be abundant, but their expected sulfide-detoxification potential did not seem to alleviate the predicted inhibition of organoheterotrophic denitrifiers and anammox by sulfide. Both denitrifiers and anammox bacteria activity could be inhibited by the competition with cable bacteria and Beggiatoaceae for electron donors and acceptors (such as sulfide, nitrate and nitrite) before summer hypoxia but might be still supported by the potential sulfide detoxification of the sediment through sulfide oxidation, supporting at least a low activity. During summer hypoxia, sulfide inhibition and low nitrate and nitrite concentrations seem to limit the activity of heterotrophic and autotrophic (sulfide-dependent) denitrifiers and anammox bacteria.

Additionally, we examined the changes in activity and structure of the sedimentary chemolithoautotrophic bacterial community in Lake Grevelingen under oxic (spring) and hypoxic (summer) conditions (Chapter 5). Combined 16S rRNA gene amplicon sequencing and analysis of phospholipid derived fatty acids indicated a major temporal shift in community structure. Chemolithoautotrophy rates in the surface sediment were three times higher in spring compared to summer. Besides, the depth distribution of chemolithoautotrophy was linked to the distinct sulfur oxidation mechanisms identified through microsensor profiling, i.e., canonical sulfur ox-

idation, electrogenic sulfur oxidation by cable bacteria, and sulfide oxidation coupled to nitrate reduction by Beggiatoaceae. The metabolic diversity of the sulfur-oxidizing bacterial community suggests a complex niche partitioning within the sediment probably driven by the availability of reduced sulfur compounds and electron acceptors regulated by seasonal hypoxia.

In Lake Grevelingen sediments, we also compared the archaeal abundance and composition by DNA-based methods and the archaeal intact polar lipid (IPL) diversity in surface sediments to determine the potential biological sources of the archaeal IPLs detected in surface sediments under changing environmental conditions (Chapter 6). We observed a dramatic change in the archaeal community composition and lipid abundance in surface sediments of a seasonally hypoxic marine lake which corresponded to a switch from a Thaumarchaeota-dominated to a DPANN-dominated archaeal community, while the total IPLs detected were significantly reduced. Considering the reduced genome of the members of the superphylum DPANN and their apparent inability to synthesize their own membrane lipids, we hypothesize that they use the CLs previously synthesized by the Thaumarchaeota to form their membrane. Results indicate an active recycling of fossil IPLs in the marine surface sediment.

Overall, the research presented in this thesis has provided new insights on the diversity, abundance and activity of chemolithoautotrophic microorganisms, such as AOA, AOB, anammox and denitrifying bacteria, as well as sulfate reducing and sulfur oxidizing microorganisms, including sulfide-dependent denitrifiers in coastal marine sediments. Hypoxia and increasing sulfide concentration have proven to be key factors restricting the presence and activity of the above mentioned groups. In addition, the interactions with other microbial groups and bioturbation seem to favor the presence of specific chemolithoautotrophs in unexpected sediment depths, such as the presence of AOA in depths where the oxygen concentration was expected to be undetectable, or the presence of anammox bacteria in bioturbated sediments where nitrate reducers could thrive and provide nitrite to fuel the anammox process. In general, the methods employed in these studies regarding the gene expression and abundance of key metabolic genes of chemolithoautotrophs under different environmental and physiological conditions have shed further light on the role of this group in the carbon and nitrogen cycle in coastal sediments. However, the connection between gene expression and the activity of the actual process is still be problematic and future studies should address the regulation of specific metabolic genes under different physiological and environmental conditions. Finally, the application of specific lipid biomarkers to detect the presence and abundance of specific chemolithoautotrophs in coastal sediments (i.e. crenarchaeol for Thaumarchaeota, ladderane lipids for anammox) has revealed a potential preservation effect of these biomarkers in deeper sediments potentially invalidating their use as proxies of living biomass. Further studies are needed to fully assess this effect as well as the recycling of intact polar lipids by specific archaeal groups.

Samenvatting

De rol die chemolithoautotrofe micro-organismen spelen in sedimenten van kustzeeën is nog steeds onduidelijk. Met name is het effect van het optreden van seizoensgebonden hypoxische/anoxische condities op de microbiële chemolithoautotrofe gemeenschappen niet onderzocht. In dit proefschrift is de diversiteit, celaantallen en activiteit van specifieke groepen chemolithoautotrofe micro-organismen die actief zijn in de stikstof- en zwavelcyclus, in relatie tot de totale microbiële gemeenschap, in kustsedimenten onderzocht door gebruik te maken van op DNA/RNA en lipiden gebaseerde biomarker benaderingen. Specifiek gaat het om ammonia-oxiderende archaea (AOA) en bacteriën (AOB), anammox en denitrificerende bacteriën, sulfaat-reducerende en zwavel-oxiderende micro-organismen (waaronder sulfide-afhankelijke denitrificerende bacteriën). Ook werden de diversiteit en activiteit van chemolithoautotrofe micro-organismen betrokken bij de CBB- en rTCA-cycli bestudeerd. De studie richtte zich op sedimenten van kustzeeën, d.w.z. (1) de Oestergronden in de centrale Noordzee, die gedurende de zomer blootgesteld zijn aan verlaagde zuurstofconcentraties, (2) verschillende soorten sedimenten (zand, klei en modder) van de IJslandse kustzeeën, en (3) hypoxische en sulfidische sedimenten van het zoute Grevelingenmeer (Nederland), blootgesteld aan seizoensgebonden hypoxia. De resultaten laten het belang van chemolithoautotrofe micro-organismen in de biogeochemische cycli van koolstof, stikstof en zwavel zien. Seizoensgebonden veranderingen in zuurstofconcentratie leiden tot grote tijdelijke veranderingen in het aantal, de diversiteit, en activiteit van chemolithoautotrofe micro-organismen.

In de gebioturbeerde sedimenten van de Oestergronden in de zuidelijke Noordzee zijn de seizoensvariaties en diepteverdeling in concentratie van AOA en AOB en anammox-bacteriën in relatie tot omgevingsfactoren bestudeerd (hoofdstuk 2). Specifieke intacte polaire lipiden (IPL) zijn gekwantificeerd. Daarnaast werden specifieke 16S rRNA-genen, het ammoniakmonooxygenase subeenheid A (*amoA*) gen van AOA en AOB, en het hydrazine synthase (*hzsA*) gen van anammox bacteriën en hun expressie gekwantificeerd. AOA, AOB en anammox bacteriën werden gedetecteerd en waren actief tot 12 cm diepte in het sediment. Het metabolisme van deze microbiële groepen is ruimtelijk gekoppeld op basis van zuurstofverbruik, hetgeen de coëxistentie van aërobe en anaërobe ammonia-oxiderende bacteriën mogelijk maakt. AOA waren gedurende het hele jaar meer dominant aanwezig dan AOB, hetgeen waarschijnlijk veroorzaakt wordt door de hogere zuurstofaffiniteit van AOA in vergelijking met AOB. De celaantallen en activiteit van anammox bacteriën waren hoger in de zomer. Dit wordt waarschijnlijk veroorzaakt door hogere temperaturen en lagere zuurstofbeschikbaarheid in de sedimenten gedurende de periode van zomerstratificatie. Gedurende de zomer zijn de anammox bacteriën waarschijnlijk niet in concurrentie met AOA en AOB voor ammonia omdat de concentraties betrekkelijk hoog waren in het poriewater van het sediment, waarschijnlijk als gevolg van ammonificatie processen.

In sedimenten van IJslandse kustzeeën werden de activiteit van ammonia-oxiderende Thaumarchaeota onderzocht met behulp van verschillende ^{13}C-gelabelde substraten, die in eerdere studies met incubatie met verrijkingsculturen en zeewater succesvol bleken (hoofdstuk 3). Sedimentkernen werden gedurende 4-6 dagen geïncubeerd met ^{13}C-gelabeled bicarbonaat, pyruvaat, glucose en aminozuren en opname van het label in Thaumarchaeale in de membraanlipiden (intacte polaire glyceroldibiphytanylglyceroltetraethers; IPL-GDGTs) werd bepaald. De aanwezigheid van labiele IPL-GDGT membraanlipiden karakteristiek voor Thaumarchaeota, zoals

hexosefosfoshexose (HPH) -crenarchaeol, duidde op de aanwezigheid van levende Thaumarchaeota. Incubatie met de ^{13}C-gemerkte substraten leidde echter niet tot een substantiële inbouw van het ^{13}C label in de bifytanylketens van deze membraanlipiden. Omzettijden en generatietijden van thaumarchaeale lipiden werden geschat op ten minste enkele jaren, hetgeen duidt op een trage groei van sedimentaire Thaumarchaeota of een langzame afbraak van IPL-GDGTs. Dit contrasteert met de bacteriële of eukaryotische membraanlipiden (vetzuren afkomstig van polaire lipiden) waar het label wel in terecht kwam.

In het sediment van het Grevelingenmeer werden de diversiteit, celaantallen en potentiële activiteit van denitrificerende, anammox en sulfide-afhankelijke denitrificerende bacteriën bestudeerd (hoofdstuk 4). De concentratie van 16S rRNA-genen, het *nirS*-gen van denitrificerende en anammox bacteriën, het *aprA*-gen van zwavel-oxiderende (SOB) en sulfaat-reducerende (SRB) bacteriën en ladderaanlipiden van anammox bacteriën werd bepaald in sedimenten tot 5 cm diepte op drie verschillende plaatsen en op twee verschillende momenten in het jaar. De condities in de sedimenten van het Grevelingenmeer worden sterk beïnvloed door het seizoensgebonden optreden van hypoxia in het bodemwater. Deze studie toont de coëxistentie en potentiële activiteit van heterotrofe en autotrofe denitrificerende bacteriën, anammox bacteriën en SOB/SRB aan. De heterotrofe denitrificerende bacteriën van het *nirS*-type vormen een stabiele gemeenschap, die onafhankelijk is van de veranderingen in zuurstof- en sulfideconcentratie gedurende de verschillende seizoenen. Hun celaantallen zijn even hoog als in sedimenten die niet gekenmerkt werden door hoge sulfideconcentraties. *NirS*-type denitrificerende bacteriën overtroffen in aantal de anammox bacteriën, hetgeen suggereert dat anammox niet significant bijdraagt aan stikstofverwijdering in sedimenten van het Grevelingenmeer. De gentranscriptie activiteit van denitrificerende en anammox bacteriën evenals van zwaveloxiderende, waaronder sulfide-afhankelijke denitrificerende en sulfaat-reducerende bacteriën, werden mogelijk door de accumulatie van sulfide in de zomer geïnhibeerd. Zowel de denitrificerende en anammox bacteriën kunnen worden belemmerd door de concurrentie voor NO_3^- en NO_2^- met kabel- en *Beggiatoa*-achtige bacteriën voor zomerhypoxia. Resultaten suggereren dat sulfide verwijdering door zwavel-oxiderende bacteriën onvoldoende is om de activiteit van denitrificerende en anammox bacteriën in de sedimenten van het Grevelingenmeer te ondersteunen.

In hoofdstuk 5 is het effect van seizoensgebonden hypoxia op de activiteit en de gemeenschapsstructuur van chemolithoautotrofe bacteriën onderzocht in sedimenten van het Grevelingenmeer voor (Maart) en tijdens (Augustus) het optreden van zomerhypoxia. Gecombineerde 16S rRNA gen amplicon sequencing en PLFA analyse onthulde een belangrijke temporale verschuiving in de gemeenschapsstructuur. Aërobe zwavel-oxiderende Gamma- en Epsilonproteobacteriën overheersten de chemolithoautotrofe gemeenschap in het voorjaar, terwijl Deltaproteobacteriën veel meer voorkwamen tijdens de zomer. De bijdrage van chemolithoautotrofe bacteriën, bepaald m.b.v. PLFA-SIP, was drie maal hoger in de lente dan tijdens de zomer, vooral in oppervlaktesedimenten. De dominantie van *cbbL* (RuBisCO) en *aclB* (ATP-citraat lyase) genen liet een overheersing, onafhankelijk van het seizoen en plaats, van micro-organismen die de CBB cyclus gebruiken in vergelijking met de rTCA cyclus zien. De diepteverdeling in het sediment van chemolithoautotrofie was gekoppeld aan de verschillende zwavel-oxidatiemechanismen, die werden geïdentificeerd door middel van microsensorprofielen, d.w.z. kanonische zwavel-oxidatie, elektrogenische zwavel-oxidatie door kabelbacteriën en sulfide-oxidatie gekoppeld aan nitraatreductie door *Beggiatoaceae*. De metabolische diversiteit van de zwavel-oxiderende bacte-

riële gemeenschap suggereert een complexe verdeling van niches in het sediment, waarschijnlijk veroorzaakt door de beschikbaarheid van gereduceerde zwavelverbindingen (H_2S, S^0 en $S_2O_3^{2-}$) en elektronacceptoren (O_2 en NO_3^-), gereguleerd door het optreden van seizoensgebonden hypoxia.

In de oppervlakte sedimenten van het Grevelingenmeer is de op DNA-gebaseerde archaeale diversiteit en distributie vergeleken met die op basis van archaeale intacte membraanlipiden om de potentiële biologische bronnen van de archaeale IPLs te detecteren (hoofdstuk 6). De archaeale levensgemeenschap veranderde ten gevolge van de hypoxia van het bodemwater van een door Thaumarchaeota-gedomineerde in een door DPANN-gedomineerde archaeale levensgemeenschap. Tegelijkertijd nam de concentratie IPL-GDGTs met een orde van grootte af. Archaea in het superphylum DPANN worden gekenmerkt door een klein genoom en bezitten geen genen die coderen voor eiwitten die de synthese van membraanlipiden katalyseren. Op basis hiervan is de hypothese geponeerd dat zij zogenaamde core lipiden gebruiken die eerder door de Thaumarchaeota zijn gesynthetiseerd om hun membraan te vormen. Dit zou duiden op een actieve recycling van fossiele IPL-GDGTs in oppervlaktesedimenten.

Samenvattend heeft het onderzoek in dit proefschrift nieuwe inzichten verschaft met betrekking tot de diversiteit, celaantallen en activiteit van chemolithoautotrofe micro-organismen. Hypoxia en sulfideconcentratie zijn belangrijke factoren die hun aanwezigheid en activiteit beperken. Bovendien lijken de interacties met andere microbiële groepen en bioturbatie de aanwezigheid van specifieke chemolithoautotrofe microben op onverwachte sedimentdiepten te bevorderen, zoals de aanwezigheid van AOA in sedimentlagen waar zuurstof niet detecteerbaar was. In het algemeen hebben de methoden die in deze studies worden toegepast met betrekking tot de genexpressie en de overvloed aan belangrijke metabolische genen van chemolithoautotrophs onder verschillende milieu- en fysiologische omstandigheden, de rol van deze groep in de koolstof- en stikstofcyclus in kustsedimenten verder benadrukt. De directe koppeling tussen genexpressie en activiteit is echter nog steeds niet eenduidig en toekomstige studies zullen de regulering van specifieke metabolische genen onder verschillende fysiologische en milieuomstandigheden verder moeten bestuderen. Tenslotte heeft het onderzoek aangetoond dat specifieke lipide biomarkers de potentie hebben om specifieke chemolithoautotrofe microben in sedimenten van kustzeeën te detecteren (bijv. crenarchaeol voor Thaumarchaeota, ladderaan lipiden voor anammox bacteriën). Omdat deze lipiden bewaard kunnen blijven in sedimenten hebben zij potentie om gebruikt te worden in de reconstructie van vroegere microbiële activiteit. De mogelijke recycling van intacte polaire lipiden door specifieke archaea is een proces dat dit gebruik mogelijk zou kunnen compliceren.

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About the Author

Yvonne Antonia Lipsewers was born on the 18th of July 1977 in Salzkotten, Germany. During her Abitur she gained interest in aquatic biology and was motivated by her biology teacher with a seminar about hydrology. After leaving secondary school, she first started studying food technology before finally, studying Biology at the University of Bielefeld from 2003 until 2007. Afterwards, she was specializing in Marine Microbiology during her Master's at the Institute of Chemistry and Biology of the Marine Environment associated to the Carl-von-Ossietzky Universität in Oldenburg from 2008 until 2010. She completed a Master's research project investigating the shift of the heterotrophic bacterial community composition during the aggregation of a diatom bloom in the North Sea. After graduating in 2010, Yvonne started a PhD project at NIOZ in 2011, focused on tracing chemolithoautotrophic microorganisms in coastal sediments, under the supervision of Dr. Laura Villanueva, which finally led to the composition of this thesis.