Competition in nitrate-reducing microbial communities

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Summary

The biogeochemical nitrogen cycle, including nitrate reduction processes, is highly affected by human activity such as fertilization and ammonia deposition caused by fossil fuel burning. Consequently, gaining a better understanding about the ecophysiology of nitrate-reducing microbial communities is crucial for inferring the impact of anthropogenic nitrogen input.

Different nitrate-reducing pathways compete with each other for the electron acceptor nitrate: Denitrifiers reduce nitrate to dinitrogen and nitrous oxide while dissimilatory nitrate reducers reduce nitrate to ammonium. The outcome of this competition has important environmental consequences: denitrification removes fixed nitrogen from the ecosystem, while dissimilatory nitrite reduction to ammonium (DNRA) keeps fixed nitrogen bioavailable. Although a lot of studies have been performed on this topic, no conclusive factors responsible for the dominance of one or the other process could be identified so far.

In this thesis, the competition between nitrate reduction pathways was addressed by combining continuous culture incubations of natural microbial communities with stable isotope labeling and metagenomics, complemented with metatranscriptomics and metaproteomics in order to gain insight into the identity, function and interaction of the enriched microbial populations. To be able to make the best use of the obtained metagenomic data a new metagenomic binning procedure was developed.

Before the competition between two different nitrate reduction pathways was studied, the relationship between functional and compositional stability over time within one nitrate reduction pathway was investigated: In a heterotrophic denitrifying microbial community, enriched from a marine intertidal flat, strong community dynamics were occurring under constant conditions and during stable conversion of substrates. A stable metabolic interaction between the denitrifying populations and co-enriched fermenting microbes persisted throughout the experiment unaffected by the ongoing population dynamics. This indicated that functional stability was independent of the community composition. Apparently, only the persistence of the overall metabolic potential was important to maintain functional stability. This suggested that stochastic as well as deterministic processes are responsible for the observed community composition.

Once the functional stability of denitrification was confirmed and interactions with other microbial guilds were known the competition between DNRA and denitrification was addressed. Several parallel continuous culture incubations that differed in one condition but

were otherwise constant led to the identification of the generation time as most important control on the competition between DNRA and denitrification. The organic carbon to nitrate ratio and the kind of electron acceptor supplied (nitrate or nitrite) were identified as further controlling factors that together with the generation time discriminated between the two pathways. The metabolic interaction between nitrate- reducing and fermenting populations was stable under both pathways.

One quarter of the nitrate reduction was coupled to the oxidation of sulfide, which was produced in the enrichment culture by microbial sulfate reduction, constituting a strong link between the nitrogen and sulfur cycle.

All in all, this thesis provides new insights into the ecophysiology of microbial nitrate reducers by unraveling the driving forces of the competition between different nitrate reduction pathways and by revealing important metabolic interactions with other microbial guilds.

Zusammenfassung

Der biogeochemische Stickstoffkreislauf wird stark durch den Menschen über einen hohen Gebrauch an Düngemitteln und Ammoniumeintrag durch das Verbrennen fossiler Brennstoffe belastet. Dies gilt auch für die Nitratreduktion. Um die Auswirkungen zu verstehen, die solch eine Einflussnahme mit sich bringt, ist es wichtig ein besseres Verständnis über die Ökophysiologie von nitratreduzierenden mikrobiellen Gemeinschaften zu gewinnen.

Verschiedene Nitratreduktionswege konkurrieren mit einander um den Elektronakzeptor Nitrat: Während Denitrifizierer Nitrat zu molekularen Stickstoff oder Distickstoffmonoxid reduzieren, reduzieren Nitratammonifizierer es zu Ammonium. Daher hat es wichtige Konsequenzen für die Umwelt, welcher der beiden Prozesse sich durchsetzt: Durch Denitrifikation wird dem Ökosystem bioverfügbarer Stickstoff entzogen. Bei Nitratammonifizierung hingegen bleibt dieser Stickstoff dem Ökosystem erhalten. Obgleich viele verschiedene Studien die Konkurrenz zwischen diesen beiden Prozessen mit verschiedenen Methoden untersucht haben, konnten keine Schlüsselfaktoren, die den einen oder anderen Prozesses eindeutig fördern würden, erkannt werden.

In dieser Dissertation wird die Suche nach solchen Schlüsselfaktoren adressiert, und zwar durch die Kombination der Inkubation von natürlichen mikrobiellen Gemeinschaften in kontinuierlichen Kulturen mit der Markierung von Substraten durch stabile Isotope und mit Metagenomik. Das Ziel besteht darin einen Einblick in die Identität, Funktion und Interaktion der verschiedenen angereicherten mikrobiellen Populationen zu bekommen. Komplementiert wird dieser Ansatz mit Metatranskriptomik und Metaproteomik. Um die Metagenomedaten bestmöglich nutzen zu können, wurde ein neues Verfahren zur Klassifizierung von Metagenomedaten, dem sogenannten „Binning“ entwickelt.

Bevor die Konkurrenz zwischen zwei verschiedenen Nitratreduktionswegen untersucht wurde, wurde zunächst der Zusammenhang zwischen funktioneller und kompositioneller Stabilität über die Zeit für einen Nitratreduktionsweg adressiert: Eine heterotrophe denitrifizierende mikrobielle Gemeinschaft aus dem Watt, die unter konstanten Bedingungen angereichert wurde, zeigte eine starke Dynamik in den angereicherten Populationen über die Zeit, während die Umwandlung der Substrate stabil war. Eine stabile metabolische Interaktion bestand zwischen den denitrifizierenden Populationen und ko-angereicherten fermentierenden Mikroben, ungeachtet der starken Populationsdynamik. Dieses zeigt, dass

die funktionale Stabilität unabhängig von der Zusammensetzung der mikrobiellen Gemeinschaft war und nur die Erhaltung des genetischen Potentials für Nitratrduktion und Fermentation wichtig ist, um die Stabilität aufrecht zu erhalten. Die Populationsdynamik fand zwischen wenigen verschiedenen denitrifizierenden und fermentierenden Populationen statt. Insgesamt deuten die Ergebnisse darauf hin, dass sowohl stochastische als auch deterministische Prozesse die Zusammensetzung der mikrobiellen Gemeinschaft beeinflussten.

Nachdem die funktionelle Stabilität von Denitrifikation und die Interaktionen von Denitrifizierern mit anderen mikrobiellen Gruppen bekannt waren, wurde die Konkurrenz zwischen DNRA und Denitrifikation adressiert. Das Anreichern von mikrobiellen Gemeinschaften in mehreren kontinuierlichen Kulturen, die sich nur in einem Faktor unterschieden, führte zu der Identifikation der Generationszeit als wichtigsten Kontrollparameter für die Selektion zwischen DNRA und Denitrifikation. Im Zusammenspiel mit dem Verhältnis von organischem Kohlenstoff zu Nitrat und der Art des verfügbaren Elektronakzeptors (Nitrat oder Nitrit) entschied die Generationszeit über den Ausgang der Konkurrenz zwischen Denitrifikation und DNRA.

Ein Viertel der Nitratreduktion war an die Oxidation von Sulfid gekoppelt. Sulfid wurde durch mikrobielle Sulfatreduktion produziert und direkt oxidiert ohne zu akkumulieren. Dieses zeigt eine starke Verknüpfung zwischen dem Stickstoff- und Schwefelkreislauf.

Durch die Aufdeckung der Kontrollparameter, die den vorherrschenden Nitratreduktionsweg vorgeben, und durch das Aufzeigen von wichtigen metabolischen Interaktionen mit anderen mikrobiellen Gruppen trägt diese Dissertation zu dem Verständnis der Ökophysiologie von mikrobiellen Nitratreduzierern bei.

List of abbreviations

ARMAN	Archaeal Richmond Mine acidophilic nanoorganisms
ATP	Adenosine triphosphate
ARISA	Automated ribosomal intergenic spacer analysis
Ca.	*Candidatus*
CARD-FISH	Catalyzed reporter deposition-fluorescence *in situ* hybridization
C/N ratio	Carbon to nitrogen ratio
COD	Chemical oxygen demand
CSCG	Conserved single copy genes
DAPI	4,6-diamidino-2-phenylindole
DOC	Dissolved organic carbon
DNA	Deoxyribonucleic acid
DNRA	Dissimilatory nitrate reduction to ammonium
Mbp	Mega base pairs
MCC	Multiheme cytochrome *c*
N	Nitrogen
OCC	Octaheme cytochrome *c*
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
RAxML	Randomized Accelerated Maximum Likelihood
RING-FISH	Recognition of individual genes-FISH
RFI	Relative fluorescence intensity
RNA	Ribonucleic acid
RNA-SIP	RNA-based stable isotope probing
rRNA	ribosomal RNA
RT-PCR	Reverse transcription-PCR
TOC	Total organic carbon

Chapter 1

1 Introduction

1.1 Nitrogen in the environment

Nitrogen is essential to all life on earth as it is a key component of fundamental building blocks of a cell such as nucleic acids and proteins. That is the reason why nitrogen limitation may limit the productivity of an ecosystem and nitrogen fertilization is used to enhance biomass production in agriculture. Biogeochemical cycling of nitrogen almost entirely depends on diverse redox reactions of nitrogen compounds primarily sustained by microbial energy metabolism (Canfield, 2010). Thereby, nitrogen compounds get transformed and, depending on the redox reaction performed, different end-products may accumulate.

This thesis addresses competition within nitrate reducing communities. Different aspects of such competition are treated: on the one hand different populations that carry out the same nitrate-reducing pathway may compete among each other. On the other hand different nitrate reducing pathways may be in competition with each other:

1.1.1 The Nitrogen Cycle

When reactive nitrogen enters an ecosystem mostly in the form of ammonium most of it gets converted to nitrate and nitrite by nitrification. These compounds can be used as electron acceptor for microbial anaerobic respiration. Nitrate and nitrite can be reduced to ammonium by a process called dissimilatory nitrate reduction to ammonium (DNRA) or to N_2 by the process of denitrification (Lam and Kuypers, 2011). In anammox, dinitrogen is formed from nitrite and ammonium. The microorganisms performing the different nitrate reduction pathways compete for electron acceptors. To understand the outcome of such competition and the impact that it may have, it is crucial to examine the different sources and sinks of nitrate and nitrite and the links and interplays of nitrate reducing pathways with the different microbial processes of the nitrogen cycle. Therefore, the following paragraphs give a short overview of the nitrogen cycle (figure 1.1). The different nitrate-reducing processes are only briefly summarized. A more detailed description can be found in chapter 2, which is part of

the introduction, but it is included in this thesis as an independent chapter, because it represents a self-contained, published manuscript.

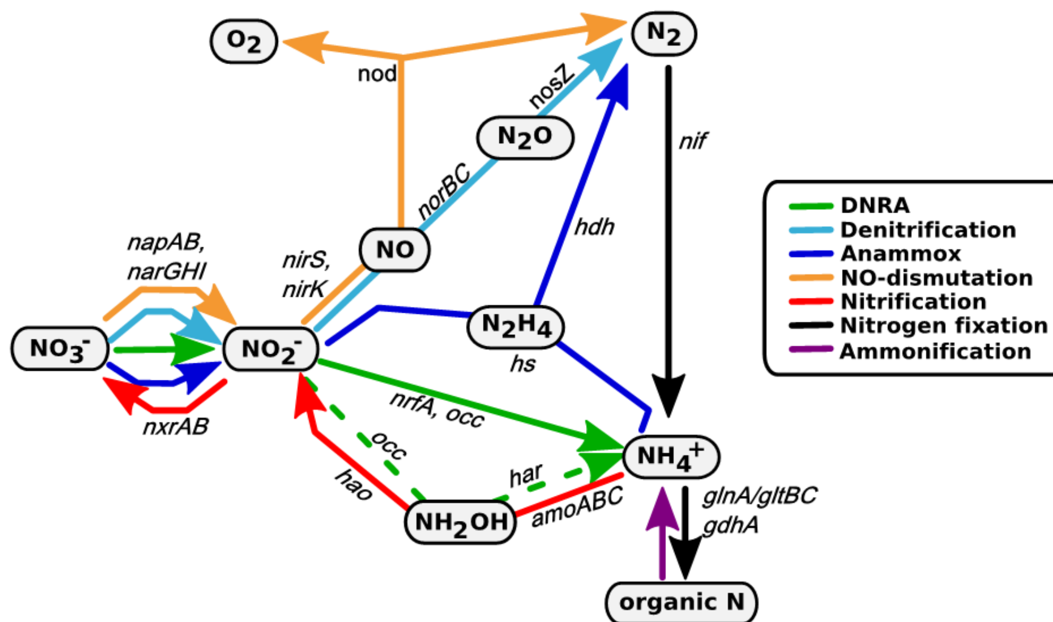


Figure 1.1: The nitrogen cycle: Biological transformation and associated genes.

Denitrification

During denitrification, nitrate is reduced via nitrite, nitric oxide and nitrous oxide to dinitrogen. The enzymes catalyzing the different steps of denitrification are the nitrate reductases NarGH and NapAB, the nitrite reductases NirS and NirK, the nitric oxide reductase NorB and the nitrous oxide reductase NosZ. A single denitrifying bacterium may not possess all the enzymes of the complete denitrification pathway and only carry out certain steps of denitrification (Zumft, 1997). Denitrification can be performed by a wide range of microorganisms from all three domains of life: more than 60 genera of bacteria and archaea and several eukaryotes. (Canfield, 2010, Hayatsu *et al*., 2008, Risgaard-Petersen *et al*., 2006). In the marine environment, among others, members of the genera *Arcobacter* and *Sulfurimonas* and the SUP05 group have been identified as important denitrifiers (e.g. Canfield *et al.,* 2010, Fuchsman *et al.*, 2012, Walsh *et al.*, 2009, Lavik *et al.*, 2009). Marine denitrification (including anammox) is responsible for 50-70% of loss of fixed nitrogen to the atmosphere, about 50% of which is occurring in coastal sediments (Fowler *et al.,* 2013,

Gruber *et al.,* 2004). Denitrification is considered to be one of the major sources of nitrous oxide emissions, a greenhouse gas that has 300 times higher warming potential than CO_2 and scavenges ozone in the stratosphere (Jetten, 2008, Canfield *et al*., 2010, Ravishankara *et al*., 2009). Especially in the presence of oxygen and in the transition from anoxic to oxic conditions, denitrification leads to the production of nitrous oxide because nitrous oxide reductases seem to be particularly sensitive to oxygen (Otte *et al*., 1996, Schreiber *et al*., 2012).

Dissimilatory Nitrate Reduction to Ammonia

Dissimilatory nitrate reduction to ammonium (DNRA), also named dissimilatory ammonification or nitrate ammonification converts nitrate to nitrite and then further to ammonium. In contrast to assimilatory nitrate reduction to ammonium, DNRA is coupled to proton translocation across the cytoplasmic membrane. Many different unrelated bacteria are capable of this process, but biochemically it has been best studied in *Wolinella succinogenes*, *Escherichia coli* and *Shewanella oneidensis* (Einsle *et al*., 2000, Bamford *et al*., 2002, Simon, 2002). The key enzyme that mediates the reduction of nitrite to ammonium is the pentaheme cytochrome *c* NrfA (Simon, 2002).

Furthermore, octaheme cytochrome *c* enzymes evolutionary related to hydroxylamine oxidoreductases also have been proposed to be involved in DNRA (Atkinson *et al.*, 2007; Klotz *et al.*, 2008). Based on the genome of *Nautilia profundicola*, an Epsilonproteobacterium from a submarine hydrothermal environment, such an octaheme (reverse) hydroxylamine:ubiquinone reductase module has been proposed to reduce nitrite to hydroxylamine (Campbell *et al*., 2009). Hydroxylamine is then further reduced to ammonium by the hybrid cluster protein hydroxylamine reductase (har) (Hanson *et al*., 2013, Cabello *et al*., 2004). The proposed pathway was confirmed for this organism based on evidence from physiological experiments and transcriptomics (Hanson *et al*., 2013). However, its distribution or relevance in the environment remains unknown.

Recently, different eukaryotes such as diatoms and the fungus *Aspergillus terreus* have been shown to also perform DNRA (Kamp *et al*., 2011, Stief *et al*., 2014). Which enzymes are responsible for this pathway in eukaryotes remains to be identified.

Denitrification as well as DNRA may be coupled to the heterotrophic oxidation of organic carbon or to the lithotrophic oxidation of inorganic compounds such as sulfide (Burgin and Hamilton, 2007) The re-oxidation of sulfide produced by sulfate-reduction (Fossing *et al.,* 1995, Canfield *et al.,* 2010) prevents the build-up of harmful sulfide concentrations (Lavik *et al.,* 2009). As sulfide oxidation and sulfate reduction are often closely coupled, this cryptic sulfur cycling is easily overlooked and may be widespread in marine habitats (Canfield *et al.,* 2010). So far, sulfide oxidation has mainly been associated with denitrification, but not with DNRA, with members of the SUP05 group and the genera *Arcobacter* and *Sulfurimonas* as important key players (e.g. Canfield *et al.,* 2010, Fuchsman *et al.,* 2012, Walsh *et al.,* 2009), while the importance of DNRA coupled to sulfide oxidation remains largely undetermined.

Anammox

Dinitrogen is also released to the atmosphere by anaerobic ammonium oxidation (anammox, Strous *et al.* 1999). Anammox is performed by a specialized monophyletic group of chemolithoautotrophic bacteria within the phylum of Planctomycetes (Kuenen, 2008). Anammox bacteria have long generation times of 7 to 22 days (Kartal *et al*., 2013). During the anammox process, dinitrogen is produced by coupling the reduction of nitrite with the oxidation of ammonium. Nitrite is reduced to nitric oxide by the nitrite reductase NirS and the enzyme hydrazine synthase forms hydrazine from nitric oxide and ammonium (Kartal *et al*., 2011). Hydrazine is oxidized to dinitrogen by the hydrazine dehydrogenase, an octaheme oxidoreductase (Schmidt *et al.* 2008). The enzymes carrying out the anammox pathway are located inside the anammoxosome, an intracytoplasmic compartment carried by all anammox bacteria.

Anammox is another pathway that competes for nitrite and in certain habitats may be the main process responsible for dinitrogen generation (Kartal *et al.,* 2013). When encountering ammonium limitation, anammox bacteria are able to perform DNRA and thus produce ammonium that is subsequently consumed again in the anammox reaction (Kartal *et al*., 2007, Van Niftrik and Jetten, 2012).

NO-dismutation

The reduction of nitrite to dinitrogen may proceed via a third pathway. Instead of reducing nitric oxide to nitrous oxide as in canonical denitrification, the bacterium *Candidatus* Methylomirabilis oxyfera, dismutates nitric oxide to dinitrogen and oxygen (Ettwig *et al.*, 2010). The generated oxygen is used to oxidize methane (Ettwig *et al*., 2010). *Candidatus* M. oxyfera and other nitrite reducing methanothrophs of the NC10 phylum, potentially performing NO-dismutation, have been found in and enriched from different methane and nitrite rich freshwater habitats such as peatlands, ditches, lakes and wastewater treatment plants (Ettwig *et al.*, 2009, Zhu *et al.*, 2012). A newly discovered characteristic bacteriohopanepolyol may serve as diagnostic biomarker for detecting *Methylomirabilis sp*., and possibly other related intra-aerobic methanotrophs in the environment (Kool *et al.*, 2014). Environmental rates of this process have not been determined yet; in stable isotope tracer experiments that use ^{15}N-labelled nitrate or nitrite NO-dismutation produces $^{30}N_2$ and thus is not distinguishable from denitrification. Consequently, its ecological significance remains unknown.

Nitrogen fixation

Microbial nitrogen fixation is the only relevant natural nitrogen fixation process replenishing the pool of fixed nitrogen lost via denitrification, anammox and NO-dismutation. The second known natural N_2-fixing process, lightning, only makes up ca 2% of natural nitrogen fixation (Fowler *et al*., 2013). The conversion of dinitrogen to ammonium is an exergonic reaction, but high activation energy is required to break the very strong triple bond of dinitrogen. In microbial nitrogen fixation this reaction is catalyzed by the oxygen sensitive heterodimeric enzyme complex nitrogenase, which requires 16 ATP per N_2 fixed to perform this reaction (Howard and Rees, 1996). Nitrogenases and thus the ability to fix nitrogen are found in different microbial groups that are widespread over the phylogenetic tree including among others Cyanobacteria, Proteobacteria, Firmicutes as well as archaea (Zehr *et al.*, 2003, Murray *et al*., 1984). Nitrogen fixation can be linked to a variety of energy metabolisms including oxygenic and anoxygenic photosynthesis, fermentation, sulfate reduction, methanogenesis (Murray *et al*., 1984, Raymond *et al*., 2004, Henderson and Wilson 1969, Carpenter and Capone, 2008). It has been detected in a variety of habitats including the open ocean, deep ocean sediments, hydrothermal vents, seagrass rhizosphere and in symbioses

with eukaryotes in e.g. root nodules of legume or termite guts (Zehr *et al.*, 2003, Dekas *et al.*, 2009, Mehta and Baross 2006). High rates of marine nitrogen fixation appear to be spatially coupled to regions with high rates of water column denitrification, while the presence of fixed nitrogen, especially ammonium, has an inhibitory effect (Dixon and Kahn, 2004). This suggests that an oceanic feedback loop stabilizes the ocean's fixed nitrogen content (Deutsch *et al.*, 2007, Brandes *et al.*, 2007).

Nitrification

In the presence of oxygen, ammonia can be oxidized to nitrite and further to nitrate. This process is called nitrification and constitutes the link between nitrogen fixation and the processes that convert fixed nitrogen to N_2, e.g. denitrification and anammox. Nitrification is performed by chemolithoautotrophic bacteria and archaea (Ward, 2008) and is divided into two different steps carried out by distinct microbial groups. The first step, ammonia oxidation is performed by bacteria including Betaproteobacteria of the genera *Nitrosomonas and Nitrosospira* and Gammaproteobacteria of the genus *Nitrosococcus,* and by archaea belonging to the phylum Thaumarchaeota e.g. *Nitrosopumilus maritimus* (Koops *et al*., 2003, Könneke *et al.,* 2005). The enzyme complex carrying out the oxidation of ammonia to hydroxylamine is the ammonia monooxygenase. The genes encoding this complex are *amoABC*. In bacteria, hydroxylamine is oxidized to nitrite by the octaheme enzyme hydroxylamine oxidoreductase, encoded by the gene *hao* (Klotz *et al.,* 2008). For the archaeal ammonia oxidizers (Walker *et al.,* 2010) the pathway and enzymes responsible for hydroxylamine oxidation still need to be identified. The oxidation of nitrite to nitrate is catalyzed by the nitrite:nitrate oxidoreductase, a molybdopterin oxidoreductase. The genes *nxrAB* encoding this enzyme complex are homologous to the nitrate reductase *narGH* performing the reverse reaction (Simon and Klotz, 2013). Known nitrite oxidizers have been found within the phylum Nitrospirae and within the Proteobacteria. Ammonium oxidation is thought to be the rate-limiting step of nitrification (Biller *et al*., 2012, Stein *et al*., 2012). The nitrite produced by ammonia-oxidizers is consumed by either aerobic nitrite oxidizing bacteria or nitrite reducers, which are usually found to occur in interaction with nitrite-producing microbes (Stein *et al.,* 2012). Furthermore, ammonia oxidizing microbes are capable of performing (partial) denitrification, which enables them to convert ammonia to N_2O or N_2 (Wrage *et al*., 2001, Schleper and Nicol, 2010).

Nitrogen assimilation and dissimilation

During biomass build-up, ammonium gets assimilated and converted to particulate organic nitrogen. Nitrate and nitrite are converted to ammonium first. Some organisms can use dissolved organic nitrogen compounds such as urea and amino acids for assimilation (Mulholland and Lomas, 2008, Bradley *et al*., 2010). When biomass gets degraded again by heterotrophic organisms of all trophic levels of the food chain the organic nitrogen contained in biomass is released as ammonium and dissolved organic nitrogen (Canfield *et al.,* 2005).

Summarizing, the availability of reactive nitrogen and its distribution among the different pools (e.g. ammonium, nitrate, nitrite) results from the balance and coupling of nitrogen fixation, transformations within the reactive nitrogen pools and processes that remove reactive nitrogen such as denitrification and anammox. In this complex network of redox reactions nitrite is an important hub and, depending on the predominating transformation process of nitrite nitrogen is lost from the reactive pool or transformed within.

1.1.2 Human impact

About 200 years ago, researchers started to understand the importance of nitrogen availability for crop yield and got interested in possibilities to influence the fate of nitrogen in agriculture. Soon after, fixed nitrogen was identified as an important nutrient for plants (Von Liebig, 1840, Galloway, 2013). Further studies driven by the motivation to feed the growing population led to the discovery of nitrification (Schloesing and Muntz, 1877) and biological nitrogen fixation (Hellriegel and Wilfarth, 1888). Once denitrification was recognized as loss of fixed nitrogen from agricultural soils, the motivation of research was the inhibition of this process (Deherain and Maquenne, 1882). In 1886, the first denitrifiers were isolated by Gayon and Dupetit (Galloway, 2013). In 1909, Fritz Haber developed a process that made it industrially feasible to produce ammonium from dinitrogen and hydrogen. In this energy–intensive process N_2 and H_2 are combined at high pressure and temperature in the presence of iron as catalyst (Erisman *et al.*, 2008). Today, anthropogenic impact on the nitrogen cycle is tremendous and has been steadily increasing during the last century. The steadily increasing demand for food leads to increasing fertilizer and land use. The demand for fixed nitrogen as fertilizer is satisfied by the Haber-Bosch process. Nowadays, approximately every third reactive nitrogen atom originates from this process and

together with ammonia deposition it exceeds biological nitrogen fixation (Fowler *et al.*, 2013, Galloway *et al.*, 2003). In agricultural soils only 17% of the synthetic fertilizer nitrogen supply is recovered in crop or food products (Fowler *et al.*, 2013). Denitrification may account for a loss of about 40% of the nitrogen input (Galloway, 2004). This equals a waste of energy invested in the Haber–Bosch process of about 1% of the global primary energy supply (Erisman *et al.*, 2008). The rest is transported to adjacent freshwater habitats and rivers and still up to 25% of the fertilizer is transported to marine coastal areas (Mulholland *et al.* 2008), where it affects ocean chemistry, both regionally in coastal waters and globally in the open ocean (Doney *et al.*, 2010).

In ecosystems with low buffering capacity this additional nitrogen supply can cause acidification (Erisman *et al.*, 2013). In nitrogen limited habitats, a surplus in nitrogen input may lead to eutrophication with further consequences such as hypoxia, loss of biodiversity and changes in species composition (Camargo and Alonso, 2006, Rabalais, 2002). The nitrogen cycle is further affected by human activity such as atmospheric deposition of ammonia and nitrogen oxides caused by combustion processes and fossil fuel burning, (Galloway *et al.*, 2008, Vitousek *et al.*, 1997).

About 1-5% of the fertilizer nitrogen is released to the atmosphere as nitrous oxide, where it acts as a greenhouse gas absorbing infrared radiation given off by the earth's surface that could otherwise escape to space. The increasing emission of nitrous oxide reinforces global warming (Fowler *et al.*, 2013, Crutzen *et al.*, 2008, Bouwman *et al.*, 2013). It has been estimated that current anthropogenic nitrogen input contributes 5-10% to the enhanced greenhouse effect (Hanke and Strous, 2010). Additionally, the extremely energy-intensive Haber-Bosch process indirectly contributes to the enhanced greenhouse effect by CO_2 emission occurring during the generation of the required energy. Furthermore, N_2O emissions are presently the most important emissions responsible for ozone depletion in the stratosphere and are projected to remain the largest during the 21st century (Ravishankara *et al.*, 2009).

The anthropogenic impact on the earth's nitrogen budget has been estimated to further increase with increasing need for food, and consequently, nitrogen fertilizer (Schlesinger, 2009). Fertilization of crops for energy production (fuel crops) would lead to additional contributions to global warming, potentially offsetting the benefits. To be able to evaluate potential consequences for different ecosystems and implication for the climate it is essential

to understand the factors that control the complex interactions of the biogeochemical nitrogen network.

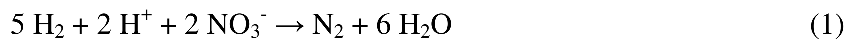
1.1.3 Competition between DNRA and Denitrification

The outcome of the competition between denitrification and DNRA has important environmental consequences. Denitrification and anammox convert fixed nitrogen to gaseous products (mainly nitrogen) and hence lead to a loss of fixed nitrogen from the system. Denitrification also contributes to the emission of the greenhouse gas nitrous oxide to the atmosphere. In contrast, DNRA produces ammonium and thus keeps fixed nitrogen available for primary production. Different natural and engineered systems sustain or require a different fate of nitrate and nitrite. Wastewater treatment plants aim for complete nitrogen removal because the discharge of ammonium is undesired causing elevated oxygen consumption and eutrophication in the receiving waters (Bernet *et al.*, 2000). In contrast, in agriculture, retention of nitrogen in the system is aspired in order to optimize fertilization effectiveness. Accordingly, a great interest lies in the question how DNRA could be favored at the expense of denitrification. Natural habitats are highly affected by elevated anthropogenic nitrogen load (Fowler *et al.*, 2013). Furthermore, the availability of nitrogen directly affects key processes in biogeochemical carbon cycling such as primary production and decomposition of organic matter. Consequently, understanding the factors that govern the outcome of the competition between nitrate reducing pathways is crucial for understanding the fate of nitrate in natural and engineered systems and hence for inferring the consequences of anthropogenic alteration of the nitrogen cycle's budgets.

Nitrate reduction rates attributed to denitrification, anammox and DNRA have been assessed in a variety of different habitats ranging from wastewater treatment plants to the open ocean: In aquatic marine environments such as marine oxygen minimum zones either denitrification (Ward *et al.*, 2009; Bulow *et al.*, 2010) or anammox (Kuypers *et al.*, 2005, Thamdrup *et al.*, 2006, Hamersley *et al.*, 2007, Kalvelage *et al.*, 2013) seem to be the predominating nitrate reduction processes. Nevertheless, recent studies showed that DNRA may also be important in such habitats being coupled to anammox (Jensen *et al.*, 2011, Lam *et al.*, 2009). In marine coastal sediments and freshwater or terrestrial habitats mainly denitrification (Jäntti *et al.*, 2011, Kaspar, 1983, Gao *et al.*, 2012, Porubsky *et al.*, 2009, Scott *et al.*, 2008, Washbourne *et al.*, 2011) or DNRA (Dong *et al.*, 2011, An and Gardner, 2002,

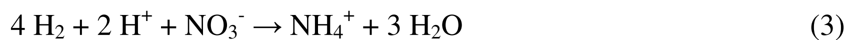
Silver *et al.*, 2001) or both together (Jørgensen, 1989, Koop-Jakobsen and Giblin 2010) have been identified as the dominating processes, while the relative importance of anammox mostly seems to be smaller (Burgin and Hamilton, 2007, Thamdrup *et al.*, 2012, Schubert *et al.*, 2006). In such habitats the fate of fixed nitrogen is of special interest because marine coastal sediments and freshwater habitats are often especially affected by anthropogenic nitrogen input (Voss *et al.*, 2013). Furthermore it has been estimated that up to 70% of marine nitrogen loss occurs in coastal shelf sediment (Codispoti, 2007).

Accordingly, the focus of this thesis is on the competition between denitrification (reaction 1 and 2, exemplary with hydrogen and glucose as electron donor) and DNRA (reaction 3 and 4).

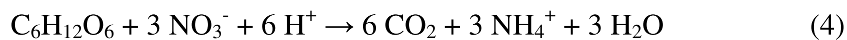


$$5\ H_2 + 2\ H^+ + 2\ NO_3^- \rightarrow N_2 + 6\ H_2O \qquad (1)$$

$$5\ C_6H_{12}O_6 + 24\ NO_3^- + 24\ H^+ \rightarrow 30\ CO_2 + 12\ N_2 + 42\ H_2O \qquad (2)$$



$$4\ H_2 + 2\ H^+ + NO_3^- \rightarrow NH_4^+ + 3\ H_2O \qquad (3)$$



$$C_6H_{12}O_6 + 3\ NO_3^- + 6\ H^+ \rightarrow 6\ CO_2 + 3\ NH_4^+ + 3\ H_2O \qquad (4)$$

It is generally assumed that the outcome of this competition is determined mainly by the relative supplies of electron donors and acceptors (Tiedje *et al.*, 1982, Schmidt *et al.*, 2011, Tugtas and Pavlostathis 2007, Strohm *et al.*, 2007, Van de Leemput *et al.*, 2011). If nitrate is supplied in excess, denitrifiers would outcompete dissimilatory nitrate reducers and vice versa: Under nitrate limitation dissimilatory nitrate reducers would outcompete denitrifiers. Biochemically this makes sense because denitrifiers conserve more energy per electron at low electron turnover. Dissimilatory nitrate reducers conserve less energy per electron but turn over more electrons (table 1.1). With the supply of nitrite instead of nitrate these bioenergetic differences get even more pronounced.

It has been proposed that the chemical nature of the electron donor also contributes to the relative success of denitrifiers versus dissimilatory nitrate reducers: The supply of fermentable carbon substrates such as glucose and glycerol are supposed to favor DNRA, while fermentation products such as acetate or lactate are assumed to favor denitrification. (Akunna *et al.*, 1993, Tugtas and Pavlostathis, 2007). Contrastingly, formate and hydrogen have been supposed to be preferred electron donors for DNRA (Simon, 2002).

Table 1.1: Theoretical energy yield of denitrification and DNRA and the electrons transferred with nitrate and nitrite as electron acceptor. ($G^{0'}$ values were taken from Thauer *et al.*, 1977.)

Reaction	**Electron acceptor**	**Electron donor**	**$\Delta G^{0'}$ (kJ/mol) per e^--donor**	**$\Delta G^{0'}$ (kJ/mol) per e^--acceptor**	**Electrons transferred per e^--acceptor**
Denitrification	NO_3^-	H_2	-224	-560	5
	NO_3^-	Glucose	2723	567	5
	NO_2^-	H_2	-159	-397	3
	NO_2^-	Glucose	-3212	-402	3
DNRA	NO_3^-	H_2	-150	-600	8
	NO_3^-	Glucose	-1828	-609	8
	NO_2^-	H_2	-109	-437	6
	NO_2^-	Glucose	-1774	-443	6

Environments where high concentrations of sulfide accumulate are supposed to favor DNRA (Brunet and Garcia-Gil, 1996, Wong *et al.*, 2011, Mazéas *et al.*, 2008, Ann and Gardner, 2002) because sulfide has been shown to inhibit the enzymes responsible for the last steps of denitrification, the reduction of nitric oxide to nitrous oxide and nitrous oxide to dinitrogen (Sørensen *et al.*, 1980, Pan and Yuan, 2013). Albeit, sulfide can also serve as electron donor for both denitrification and DNRA (Fossing *et al.,* 1995, Canfield *et al.,* 2010). The ambient temperature has been proposed as a further factor differentiating between nitrate-reducing processes. High temperatures have been shown to favor DNRA over denitrification, while at low temperatures denitrification is favored (Dong *et al.*, 2011, Ogilvie *et al.*, 1997). DNRA is bioenergetically more favorable than denitrification under electron acceptor limitation over the range of environmentally relevant temperatures. However, the bioenergetic advantage of DNRA over denitrification becomes slightly smaller at higher temperatures (Dong *et al.*, 2011, figure 1.2). A higher affinity for nitrate especially at higher temperature has been proposed for DNRA (Dong *et al.*, 2011).

The pH of the environment has also been proposed to influence the ongoing nitrate reducing process (Schmidt *et al.*, 2011, Nägele and Conrad 1990). A low pH seems to inhibit the last steps of denitrification, NO and N_2O reduction (Nägele and Conrad 1990).

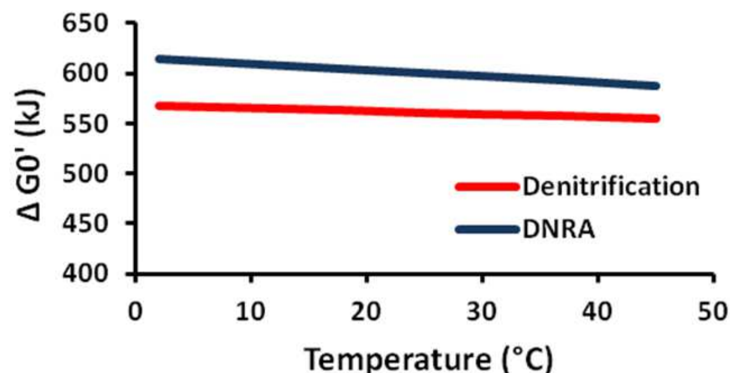


Figure 1.2: Gibbs free energy changes per mol nitrate [$\Delta G^{0'}$ (kJ)] for denitrification and DNRA at different temperatures exemplarily for hydrogen as electron donor (according to Dong *et al.,* 2011).

Furthermore, the absolute nitrate and nitrite concentrations seemed to have an effect on the competition (Dong *et al.*, 2011; Schmidt *et al.*, 2011). Lower nitrate concentrations were reported to favour denitrification and higher concentrations DNRA (Dong *et al.*, 2011). Furthermore, the affinity for the electron acceptor has been discussed as potential differentiating factor (Tiedje *et al.*, 1982).

The determination of the potential influence of environmental parameters on rates of denitrification and dissimilatory nitrate reduction in natural ecosystems is difficult because natural ecosystems are dynamic and differ in more than one factor simultaneously (Wallenstein *et al.*, 2006). Factors such as dissolved and particulate organic carbon content and sulfide concentrations frequently remain undetermined. Consequently, it is not surprising that factors responsible for the dominance of one or the other process could not be conclusively identified so far. Biochemical and physiological investigations have been restricted to very few model organisms (e.g. Strohm *et al.*, 2006) with unresolved environmental significance.

1.2 Population dynamics in nitrate-respiring communities

For evaluating ecosystem stability it is not only important to get a clear picture of the competition between different nitrate reducing processes but also crucial to understand how a microbial community maintains stable performance of a process and responds to

perturbations such as invasion. To what extent does the stable persistence of a nitrate respiring pathway, e.g. denitrification, depend on the microbial species that perform this pathway in a given habitat? Is the stable performance linked to the persistence and abundance of single microbial populations or the composition and stability of the whole microbial community?

Regarding the strong human influenced alteration of biogeochemical nitrogen budgets it is furthermore important to understand how microbial communities and especially the populations directly involved in nitrogen cycling react to such environmental changes.

1.2.1 Interactions between different populations of a microbial community

Interaction between different populations can be mutualistic (both populations benefit), commensalistic (one population benefits, while the other does not encounter any advantages or disadvantages) and antagonistic. Examples for mutualistic interactions are obligate or facultative syntrophy (Schink, 2002). In an antagonistic relationship both populations can harm each other, e.g. when competing for nutrients or space, or one population benefits, while the other is harmed, e.g. parasitism or predation (Little *et al*., 2008). A further example for antagonistic behavior is the production of toxins (Riley and Wertz, 2002, Czárán *et al*., 2002). In a microbial community with multiple members these relationships become intertwined and a complex network of interactions is created.

Furthermore, viral lysis of most abundant populations of a community can introduce community dynamics and benefit less abundant populations (Thingstad and Lignell, 1997, Thingstad, 2000).

1.2.2 Niche or neutral theory and population dynamics

Neutral theory assumes that all members of a particular functional group are equally competitive and that the community structure results from stochastic dynamics (Hubbell, 2001). Once a community has adapted to the environmental setting of its habitat, in steady state its composition may still change, but these would be random ecologically neutral

changes. Accordingly, replicates of the same habitat would never have the same community composition even if they were inoculated with the same source community (Curtis and Sloan, 2004). The extent of reproducibility would depend on the biodiversity of the source community: A higher diversity would lead to lower reproducibility. A higher diversity is also expected to lead to a higher stability of the community performance and community assembly is supposed to be a stochastic process (Curtis and Sloan, 2004).

In contrast, niche–based theory assumes that coexistence of different populations originates from their occupancy of different niches. According to the niche concept developed by Hutchinson (Hutchinson, 1953) the combination of a set of biotic and abiotic parameters in an environment creates a niche. Populations within a community are adapted to different sets of conditions and therefore occupy different niches and can co-exist without competition, while species that occupy the same or overlapping niches compete with each other (Whittaker, 1972). Changes in species composition can be explained by changes in environmental parameters. Populations that are best adapted to the new conditions succeed (Dumbrell *et al.*, 2010).

Nowadays, it is well accepted that deterministic niche-based processes and stochastic-neutral processes complement each other and together structure community composition (Chave, 2004). Nevertheless, the contribution of one or the other process to shaping community dynamics in natural habitats is difficult to untangle and studies have come to different results when trying to assign relative importance to the two mechanisms (van der Gast *et al*., 2008, Langenheder and Székely, 2011, Ofiteru *et al*., 2010).

The assumption that all microbial populations are ubiquitously distributed led to the statement of Lourens Baas Becking ‘alles is overal, maar het milieu selecteert’ (Translation: Everything is everywhere, but the environment selects) (Baas Becking, 1934). This implies that all microbial populations are mostly present at very low abundances often below the detection limit. In an environment that a microbial population is well adapted to it gains in abundance (De Wit and Bouvier, 2006). Contrastingly, further geographic distance between habitats is believed to lead to higher differences in species composition due to limitation of dispersal (McGill *et al.*, 2006).

1.3 Methodical approach

1.3.1 Continuous cultivation

Information about the physiology of isolated microbial strains is often obtained by pure culture studies. As microbes are isolated from their natural habitat and from interactions within a microbial community, little information can be gained about their ability to compete with other microbial populations. The composition of a microbial community and the ecophysiological role of each population arise from synergistic or antagonistic interactions between the different populations present e.g. cross feeding and competition. Hence, the success of a certain microbial population can only be studied in the context of a microbial community and the competition between different microbial guilds and/or populations needs to be studied under a setting that most closely approaches *in situ* conditions.

Natural environments are dynamic systems: Perturbations may lead to changes in the biogeochemical settings and thereby directly influence the microbial community and the processes it is performing. Multiple variables may change concurrently at one time point and may have changed significantly in the recent past. To disentangle the effect that a certain factor may have *in situ,* all variables would need to be captured at and before the time of sampling. Thus it is difficult if not impossible to link the abundance and activity of microbial populations to specific environmental factors (Curtis and Sloan, 2004). Consequently, the questions formulated above can only be answered with a controlled experiment in the laboratory with constant conditions, in which only the factor, whose influence is addressed, is varied, while all other variables are kept constant. The requirement for a controlled experimental set-up that approximates *in situ* conditions is met by a chemostat. In contrast to batch cultures, in which initially supplied substrates are consumed and thus concentrations change over time, the conditions in a chemostat are stable. The chemostat consists of culture vessel with continuous supply of medium from a reservoir vessel and continuous removal of cells and metabolic products at the same rate (figure 1.3; Novick and Szilard, 1950). Thus, the volume of the culture and, in steady state, the overall sum of all cells (biomass) is constant and so are the substrate and product concentrations in the culture. Furthermore the growth rate and cell numbers are under experimental control: The cells within the continuous culture are permanently dividing at a rate that equals the volume turnover rate of the chemostat (Dykhuizen and Hartl 1983). If the maximum growth rate of a microbial population present in the inoculum lies below the growth rate defined by the experimental

setting, it is washed out. The cell number only depends on the concentration limiting nutrient in supplied medium (Novick and Szilard in 1950).

In combination, the chosen experimental conditions (e.g. medium composition, growth rate, pH) lead to the natural selection of a microbial community that is well adapted to the conditions applied from a more complex inoculum. The selective forces applied can be translated into environmental factors and their influence on nitrate respiration can be studied. The close monitoring of substrate turnover by measuring the consumption of substrates and generation of products including gases enables a mass balancing of the overall ongoing metabolic processes. Constantly low substrate concentrations in the culture vessel in combination with high substrate turnover rates reflect conditions as they are often found in natural habitats (Hanke *et al*., 2014).

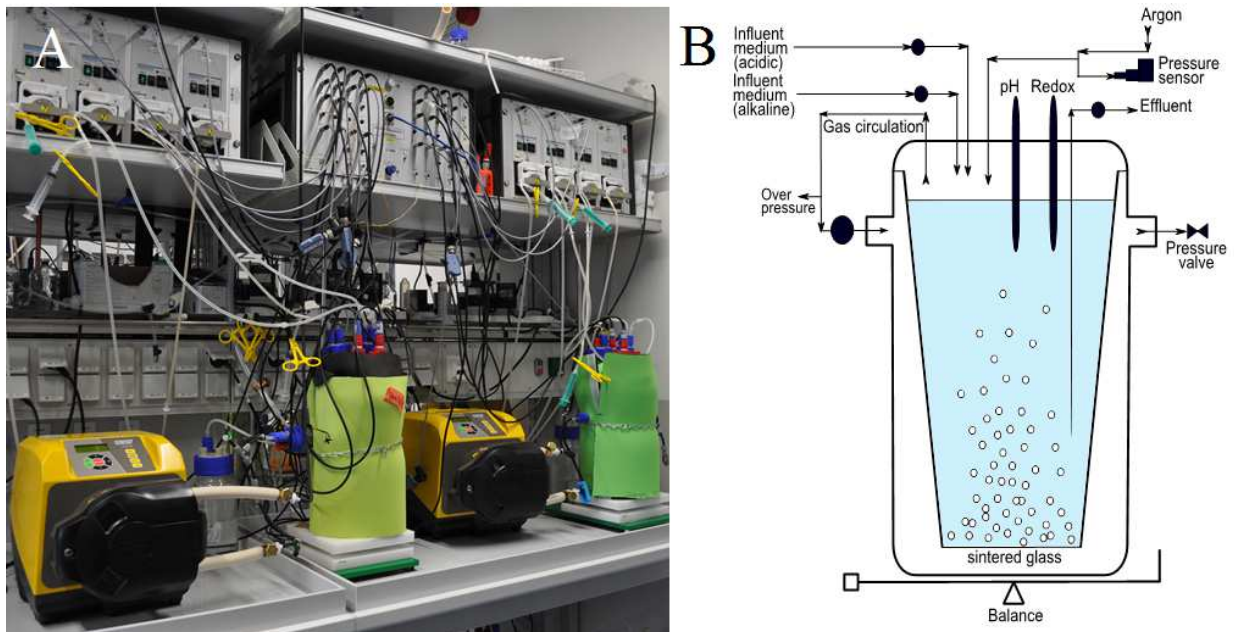


Figure 1.3: Experimental set-up. A) Implementation in the laboratory B) Schematic overview, adapted from Hanke *et al*., 2014.

Different microbial populations within the selected community members can be identified and information about their specific role within the community can be obtained by the application of microscopic methods such as CARD-FISH and metagenomic, transcriptomic and proteomic approaches.

In the continuous culture enrichments described in this thesis, the electron acceptor in the supplied medium was either nitrate or nitrite. The electron donors and carbon source of the supplied media consisted of a mixture of organic carbon compounds, except in some experiments that addressed the nature of electron donor itself. The supply of a mixture of organic carbon substrates was important to prevent selection based on the electron donor itself when other factors were addressed. The mixture was composed of glucose, acetate and different amino acids in order to mimic the monomeric composition of decaying biomass, the main carbon and electron source in marine environments (Oevelen *et al*., 2006, Novitsky, 1986) including the sampling site for the source communities used to inoculate the enrichment cultures (Seidel *et al*. 2014).

The inoculum for the chemostat enrichments originated from the upper sediment layer of the intertidal flat "Janssand" in the German Wadden Sea (figure 1.4). The sampling site has been shown to be active in nitrate reduction: The two nitrate reduction processes addressed here, denitrification as well as DNRA, occur *in situ* (Gao *et al*., 2010, Behrendt *et al*., 2013). Thus, the microbial guilds that are able to carry one or the other process were present in the inoculum, which is the prerequisite for the study of the competition between the two processes. Furthermore, as pointed out above, marine coastal sediments make an important contribution to global nitrogen cycling (Gruber *et al*., 2004, Codispoti, 2007).

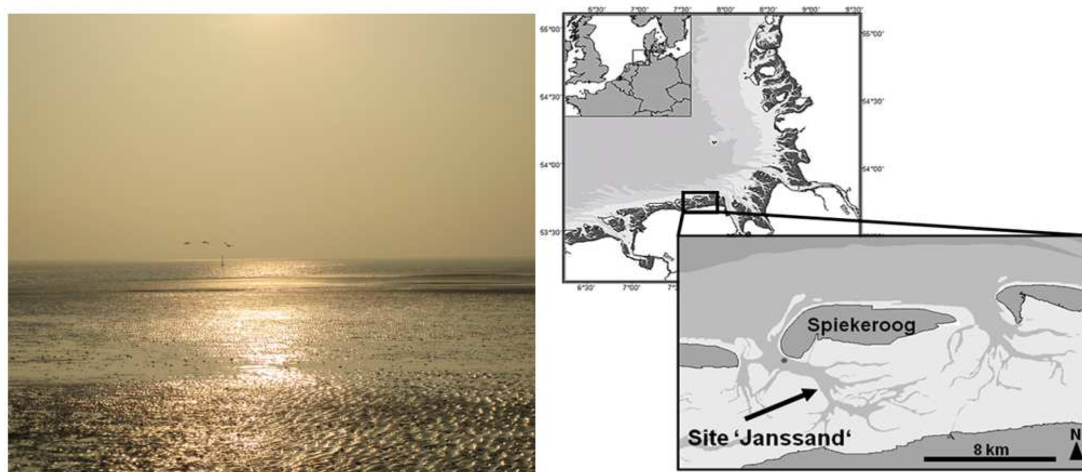


Figure 1.4: Sampling site "Janssand" in the German Wadden Sea.

1.3.2 Metagenomic sequencing

In this thesis, one mayor aim for the application of metagenomics, the sequencing of a whole community's DNA, was to obtain information on the identity and function of the members of the enriched microbial communities. To reach this aim the information contained in the sequencing data needs to be associated with the different populations present in the community. Ideally, complete genomes or nearly complete genomes can be reconstructed for the different population of a microbial community (Sharon and Banfield, 2013). To achieve this, sequencing reads need to be assembled into longer sequence contigs and subsequently binned (Kunin *et al*., 2008). During binning the contigs are sorted apart according to the different populations from which they derived (Thomas *et al*., 2012).

The additional application of metatranscriptomics and/or metaproteomics provides information about the activity of genes and their translation into proteins. By the combination of metagenomic, transcriptomic and proteomic approaches with the enrichment of well adapted microbial communities in chemostats influence of the applied selecting forces on the interaction of different populations, partitioning of metabolism, transfer of metabolites and cryptic cycling of elements within a community can be identified.

1.4 Objectives of the thesis

In view of the strong human influence on the nitrogen cycle the understanding of the fate of nitrate and its environmental control is of great importance.

Therefore, the first objective of this thesis was the evaluation of the relation between compositional and functional stability of a denitrifying community. I wanted to answer the question whether a stable performance is associated with a stable community composition or if the denitrifying populations change over time (chapter 4). A further aim of this study was the enrichment and identification of environmental relevant denitrifiers and their potential metabolic interaction with other microbial guilds, for example fermenting populations.

Several hypotheses exist on the factors that determine the controls that govern the competition between denitrification and DNRA. However, solid experimental support for these is lacking. Hence, the second mayor aim of this thesis was to systematically determine the influence of different environmental factors on the competition between denitrifiers and dissimilatory nitrate reducers. More specifically, the role of the organic carbon to nitrogen ratio, the kind of electron donor and acceptor supplied and the growth rate in controlling the nitrate respiration process are addressed (chapter 5).

Once the relevant driving forces were identified I wanted to determine how the enriched microbial communities changed along with a shift in the predominating nitrate respiration process. Furthermore, I wanted to answer the questions if the cross-feeding between nitrate-reducers and fermenting populations that was identified in chapter 4 was consistent between the two nitrate reduction processes and if the different nitrate reduction processes showed differences in the turnover of electron donors. The assessment of the electron donors that were utilized revealed an important role of sulfide as electron donor. Therefore, the contribution of sulfur cycling to the overall community metabolism was investigated (chapter 5).

Binning, the separation of sequence data according to the different populations present in a microbial community, is a bottle neck in the analysis of metagenomic sequence data. Therefore, a new binning approach that was able to also bin populations that were taxonomically only distantly related to available reference genomes, delivered reproducible results and did not require an estimate of the number of binnable populations present had to

be developed. A tool that has been developed to meet these requirements and was used for the binning of metagenomic datasets in chapter 4 and 5 is described in chapter 3.

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Chapter 2

2 Microbial nitrate respiration – Genes, enzymes and environmental distribution

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2.1 Abstract

Nitrate is a key node in the network of the assimilatory and respiratory nitrogen pathways. As one of the ‘fixed’ forms of nitrogen, nitrate plays an essential role in both nature and industry. For bacteria, it is both a nitrogen source and an electron acceptor. In agriculture and wastewater treatment, nitrate respiration by microorganisms is an important issue with respect to economics, greenhouse gas emission and public health. Several microbial processes compete for nitrate: denitrification, dissimilatory nitrate reduction to ammonium and anaerobic ammonium oxidation. In this review we provide an up to date overview of the organisms, genes and enzymes involved in nitrate respiration. We also address the molecular detection of these processes in nature. We show that despite rapid progress in the experimental and genomic analyses of pure cultures, knowledge on the mechanism of nitrate reduction in natural ecosystems is still largely lacking.

Keywords: Nitrogen cycle, Nitrate respiration, Denitrification, Anammox, DNRA

2.2 Introduction

Nitrate is one of the essential environmental components in the biosphere. It serves as nutrient for plants and microorganisms, and is used as an electron acceptor by many bacteria, archaea and also by several eukaryotes (Hayatsu *et al*., 2008; Zumft, 1997). Because of the wide distribution of nitrate respiration and the phylogenetic pattern of the involved enzymes, it has been argued that nitrate respiration was a common process in microorganisms already before the increasing concentration of oxygen in the atmosphere led to the development of oxygen respiration (Castresana and Saraste, 1995; Ducluzeau *et al*., 2009).

Several microbial processes compete for nitrate, such as denitrification, dissimilatory nitrate reduction to ammonium and anaerobic ammonium oxidation. As evidence for the widespread existence of these processes accumulated in the past two decades it became obvious that the so-called nitrogen cycle is in fact a network of pathways (figure 2.1). One of the key reactions of this network is the reduction of nitrate to nitrite, since this reaction is always the first step in the use of nitrate. Depending on the microbial community and environmental conditions nitrite is then either released or further reduced in different ways.

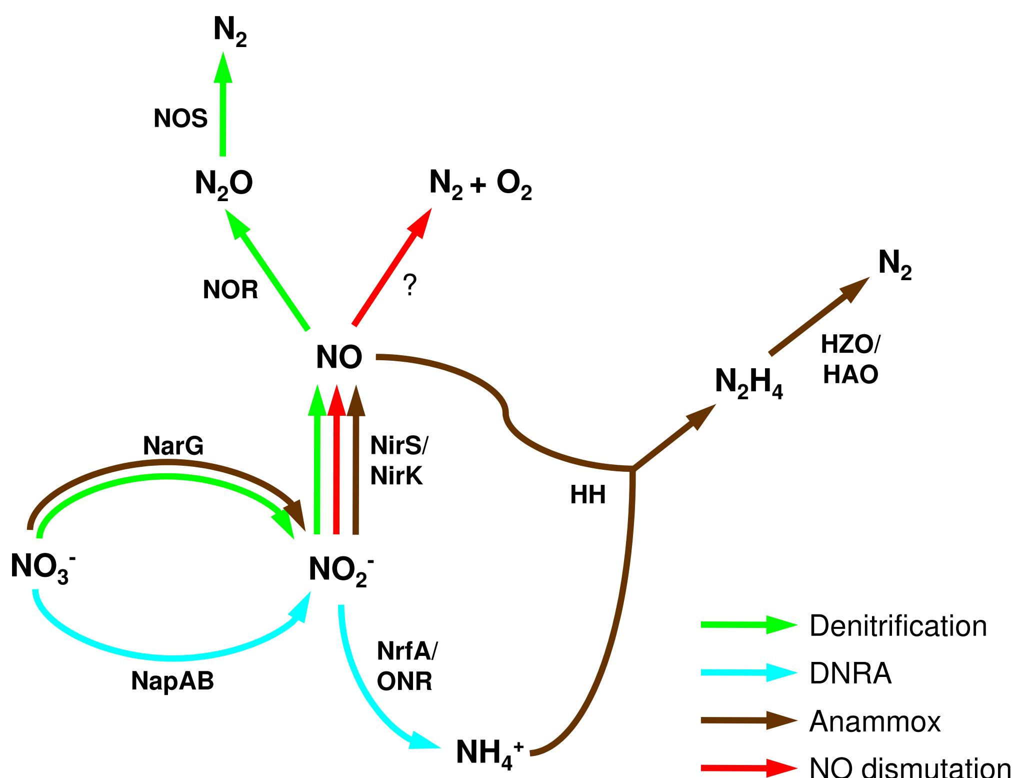

Figure 2.1: Current perspective on the network of nitrate reduction pathways: nitrogen compounds (between arrows), processes (arrows) and enzymes (next to arrows) are indicated. ? indicates not yet characterized enzyme(s). Nar: membrane-bound nitrate reductase, Nap: periplasmic nitrate reductase, HAO: hydrazine oxidoreductase, HH: hydrazine hydrolase, HZO: hydrazine-oxidizing enzyme, Nir: nitrite reductase; NOR: nitric oxide reductase, NOS: nitrous oxide reductase, ONR: octaheme cytochrome c reductase, NrfA: nitrite reductase, DNRA: dissimilatory nitrate reduction to ammonium.

Current human impact on the global nitrogen cycling is substantial (Galloway *et al*., 2008; Schlesinger, 2009). The use of nitrogen as fertilizer in agriculture often causes changes in the adjacent habitats, mostly due to nitrite pollution or rapid eutrophication (Vitousek *et al*., 1997). Intense agricultural fertilization may lead to increased concentrations of nitrate in the groundwater (Almasri and Kaluarachchi, 2004). This constitutes a risk for public health, given that groundwater is an important drinking-water supply (Ward *et al*., 2005).

Increased input of fixed nitrogen has also demonstrated impacts on more distant terrestrial (Brooks, 2003; Clark and Tilman, 2008) and marine ecosystems (Duce *et al*., 2008), where fixed inorganic nitrogen, one of the key nutrients, often is a limiting factor for primary productivity (Arrigo, 2005). Furthermore, fertilization increases the atmospheric

concentrations of methane and nitrous oxide and thus contributes to global warming (reviewed by Hanke and Strous, 2010). In waste water treatment plants the conversion of nitrate to gaseous nitrogen and thus the loss of fixed nitrogen from the water is the aim. Complete denitrification to N_2 and anammox are usually the desired processes (Kartal *et al*., 2010; Kumar and Lin, 2010; Strous *et al*., 1997).

Although a fair amount of studies on pure cultures have been performed, little is known about how the natural microbial communities of terrestrial and aqueous habitats react to changing nitrate concentrations and nitrogen speciation. Application of high throughput tools for DNA, RNA and protein analysis showed that only a small fraction of the entire natural microbial diversity has been discovered and described so far (Pace, 2009; Rappe and Giovannoni, 2003). All known processes of microbial nitrate and nitrite reduction appear to be globally widespread and it is likely that most microbial communities in nature are able to use different nitrogen compounds in different ways. This interaction of nitrogen reaction pathways will probably be affected as a whole as soon as the concentration or fluxes of one of the involved compounds changes. The same might hold true for the composition of the affected microbial community, more precisely, for the presence of specific genes and enzymes. This review summarizes the current view on the network of respiratory nitrate reduction pathways and the enzymes involved, as well as their environmental distribution and impact.

2.3 Denitrification

Among the different pathways of microbial nitrate reduction, bacterial denitrification is most extensively described and numerous studies have been undertaken to elucidate this pathway. A number of comprehensive reviews have been published during the last years discussing bacterial and archaeal denitrification and the influencing factors and enzymes involved (Berks *et al*., 1995; Cabello *et al*., 2004; Hermann *et al*., 2000; Moura and Moura, 2001; Philippot, 2002; Shapleigh, 2006; Wallenstein *et al*., 2006; Zumft, 1997).

The trait of denitrification is phylogenetically wide spread. Denitrifiers are found among bacteria, archaea and eukaryotes. However, so far most of the isolated and studied denitrifying organisms belong to the phylum Proteobacteria (Alpha, Beta, Gamma, and

Epsilon Divisions) (Cabello *et al*., 2004; Green *et al*., 2010; Hayatsu *et al*., 2008; Heylen *et al*., 2006; Jones *et al*., 2008; Kern and Simon, 2009; Risgaard-Petersen *et al*., 2006).

Briefly, denitrification is a respiratory process (Conrado and Stuart, 1998) in which nitrate is reduced stepwise to dinitrogen ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$). In bacteria, this process is used as an alternative to oxygen respiration under low oxygen or anoxic conditions. Like in aerobic respiration, the reaction chain is split over the periplasmic and cytoplasmic compartment, and it allows for the generation of a proton motive force across the bacterial membrane, which is exploited for ATP synthesis.

Low oxygen tension is detected by the global oxygen-sensing regulator FNR or its homologues. A nitrate sensor detects the presence of nitrate in the periplasm and, for nitrate reduction on the cytoplasmic side of the membrane, activates nitrate transport through the cytoplasmic membrane via transporters NarK1, and later in the process NarK2. NarK1 is a proton motive force driven nitrate importer (nitrate/proton symporter) and NarK2 is a nitrate/nitrite antiporter (Moir and Wood, 2001; Wood *et al*., 2002). The presence of nitrate initiates the expression of the denitrification machinery via transcriptional regulators, such as FNR and its homologues (Berks *et al*., 1995; Heinz *et al*., 2003) or NarR in two *Paracoccus* species (Wood *et al*., 2001). The four enzymes of the denitrification pathway are best studied in gram-negative bacteria: A membrane bound nitrate reductase catalyzes the reduction of nitrate to nitrite in the cytoplasm (figure 2.2a). Nitrite is then transported into the periplasm (via NarK2), and a periplasmic nitrite reductase catalyzes the reduction of nitrite to nitric oxide. Reduction of nitric oxide to nitrous oxide is catalyzed by nitric oxide reductase, an integral membrane protein with its active site in the periplasm. The final step, reduction of nitrous oxide to dinitrogen is catalyzed by the periplasmic nitrous oxide reductase (figure 2.3). In gram-positive bacteria and in archaea that lack a periplasmic space evidence suggests that all four enzymes are membrane bound (Cabello *et al*., 2004; Suharti and de Vries, 2005). Furthermore there is evidence that in archaea nitrate reduction takes place on the 'outer' side of the cytoplasmic membrane (Cabello *et al*., 2004; Martínez-Espinosa *et al*., 2007).

Enzymes in bacterial denitrification

Nitrate reductase

In denitrification the reduction of nitrate to nitrite is catalyzed by the membrane bound complex nitrate reductase NarGHI (González *et al*., 2006; Moreno-Vivian *et al*., 1999). In several non-denitrifying bacteria membrane bound Nar can also function as respiratory nitrate reductase (Richardson *et al*., 2001; Zumft, 1997). The trimeric enzyme complex belongs to the family of molybdopterine oxidoreductases.

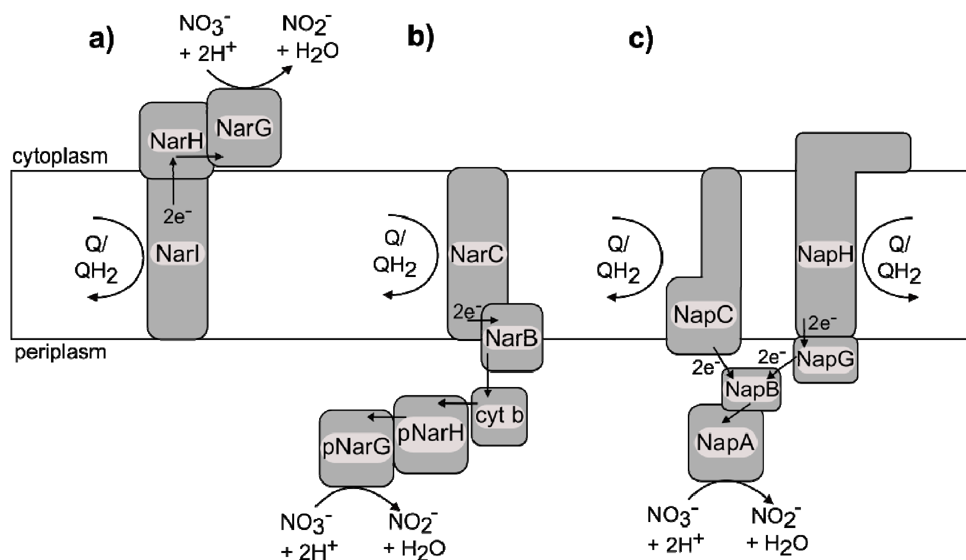

Figure 2.2: Comparison of the organization of nitrate reductases. (a) Bacterial membrane-bound nitrate reductase (Nar). (b) Model of archaeal form of membrane-bound nitrate reductase as proposed by Martínez-Espinosa *et al*. (2007) (Nar/pNar). (c) Periplasmic nitrate reductase (Nap) and membrane bound electron donors. For more detailed explanations see Sections 2.1, 2.2 and 3.1. cyt b: cytochrome b.

NarG, the subunit facing the cytoplasm, contains the active site: the molybdenum cofactor or Mo-bis-MGD factor, in which a Mo atom is bound to molybdopterin guanine dinucleotide (Hille, 1996; Jormakka *et al*., 2004; Lalucat *et al*., 2006; Richardson *et al*., 2001). NarI, the subunit that anchors the enzyme to the membrane, receives electrons from the quinone pool in the membrane. The electrons are passed via two hemes *b* to NarH, the subunit that links NarI to NarG. In NarH, electrons are further transported through the enzyme via four Fe–S clusters. NarG also contains an Fe–S cluster that receives electrons from NarH and passes them to the active site of the enzyme. At the same time as electrons flow to the cytoplasm, positive charges are translocated to the periplasm. Protons are consumed in the cytoplasm by

formation of H_2O in the course of nitrate reduction and protons are released into the periplasm from the quinone pool that passes electrons to NarI (Richardson and Sawers, 2002; Simon *et al*., 2008) (Figure 2.2a).

Periplasmic nitrate reductase

In several bacteria, the nitrate reduction step in the denitrification process is not dependent on the presence of NarGHI, but is performed by a periplasmic nitrate reductase. For example, *Bradyrhizobium japonicum* lacks a *nar* gene cluster, but is capable of denitrification, due to the presence of periplasmic nitrate reductase NapAB (*nap* gene cluster *napEDABC*) (Bedmar *et al*., 2005). NapA, the large subunit of NapAB, contains the molybdenum cofactor and a [4Fe–4S] center. The NapB subunit is a *c*-type cytochrome. Liu *et al*. (1999) showed the involvement of the nap gene *cluster (napKEFD-ABC)* in denitrification in *Rhodobacter sphaeroides* f. sp. *denitrificans*.

Another example is *Pseudomonas sp.* strain G-179, equipped with all functional genes for denitrification given that nitrate reduction is mediated by NapA, which has been shown by Bedzyk *et al*. (1999). Also in the genome of *Sulfurimonas denitrificans*, a *nap* gene cluster is present along with all other functional genes of the denitrification pathway, yet a *nar* gene cluster is absent (Sievert *et al*., 2008).

Nitrite reductase

Two isofunctional periplasmic enzymes, which are evolutionary unrelated, catalyze the reduction of nitrite to nitric oxide in gram-negative bacteria: a homotrimeric copper-containing enzyme, NirK (or CuNIR), and a homodimeric cytochrome cd_1 nitrite reductase, NirS (or cd_1 NIR) (Moura and Moura, 2001; Zumft, 1997). In the gram-positive denitrifying bacterium *Bacillus azotoformans* a copper-containing, membrane bound nitrite reductase was detected, and it is hypothesized that also in gram-positive bacteria respiratory nitrite reduction occurs on the outer face of the cytoplasmic membrane (Suharti and de Vries, 2005).

In NirK each monomer contains two copper centers, type 1 and type 2 (Nojiri *et al*., 2009). Nitrite binds to the copper ion in the type 2 center, replacing an exogenous ligand (water or chloride), and by electron transfer from the type 1 copper site, nitrite is reduced to

nitric oxide. Based on sequence similarity, NirK can be divided into at least two subfamilies. Compared to the well characterized group I NirK types, NirK types belonging to group II carry an additional domain at the N-terminus (Nojiri *et al*., 2007), and database searches revealed further NirK variants with a C-terminal *c*-type heme domain extension (Ellis *et al*., 2007). Although group I NirK are most frequently detected among denitrifying bacterial isolates, there is so far no evidence that this NirK group is most frequently found in denitrifiers in general (Jones *et al*., 2008), and the particular functions of the additional domains in other NirK groups have yet to be investigated.

In NirS each of the two cytochrome cd_1 monomers binds two prosthetic heme groups, heme *c* and heme d_1. Electrons are known to be transferred from the electron donor cytochrome c_{551} via the heme *c* of NirS further to heme d_1, where nitrite binds and is reduced to nitric oxide (Baker *et al*., 1997; Fülöp *et al*., 1995).

Typical electron donors to NirK type I site copper are azurins or pseudoazurins (Murphy *et al*., 2002), in some cases cytochrome c_{551} (Glockner *et al*., 1993; Nojiri *et al*., 2009; Zumft, 1997). In vitro studies show that in some species NirS also accepts electrons from different donors, e.g. cytochrome c_{550} or azurin (Zumft, 1997).

The electrons are delivered via ubiquinone and complex III (the cytochrome bc_1 complex). The oxidation of quinones by this complex allows proton translocation from the cytoplasm to the periplasm (figure 2.3). Nitrite reductase from *B. azotoformans* accepts electrons from menaquinol via a membrane bound cytochrome *c* (Suharti and de Vries, 2005).

In several species of *Neisseria*, AniA, a nitrite reductase located in the outer membrane was identified and the crystal structure revealed that it is a NirK homologue (Boulanger and Murphy, 2002). However, none of the so far examined gene sequences revealed a complete functional denitrification pathway in any *Neisseria* species and it is assumed that each species individually adapted to its niche in the host, making use of intermittent supply of nitrite as electron acceptor (Barth *et al*., 2009).

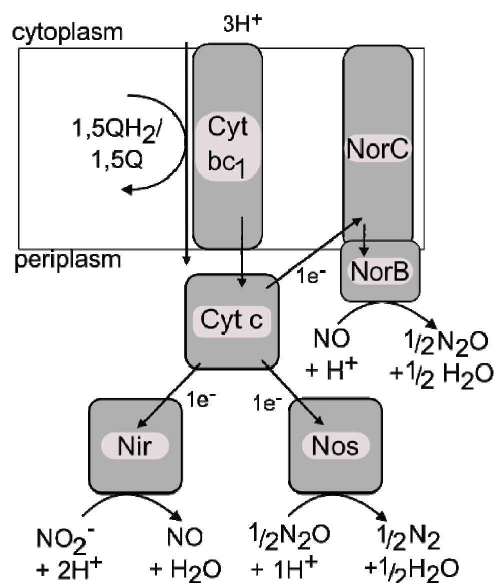

Figure 2.3: Respiratory chain in bacterial denitrification. Cyt bc1: cytochrome bc_1 complex, cyt c: cytochrome *c*, nir: nitrite reductase, Nor: nitric oxide reductase, Nos: nitrous oxide reductase, Q: co-enzyme Q.

Nitric oxide reductase

The product of NirS and NirK, nitric oxide (NO), is a radical which reacts unspecifically with many other molecules. Therefore, its accumulation has to be prevented. Several studies point to a co-regulation of respiratory nitrite reductases with the membrane bound nitric oxide reductase (NOR), via Crp-Fnr protein family regulators, responding to the presence of nitric oxide (Heinz *et al*., 2003; Spiro, 2007; Tosques *et al*., 1997; Zumft, 2005). The NOR enzyme active in denitrification is homologous to heme–copper oxidases (Garcia-Horsman *et al*., 1994), and shares the conserved structure of 12 antiparallel transmembrane domains (Zumft *et al*., 1994). However, different to other heme–copper oxidases NOR shows no proton translocation activity and the highly conserved bimetallic (binuclear) catalytic center contains a non-heme Fe instead of Cu (van der Oost *et al*., 1994; Wasser *et al*., 2002). Thus the binuclear center of NOR consists of a high spin heme *b* and Fe_B. A further (low spin) heme *b* functions in electron transfer to the binuclear catalytic center. In gram-negative bacteria two types of NOR are known, a long chain (lc)NOR (also called qNOR) and a short chain (sc)NOR. The latter functions in a complex with NorC that transfers electrons to the catalytic NOR unit, NorB. NorC is a membrane bound cytochrome *c*, to which electrons are transported from the quinone pool via cytochrome *bc*1 and soluble cytochrome *c* or cupredoxins. From the observations made so far, scNORs seem to be present almost

exclusively in denitrifying organisms, whereas lcNORs might play a role in other, non-respiratory processes, such as scavenging of nitric oxide, since this NOR type is mainly present in bacteria that do not possess a complete denitrification pathway (Hendriks *et al*., 2000).

Although it was shown earlier that nitric oxide respiration is connected with transient proton translocation (Shapleigh and Payne, 1985) the exact mechanism is not known and later studies showed that nitric oxide reductase is not a proton pump and evidence accumulated that the enzyme derives protons from the periplasm (Huang *et al*., 2008; Reimann *et al*., 2007). The structural gene of lcNOR was named *norZ* but is also sometimes referred to as *norB*. In NorZ, an N-terminal domain that does not exist in (sc)NorB takes up electrons from quinol and transfers them, via the low spin heme *b*, towards the reactive binuclear site (Tavares *et al*., 2006; Zumft, 2005). This N-terminal domain is structurally similar to NorC, but lacks a heme *c* binding site (Hendriks *et al*., 2000).

In gram-positive B. *azotoformans* a third type of membrane bound NOR, qCuA NOR, was detected. It consists of two subunits. Next to two heme *b* qCu_A NOR contains copper (Cu_A) and uses menaquinol as electron donor, but not cytochrome *c* (Suharti *et al*., 2001).

Nitrous oxide reductase

The multicopper enzyme nitrous oxide reductase, NOS, is a periplasmic enzyme in gram-negative bacteria, and membrane bound in gram-positive *B*. *azotoformans*. NOS is the last of the four enzymes required for complete denitrification. However, apart from denitrifiers also other microorganisms use nitrous oxide as electron acceptor and reduce it to dinitrogen via NOS (Zumft, 1997). The functional enzyme NosZ is a homodimer with two copper centers per monomer (Moura and Moura, 2001). Each of the catalytic Cu_Z sites contains four copper atoms, ligated by seven histidine residues, and a bridging sulfur atom (Brown *et al*., 2000a). Cu_A, the electron entry site in each monomer, is a binuclear copper center. The localization of the copper centers in the NosZ dimer suggests that electron transfer proceeds from Cu_A of one subunit to Cu_Z of the other subunit, where nitrous oxide is reduced to dinitrogen (Brown *et al*., 2000b; Tavares *et al*., 2006). For several species, such as *Paracoccus denitrificans* and R. *sphaeroides*, it was shown that electron transfer to NOS occurs via cytochrome *c*, and that the cytochrome bc_1 complex is also involved in the reduction process of nitrous oxide, which implies coupling of nitrous oxide reduction to proton transfer across the cytoplasmic

membrane (Itoh *et al*., 1989; Tavares *et al*., 2006; Zumft, 1997). NOS in *B. azotoformans* accepts electrons from menaquinol (Suharti and de Vries, 2005).

Archaeal denitrifying enzymes

Compared to bacterial denitrification, little is known about this process in archaea, and only a few archaeal denitrification genes and enzymes have been investigated so far. From the results obtained to date, it appears that all enzymes of the reaction chain are membrane bound (Cabello *et al*., 2004). The most striking difference between bacterial and archaeal denitrification appears to be that in archaea the reactive center of the nitrate reductase Nar is not located in the cytoplasm and thus the whole process takes place at the outer (potential-positive) face of the membrane (Cabello *et al*., 2004; Martínez-Espinosa *et al*., 2007; Yoshimatsu *et al*., 2002). Nevertheless, both bacterial and archaeal Nar are membrane associated protein complexes and show a high percentage of sequence similarity (Cabello *et al*., 2004; Martínez-Espinosa *et al*., 2007). Furthermore, although archaeal homologues to bacterial NarG and NarH could be identified, and some of them were characterized (Afshar *et al*., 2001; Lledó *et al*., 2004; Martínez-Espinosa *et al*., 2006 and references therein), no NarI homologue has been found in any of the so far investigated archaeal genomes. It is assumed that the function of NarI in the bacterial enzyme complex is not required in the archaeal system. In bacteria, quinones are oxidized at the periplasmic side of the membrane, and NarI takes up the electrons and transports them through the membrane to the NarH subunit. In the archaeal system NarGH is located at the outer side of the membrane, and the electrons have to be translocated to this 'periplasmic' Nar (pNar) in a different way. One proposed possibility is the existence of a quinone-cycle coupled system at least in some archaea (figure 2.2b), in which a protein complex, including a cytochrome *b*-like protein (NarC) and a Rieske iron–sulfur protein (NarB), takes up electrons from the quinone pool and transfers them to pNar (Martínez-Espinosa *et al*., 2007). If this is the case, pNar would be coupled to protonmotive activity, as is bacterial NarGHI, since the proposed quinone-cycle allows proton translocation through the membrane. This would maintain the same bioenergetic effectivity as observed for bacterial NarGHI, and differentiate the role of pNar from that of the periplasmic dissimilatory nitrate reductase Nap.

Also in some bacterial genomes, based on sequence similarity, pNar-type genes were proposed (Martínez-Espinosa *et al*., 2007), for example in the two gram-positive firmicutes *Carboxythermus hydrogenoformans* (Wu *et al*., 2005) and *Moorella thermoacetica* (Pierce *et*

al., 2008), as well as in the anammox bacterium *Candidatus* Kuenenia stuttgartiensis (Strous *et al*., 2006). For the two firmicutes denitrification is not known to be a major energy pathway, and also in *Candidatus* Kuenenia stuttgartiensis, rather than being involved in denitrification, the membrane bound nitrate reductase appears to have a role specific to anammox (Strous *et al*., 2006) (see Section 4).

These findings indicate a considerable diversity of particular enzymes active in the N-network, regarding their localization within the cell and the pathways in which they are involved. They point to a flexible evolutionary adaptation of microbial enzymes supporting the process of the adaptation of microorganisms to their respective environments. This provides chances y*et al*so poses challenges on the determination of marker genes for particular environmental microbial processes.

Both types of respiratory nitrite reductase genes, *nirK* and *nirS*, have been found in archaea (Jones *et al*., 2008). Different to their bacterial homologues, at least some archaeal respiratory nitrite reductases are membrane bound enzymes (for example in *Pyrobaculum aerophilum*). One possible explanation is the relatively small volume between the cytoplasmic membrane and the surface-layer of archaea compared to the larger periplasmic space of gram-negative bacteria (Cabello *et al*., 2004).

The characterization of archaeal nitrite reductase proteins is still at its beginnings, only two archaeal nitrite reductases have been characterized so far and both are encoded by *nirK* like genes (Bonete *et al*., 2008; Ichiki *et al*., 2001). Interestingly, their sequences are most similar to outer membrane CuNIR of *Neisseria gonorrhoeae* (*aniA*) (Hoehn and Clark, 1992), which could be an indication for lateral gene transfer (Cabello *et al*., 2004; Ichiki *et al*., 2001).

The nitric oxide reductase of the archaeon P. *aerophilum* is a qNor-type protein with menaquinol as electron donor (Cabello *et al*., 2004; de Vries and Schröder, 2002) and also other archaeal *nor* genes appear to encode qNor. By far not all investigated archaeal genomes contain a *nor* gene, raising the question whether alternative enzymes for nitric oxide reduction exist in archaea, and whether these nitric oxide reductases also participate in denitrification (Bonete *et al*., 2008; Cabello *et al*., 2004).

Unlike the bacterial nitrous oxide reductase, archaeal NOS is membrane bound and (in *P. aerophilum*) receives electrons from menaquinol. Although *nosZ* has been detected in other archaeal genomes, no other archaeal NOS has been characterized yet (Cabello *et al*., 2004; de Vries and Schröder, 2002). Within the genera Haloarcula and Haloferax, several

denitrifiers have been found. Some of these species produce mainly nitrous oxide from nitrate, however, at least in some species, nitrous oxide and dinitrogen production seems to be dependent on the growth phase (*Har marismortui, Hfx mediterranei*) or on nitrate concentration (*Hfx denitrificans*) (Bonete *et al*., 2008; Tindall *et al*., 1989).

Eukaryotic denitrification

Denitrification has been observed in fungi and in a benthic foraminifer (Hayatsu *et al*., 2008; Kobayashi *et al*., 1996; Risgaard-Petersen *et al*., 2006). In fungi, the process is located in the mitochondria and is coupled to the synthesis of ATP (Kobayashi *et al*., 1996). The foraminifer *Globobulimina pseudospinescens* accumulates nitrate in intracellular stores and is able to respire this nitrate intracellularly to dinitrogen gas (Risgaard-Petersen *et al*., 2006). Recently it has been discovered that next to several different and diverse groups of Foraminifera an additional taxon of the Rhizaria, the Gromiida, is capable of denitrification indicating that eukaryotic denitrification may also significantly contribute to the world's oceans fixed nitrogen loss (Pina-Ochoa *et al*., 2010).

Aerobic denitrification

Denitrification also occurs when oxygen is present, as has been shown for several isolated bacterial species (Bell *et al*., 1990; Robertson *et al*., 1989, 1995; Robertson and Kuenen, 1984) and also in natural aqueous habitats (Gao *et al*., 2009; Trevors and Starodub, 1987) and in soil (Trevors, 1985). Normally, under oxic conditions denitrification is repressed by inhibition of nitrate transport through the cytoplasmic membrane (Alefounder and Ferguson, 1980; Moir and Wood, 2001). Bell *et al*. (1990) could show that the sulfur bacterium *Thiosphaera pantotropha* (now *Paracoccus pantotrophus*), in the presence of oxygen, expresses a periplasmic nitrate reductase that is not dependent on nitrate transport through the cytoplasmic membrane and can thus take over the first step in denitrification. The last enzyme in the denitrification reaction chain, NOS, is oxygen sensitive in many species. Therefore, aerobic denitrification is often incomplete and an increase in N_2O formation has been observed when conditions switch from anaerobic to aerobic (Frette *et al*., 1997; Patureau *et al*., 1994).

It has been suggested that aerobic denitrification mainly occurs in environments of alternating oxic/anoxic conditions (Frette *et al*., 1997; Patureau *et al*., 2000). Microorganisms

capable of both aerobic and anaerobic denitrification would have the best chances of survival in these habitats (Gao *et al*., 2009; Lloyd *et al*., 1987).

Denitrification in the environment

Elucidating the process of denitrification in the environment was the subject of numerous studies employing a variety of approaches in many different habitats. Since denitrification is not a trait that can be tied to particular taxonomic clades (e.g. Jones *et al*., 2008), detection of denitrifiers on the genome level requires the detection of functional rather than 16S-rRNA genes. The nitrite reductase genes *nirS* and *nirK* have frequently been used as marker genes for denitrifying bacteria. For example, *nirS* was used to study the denitrifier community composition and abundance along an estuarine gradient with a microarray approach (Bulow *et al*., 2008). Ward *et al*. (2009) studied denitrifier communities in the Arabian Sea via *nirS* amplification by quantitative PCR combined with measuring denitrification rates using 15N tracers. Pratscher *et al*. (2009) used RING-FISH to detect microorganisms carrying *nirK* in activated sludge. Both genes *nirS* and *nirK* served as signature genes for analyzing the denitrifying community composition for example in the Black Sea by Oakley *et al*. (2007) who measured *in situ* nitrogen compounds, and performed sequence analysis of PCR products obtained from in situ and culture samples, and in the Arabian Sea by Jayakumar *et al*. (2009) who characterized the progression of denitrification by the presence and proportion of nitrogen compounds, and by abundance, sequence composition and diversity of the two nitrite reductase genes. Due to the apparent exclusivity of Nir-types in denitrifiers it has been suggested that despite the functional equivalence of NirK and NirS, a niche differentiation between bacteria carrying one or the other reductase exists (Jones *et al*., 2008; Smith and Ogram, 2008). Abell *et al*. (2010) combined nutrient and N flux measurements with determination of abundance and diversity of *nirS*, *nirK* and the nitrification marker gene *amoA* in a subtropical estuary. The authors emphasize the interconnection between the environmental factors influencing denitrification and point out that the verification of links between dominance and diversity of particular genes and physical and chemical variables requires further efforts in both field and laboratory work.

Several studies include further functional genes of the denitrification pathway as marker genes (Cuhel *et al*., 2010; Demaneche *et al*., 2009; Dong *et al*., 2009; Nogales *et al*., 2002; Scala and Kerkhof, 1999; Smith *et al*., 2007). Nogales *et al*. (2002) studied the expression of *narG*, *napA*, *nirS*, *nirK* and *nosZ* in estuarine sediments. Of these five genes, only *nirS* and

nosZ could be shown on transcript level via RT-PCR. Furthermore, almost all of the obtained *nirS* sequences were novel sequences, showing a high diversity and site specificity. The failure to detect transcripts of the remaining three genes could be an indication that not all of the genes were expressed in sufficient amounts, but could also have been at least in part due to the fact that environmental sequences are still largely unknown and the generation of suitable primers for (RT-)PCR is thus difficult if possible at all. High diversity and divergence from database sequences was also shown for *narG* and *napA* sequences obtained from the same sampling site by Smith *et al*. (2007). The authors indicate that even for this restricted habitat, based on so far obtained DNA sequences, the design of "universal" primers and probes for *narG*, *napA* and *nirS* was not possible. Given the ever increasing genetic diversity that emerges with ongoing sequencing effort in the field of environmental microbiology, this might be the case for almost any of the environmental marker genes. Employing alternative methods for the detection of functional genes, such as metagenomic and genome sequencing is thus necessary, as has been pointed out for example by Wallenstein *et al*. (2006). In their review Wallenstein *et al*. discuss the difficulties of pinpointing the processes of denitrification in the environment since analysis of environmental data regularly shows that changes in denitrifier activity, abundance and community composition are generally caused by a multitude of interconnected factors. Dong *et al*. (2009) combined studying the presence of marker genes (*narG*, *napA*, *nirS* and *nrfA*) and transcripts with measuring nitrate reduction rates in sediments along an estuary. The processes of denitrification, dissimilatory nitrate reduction to ammonium and anaerobic ammonium oxidation were investigated. It was pointed out that relationships between the presence of nitrate and electron donors, nitrate reduction rates, and the corresponding reductase genes occur only on a broad scale, and that the multifactorial influence on gene expression in the environment makes a small scale relationship between nitrate reduction rates and abundance of marker genes or transcripts less likely. In their study on soil denitrifier communities, Cuhel *et al*. (2010) combined the investigation of denitrification gene abundance (*narG*, *napA*, *nirS*, *nirK*, *nosZ*) with measurements of denitrification activity. They found that abundance of *narG*, *napA* and *nirS* but not of *nirK* and *nosZ* was negatively correlated with the $N_2O/(N_2O+N_2)$ production ratio, whereas total N fluxes were not correlated with the abundance of any of the tested genes. They discuss that these results were, after all, not regularly obtained in other studies, and argue that counting functional genes is not sufficient for a comprehensive understanding of how denitrification is affected *in*

situ. The enormous diversity, abundance and widespread distribution of denitrifying microorganisms, the possibility of denitrification occurring as a community process (Wallenstein *et al*., 2006; Zumft, 1997), as well as the great variety of their habitats may explain why, despite continuing efforts, the identification of relevant environmental denitrifiers *in situ* remains a challenge.

2.4 Dissimilatory nitrate reduction to ammonium (DNRA)

In contrast to denitrification, dissimilatory nitrate reduction to ammonium (DNRA) is assumed to occur when nitrate in comparison to organic carbon is limiting (Cole and Brown, 1980). In DNRA nitrite is reduced to ammonium and eight electrons are transferred. The reduction of nitrate to nitrite is assumed to mostly being catalyzed by the periplasmic nitrate reductase complex NapAB (figure 2.2c). However, a membrane-bound nitrate reductase, NarGHI (figure 2.2a), may also be present in the same organism (Richardson *et al*., 2001; Simon, 2002). The entire reduction of nitrite to ammonium is catalyzed by NrfA, a pentaheme cytochrome c nitrite reductase without the release of any intermediate (Einsle *et al*., 1999, 2002).

Nevertheless, there are some indications that N_2O is also released as a byproduct of DNRA and not all N_2O originates from denitrification (and detoxification) as generally assumed. Enzyme bound NO and NH_2OH are probable intermediates (Cruz-Garcia *et al*., 2007; Vermeiren *et al*., 2009) of DNRA. The ability to carry out DNRA is phylogenetically widespread. The functional gene *nrfA* occurs in diverse groups of bacteria. It has been found in Gamma-, Delta- and Epsilonproteobacteria (Smith *et al*., 2007) and in members of the Bacteroides (Mohan *et al*., 2004). Many sulfate reducing Deltaproteobacteria are able to perform DNRA in the presence of nitrate (Dannenberg *et al*., 1992; Mitchell *et al*., 1986; Pereira *et al*., 1996; Seitz and Cypionka, 1986) although there has been recent evidence that sulfate reduction is preferred over DNRA if both electron acceptors are present (Marietou *et al*., 2009). NrfA has been purified and characterized from many different organisms as for example *Escherichia coli* (Kajie and Anraku, 1986; Liu *et al*., 1981), *Desulfovibrio desulfuricans* (Liu and Peck, 1981), *Wolinella succinogenes* (Blackmore *et al*., 1986; Liu *et al*., 1983) and *Vibrio fischeri* (Liu *et al*., 1988). In other cases, the corresponding enzymes

have only been predicted from the nrf genes detected in genome sequences and their structural and functional characterization remains the task of further research.

Enzymes in DNRA

Nitrate reductase

The two subunit complex NapAB binds a bis-molybdopterin guanine dinucleotide (bis-MGD) cofactor at the active site like the membrane bound NarGHI, but differing in the number and nature of cofactors. The catalytic subunit NapA receives electrons from NapB, a diheme cytochrome *c*. High resolution structures of a NapA and a NapB fragment have been determined (Dias *et al*., 1999; Brigé *et al*., 2001). Electrons are mostly transferred from the quinol pool to the membrane-bound tetraheme cytochrome *c* NapC and then to NapB (Roldan *et al*., 1998; Richardson *et al*., 2001). Next to NapC an alternative electron transport system to NapAB exists. W. succinogenes, for example, has a *napAGHBFLD* gene cluster. No NapC orthologue is encoded in the nap cluster and electrons are hypothesized to flow from the quinol pool to NapAB via NapH and NapG. NapH is proposed to be anchored in the membrane and NapG to form a complex with NapH (Kern and Simon, 2008; Simon *et al*., 2003) (figure 2.2c). In contrast to the *nar* operon, the nap operons differ in gene composition and ordering. Almost all gene clusters have the genes *napDABC* in common while the genes *napEKFGH* are found in different combinations (González *et al*., 2006; Richardson *et al*., 2001).

Nitrite reductase

In respiratory DNRA energy is obtained by generating an electrochemical proton potential across the membrane. Electrons are transported from the substrate (e.g. hydrogen, sulfide, formate) to nitrite. The electron transport chain consists of an enzyme that oxidizes the electron donor, e.g. a NADH dehydrogenase, formate dehydrogenase or a hydrogenase. Next, electrons are transferred to a respiratory menaquinone and then passed to the cytochrome *c* nitrite reductase (NrfA) (Einsle *et al*., 2002). The catalytic side of the enzyme is orientated towards the periplasm (Simon, 2002). In W. succinogenes and other Proteobacteria the membrane bound tetraheme cytochrome *c* subunit NrfH mediates the electron transport from menaquinones to NrfA and anchors the NrfHA complex to the membrane (Kern *et al*., 2008;

Rodrigues *et al*., 2006; Simon *et al*., 2001) (Figure 2.4a). NrfA and NrfH are encoded by the *nrfHAIJ* operon together with the gene *nrfI* that is involved in cytochrome *c* biogenesis (Kern *et al*., 2010; Simon *et al*., 2000). In enteric Gammaproteobacteria NrfB, a periplasmic pentaheme, is the direct electron donor to NrfA. It has been proposed that NrfB transfers electrons from the menaquinol pool to NrfA by oxidizing the membrane integrated protein complex NrfC-NrfD (Clarke *et al*., 2007; Einsle *et al*., 2000) (Figure 2.4b).

The corresponding gene cluster is the *nrfABCDEFG* operon (Hussain *et al*., 1994). The detailed function and structure of the transport chain and especially of NrfA has been reviewed by Simon (2002). The reaction mechanism has been further described by Einsle *et al*. (2002).

Most organisms that carry out DNRA also can reduce nitrate to nitrite except some sulfate reducing bacteria which are only able to use nitrite (Dannenberg *et al*., 1992; Mitchell *et al*., 1986). Recently, octaheme enzymes that can reduce nitrite to ammonia were found (Atkinson *et al*., 2007). Octaheme cytochrome *c* nitrite reductases (ONR) may represent a phylogenetic link between pentaheme cytochrome *c* NrfA and octaheme cytochrome *c* hydroxylamine oxidoreductase involved in the aerobic oxidation of ammonium and hydrazine oxidoreductase involved in anammox (Bergmann *et al*., 2005; Klotz *et al*., 2008). One ONR has been isolated from a sulfuroxidizing bacterium and characterized by Tikhonova *et al*. (2006).

Fermentative DNRA

For some fermenting bacteria nitrate reduction to ammonium allows NAD regeneration and ATP synthesis through acetate formation in contrast to ethanol production during fermentation (Cole and Brown, 1980; Polcyn and Podeszwa, 2009). In this case, the enzyme NrfA is not coupled to an energy conserving respiratory chain but the bioenergetic advantage compared to fermentation is additional ATP generation by substrate-level phosphorylation (Bonin, 1996). Fermentative DNRA has been well studied in *E. coli* (Stewart, 1988). Bacteria known to carry out this process mainly originate from gastrointestinal tracts of mammals and from wastewater treatment plants (Cole, 1996).

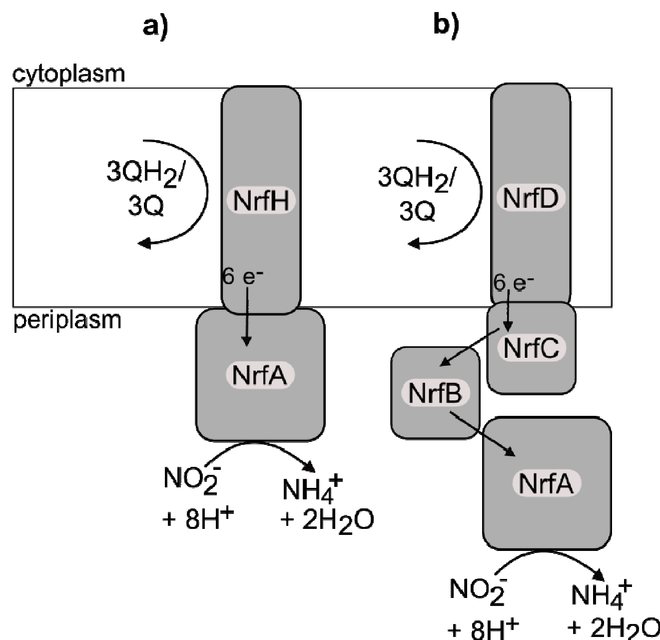

Figure 2.4: Respiratory chain in DNRA. (a) NrfHA complex. (b) NrfABCD complex. DNRA: dissimilatory nitrate reduction to ammonium.

DNRA in the environment

Until now few studies have addressed the distribution and diversity of functional genes for DNRA compared to other nitrate reducing pathways, such as denitrification and anammox. For a long time it was generally assumed that most of the nitrate in nature is mainly respired via denitrification and no greater relevance was assigned to DNRA. Recent 15N-labelling experiments indicated that DNRA has an important impact on N-cycling. For example, Silver *et al.* (2001) accounted up to 75% of the nitrate turnover in humid tropical forest soils to DNRA. In salt marshes, DNRA also contributed significantly to the total nitrate consumption (Koop-Jakobsen and Giblin, 2010; Tobias *et al.*, 2001). In the Benguela upwelling system anammox bacteria were shown to produce ammonium by carrying out DNRA (Kartal *et al.,* 2007a). A new method enabling to measure DNRA in sediments at millimeter-level resolution has recently been developed (Stief *et al.,* 2010).

Information on the physiology, phylogeny and on enzymes involved in DNRA has been obtained by characterizing isolated DNRA-performing strains (Bonin, 1996; Strohm *et al.*, 2007). Nevertheless, the key organisms carrying out DNRA in aquatic and terrestrial environments remain to be identified. Furthermore the ecological niche of DNRA in comparison to denitrification and the conditions that favor the one or the other nitrate

respiration pathway have to be determined. The ratio between the available electron donor compared to nitrate, the kind of electron donor and the prevailing redox potential in the environment are hypothesized factors for the selection of the predominating nitrate reduction process (Akunna *et al*., 1993; Kaspar, 1983; Tiedje *et al*., 1982). Nevertheless slurry incubations with 15N-nitrate showed that DNRA occurs under oxic conditions and is not inhibited by oxygen (Morley and Baggs, 2010).

The application of *nrfA* as a marker gene is difficult as currently there are only few sequences available and they come from culture collections with the majority being pathogens and probably not relevant in nature. Furthermore the microorganisms capable of DNRA are phylogenetically wide spread. Therefore the isolation of environmentally important DNRA performing species is an important step in order to obtain more sequences of the *nrfA* gene.

Mohan *et al*. (2004) designed primers for the detection of nrfA based on the alignment of six *nrfA* sequences similar to the *E. coli nrfA* sequence including the *nrfA* from *Sulfurospirillum deleyianum* and *W. succinogenes*. These primers were shown to detect *nrfA* sequences from a wide range of bacteria (Mohan *et al*., 2004). One of the rare studies that use *nrfA* as a marker gene for DNRA in the environment showed that *nrfA* is strongly expressed in the Peruvian oxygen minimum zone. Based on these results it was assumed that DNRA supplies most of the ammonium needed for anammox (Lam *et al*., 2009). Functional genes including *nrfA* and their mRNA transcripts were used as markers for nitrate respiration processes in an estuary (Dong *et al*., 2009; Smith *et al*., 2007).

Recently, the *nrfA* marker gene was used within a multidisciplinary study (Yagi *et al*., 2010) in order to characterize a contaminated terrestrial subsurface site and an apparent nitrogen redox cycling linking DNRA and ammonium oxidation was discovered.

For the investigation of denitrification and anammox in the environment the analysis of functional genes in combination with activity measurements has already been effectively applied. In future studies on nitrate reduction nrfA should also be addressed more commonly as the significance of DNRA in different environments may be higher than previously assumed. The diversity and distribution of the responsible microorganisms remain to be investigated.

2.5 Anaerobic ammonium oxidation (anammox)

Under anoxic conditions, anammox bacteria are able to gain energy by the formation of nitrogen gas from nitrite and ammonium (Jetten *et al*., 2005; Mulder *et al*., 1995; Richards, 1965; Strous *et al*., 1999; van de Graaf *et al*., 1995, 1997).

Since their identification by Strous *et al*. (1999), several bacteria that were found to be able to perform the anammox pathway have been characterized. They all belong to the order of *Planctomycetales*. They form a monophyletic order branching off deep inside the planctomycete lineage and have a large evolutionary distance among the genera described so far: *Candidatus* "Brocadia", "Kuenenia", "Scalindua", "Anammoxoglobus" and "Jettenia" (Jetten *et al*., 2009; Kuenen, 2008; Quan *et al*., 2008; Strous and Jetten, 2004). They are slow-growing organisms with a doubling time of eleven days up to three weeks (Jetten *et al*., 2005; Strous *et al*., 1999). Anammox bacteria have been detected in almost every type of aquatic habitat that contains oxygen-depleted zones, including marine and freshwater sediments, sea ice, and wastewater plants (Dong *et al*., 2009; Jetten *et al*., 2005, 2009; Kuenen, 2008; Kuypers *et al*., 2005; Lam *et al*., 2009; Schubert *et al*., 2006; Strous and Jetten, 2004; Strous *et al*., 2006).

Based on the first experimental results from studies with 15N-labeled substrates (van de Graaf *et al*., 1997), hydroxylamine was thought to be used to activate ammonium. At the same time, formation of hydrazine as an intermediate in the anammox reaction was discovered as a so far unique feature of anammox bacteria (van de Graaf *et al*., 1997). Later, the completion of the first genome sequence of an anammox organism (Strous *et al*., 2006) resulted in the revision of the previously postulated reaction pathway. The presence of the nirS gene in the genome of *Candidatus* Kuenenia stuttgartiensis suggested nitrite reduction to nitric oxide followed by hydrazine formation with nitric oxide and ammonium as the two substrates of the reaction (Strous *et al*., 2006). This 'new' pathway was consistent with the experimental data acquired until then (Schalk *et al*., 2000; Schmidt *et al*., 2002; Strous and Jetten, 2004; van der Star *et al*., 2008; van de Graaf *et al*., 1997) (Figure 2.5).

Enzymes in anammox

Next to known enzymes for nitrate reduction (NarGH), nitrite reduction (NirS), and the oxidation of hydrazine to dinitrogen by an anammox-specific hydroxylamine oxidoreductase (HAO) (Schalk *et al.*, 2000), novel enzymes were suggested by the genomic data that may be

involved in the anammox process. Hydrazine hydrolase (HH) produces hydrazine from ammonium and nitric oxide (Strous *et al.,* 2006). Shimamura *et al.* (2007) showed that next to expressing high amounts of the HAO enzyme that was characterized by Schalk *et al.* (2000), anammox bacteria also express an enzyme that oxidizes hydrazine but not hydroxylamine. It was named hydrazine-oxidizing enzyme (HZO). Since both anammox HAO and HZO oxidize hydrazine, are similar in their gene sequences, and can be differentiated from hao genes of aerobic ammoniumoxidizing bacteria, they are mostly referred to as HZO or HAO/HZO.

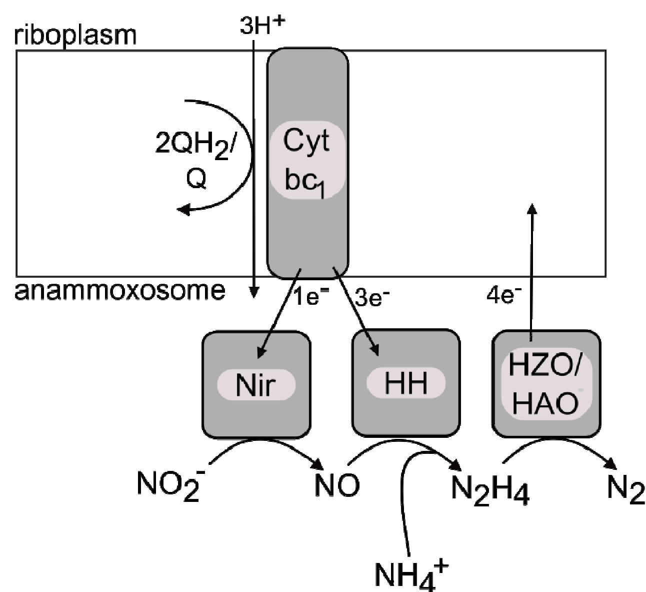

Figure 2.5: Respiratory chain in anammox. Cyt bc_1: cytochrome bc_1 complex, Nir: nitrite reductase, HH: hydrazine hydrolase, HZO/HAO: hydrazine/hydroxylamine oxidoreductase, Q: co-enzyme Q.

During hydrazine oxidation to dinitrogen by HZO electrons are proposed to be transferred to the quinone pool and further to cytochrome bc_1, generating a proton motive force. In parallel hydrazine dehydrogenase (HD) transfers high energy electrons from hydrazine to ferredoxin. This process is not connected with proton motive force generation. Instead, from ferredoxin the electrons can be fed into the acetyl-CoA pathway to enable carbon fixation (Strous *et al*., 2006). Interestingly, anaerobic nitrite oxidation to nitrate by NarGH might be a way to then replenish the quinone (and hydrazine) pool by reverse electron transport (Strous *et al*., 2006; Kuenen, 2008, figure 2.3 therein). The fact that the

anammox process takes place at the membrane of the innermost compartment of anammox bacteria, the anammoxosome, and most likely builds up a proton gradient between anammoxosome and riboplasm that is used for the generation of ATP, makes it analogous to the energy generation process in mitochondria (van der Star *et al*., 2010; van Niftrik *et al*., 2010).

Anammox in the environment

The chemolithoautotrophic anammox pathway is advantageous in oxygen-depleted environments that are limited in organic substrate. Furthermore, anammox bacteria depend on the presence of ammonium and nitrite. Ammonification, associated with the anaerobic degradation of organic matter and DNRA are possible sources for ammonium. Nitrate and nitrite can be produced by aerobic nitrifying bacteria which inhabit the interface of oxic/anoxic habitats. Here, it is likely that competing and/or beneficial interactions occur between nitrite and ammonium oxidizers (Hao *et al*., 2002; Kuenen, 2008). Co-occurrence of aerobic and anaerobic ammonium oxidizing bacteria was also observed in oxygen-limited reactors (Third *et al*., 2001). Nitrate reducers could be further advantageous coinhabitants of anammox habitats. However, it was found that by oxidation of organic compounds, anammox bacteria themselves also can reduce nitrate to nitrite (Guven *et al*., 2005; Kartal *et al*., 2007a, b).

The anammox reaction is by now considered to contribute significantly to the removal of fixed nitrogen from several natural habitats, mainly in marine anoxic basins, for example in the Black Sea (Jensen *et al*., 2008) or in Golfo Duce, Costa Rica (Dalsgaard *et al*., 2003), and in oxygen minimum zones (OMZs), for example west of Peru, Chile or Namibia (Galán *et al*., 2009; Hamersley *et al*., 2007; Kuypers *et al*., 2005; Lam *et al*., 2009).

Amplification of 16S rRNA genes is usually the first step for the identification of anammox bacteria in the environment. Of the anammox genera known to date, the most widespread genus appears to be *Candidatus* Scalindua, which has first been detected in the Black Sea (Kuypers *et al*., 2003), and later on in many further different, mainly marine, natural habitats (Amano *et al*., 2007; Dang *et al*., 2010; Dong *et al*., 2009; Li *et al*., 2010; Penton *et al*., 2006; Rich *et al*., 2008; Schmid *et al*., 2007; Woebken *et al*., 2008). Based on 16S rRNA sequences, members of the *Candidatus* Brocadia clade were detected in a temperate stratified lake and in river sediments (Hamersley *et al*., 2009; Zhang *et al*., 2007),

and 16S rRNA sequences of the *Candidatus* Kuenenia clade were detected in a deep sea hydrothermal vent ecosystem (Byrne *et al*., 2009). A great diversity of 16S rRNA anammox sequences was found by Dale *et al*. (2009), who studied anammox bacterial communities in sediment samples taken along a river estuary, and detected sequences of the *C*. Scalindua, the *C*. Kuenenia, the *C*. Brocadia and the *C*. Jettenia clades.

Apart from *Candidatus* Scalindua that was first detected in nature, the other four so far known genera were first found in enrichment reactors, inoculated with samples originating from fresh water habitats, such as river sediment (Quan *et al*., 2008) or wastewater (Kartal *et al*., 2007b; Schmid *et al*., 2000; Strous *et al*., 1999). The geographical distribution of the environmental anammox gene sequences evaluated so far gave rise to theory that *C*. Scalindua is mainly present in marine habitats whereas the other known genera mainly occur in fresh water habitats (Schmid *et al*., 2007). The presence of *C*. Scalindua together with other clades of anammox bacteria in estuaries supports this theory, although the exact effect of salinity on the composition of anammox communities in this environment is not totally clear yet (Dale *et al*., 2009). Furthermore, the extent to which currently used primers cover the real anammox 16S rRNA sequence diversity might still be too limited to deduce a realistic picture of the global distribution and niche separation of anammox organisms (Dang *et al*., 2010; Li *et al*., 2010; Penton *et al*., 2006).

Next to the amplification of 16S rRNA sequences, the *hao/hzo* gene is used as an additional genetic marker for anammox bacteria (Dang *et al*., 2010; Li *et al*., 2010; Schmid *et al*., 2008). When both markers were studied, a greater phylogenetic diversity was found for *hao/hzo* genes compared to 16S rRNA genes (Dang *et al*., 2010; Li *et al*., 2010). It remains to be determined whether this indicates that the primers used for the detection of these genetic markers cover different parts of the phylogenetic diversity of anammox bacteria or whether there is indeed incongruence in the phylogenies of their 16S rRNA and *hao/hzo* genes. Furthermore, there is considerable variety among the *hao/hzo* genes. Several divergent octaheme cytochrome *c* (OCC) open reading frames were detected in the genome of *Candidatus* Kuenenia stuttgartiensis (Strous *et al*., 2006), most probably encoding HAO/HZO. Schmid *et al*. (2008) found that the anammox *hao/hzo* sequences cluster in three groups, one of which was fairly consistent in its phylogeny with anammox 16S rRNA sequences. Thus, adding a functional gene, of which several variants are present per genome, to the set of genetic markers increases information on both phylogeny and potential anammox activity (Dang *et al*., 2010; Junier *et al*., 2010; Li *et al*., 2010; Quan *et al*., 2008).

Another candidate for a functional genetic marker for anammox bacteria is their *nirS* gene, since the known sequences of anammox nirS genes are very different from the *nirS* sequences of denitrifiers (Lam *et al*., 2009). Nevertheless, further screening of environmental clones and metagenomes for new sequences, as well as enrichment and genome sequencing of yet unknown anammox species will be necessary to approach the full extent of environmental anammox diversity.

Anammox activity is normally detected via rate measurements using 15N labeled tracers (Thamdrup and Dalsgaard, 2002; Risgaard-Petersen *et al*., 2003). Since anammox 16S rRNA sequences can be amplified from sites where no anammox rates are measured, the combination of genetic marker detection and rate measurements is now a common strategy to detect active anammox communities in the environment (Dalsgaard *et al*., 2005; Jetten *et al*., 2009; Schmid *et al*., 2005). Another widely used method for anammox detection in environmental samples is fluorescence *in situ* hybridization (FISH). With this approach anammox bacteria were found in deep sea hydrothermal vents (Byrne *et al*., 2009), mangrove soils (Wickramasinghe *et al*., 2009), in several terrestrial (Humbert *et al*., 2010) and further aquatic habitats (Galán *et al*., 2009; Hamersley *et al*., 2007; Jaeschke *et al*., 2009b; Risgaard-Petersen *et al*., 2004; Tal *et al*., 2005; Zhang *et al*., 2007). Probes usually target 16S rRNA genes, and thus the same challenges as for anammox 16S rRNA targeting primers apply. Gene based techniques and activity measurements can be complemented by lipid analysis since the detection of ladderane lipids is a strong indication for the presence of anammox bacteria (Kuypers *et al*., 2003; Hamersley *et al*., 2007; Jaeschke *et al*., 2009a,b; Schmid *et al*., 2003). The phylogenetic information in the composition of these lipids has been investigated by Rattray *et al*. (2008). Ladderane core lipid compositions were found to be relatively consistent among the four studied genera, whereas ladderane phospholipid compositions varied. Whether these differences were species-related or due to different cultivation methods could, however, not be ascertained in this study.

2.6 Nitrite reduction drives methane oxidation

Recently, microbes that couple dinitrogen formation to methane oxidation were enriched (Ettwig *et al*., 2009; Hu *et al*., 2009) which let to the discovery of a new dinitrogen forming pathway. '*Candidatus* Methylomirabilis oxyfera', the dominant organism of enrichment

cultures from different drainage ditch sediments, is capable of oxidizing methane under anoxic conditions, using oxygen originating from nitric oxide (Ettwig *et al*., 2010). The pathway was deduced from metagenomic data followed by experimental verification. The genome of '*M. oxyfera*' encodes all genes for the pathway of aerobic methane oxidation, as well as an incomplete gene set for denitrification, missing a gene for nitrous oxide reductase. Nevertheless, under anoxic conditions, in the presence of methane and nitrite or nitric oxide, '*M. oxyfera*' oxidizes methane and, at the same time, produces dinitrogen gas. Nitrous oxide was produced only in very small amounts and if nitrous oxide was the only nitrogen compound present, no methane oxidation occurred (Ettwig *et al*., 2009, 2010; Raghoebarsing *et al*., 2006). Based on these observations, a new reaction pathway was proposed: In the last step of this 'alternative' denitrification pathway, a not yet known enzyme catalyzes the formation of dinitrogen and oxygen from two molecules of nitric oxide (figure 2.1, NO dismutation pathway). The oxygen is used by particulate methane monooxygenase in the first step of the 'conventional' methane oxidation pathway (Ettwig *et al*., 2010). It is unknown to what extent this alternative form of denitrification is used to oxidize other electron donors than methane and whether it competes with 'normal' heterotrophic denitrification. '*M. oxyfera*' produces a membranebound bo-type respiratory terminal oxidase, which might be an indication that residual produced oxygen may serve for respiration using reducing equivalents from the methane oxidation (Wu *et al*., 2010).

2.7 Conclusions

Regarding the numerous biochemical pathways starting from nitrate and their interactions it is obvious that the nitrogen cycle is a complex network rather than a cycle. This network provides a great variety of ecological niches that bacteria and other (micro-)organisms can occupy and linkings between the different pathways add even more complexity to the network. For instance, W. succinogenes known for carrying out DNRA also reduces nitrous oxide to dinitrogen and thus carries out the last step of denitrification (Simon *et al*., 2004). This organism has a *nos* gene cluster but no genes for nitrite reduction to nitric oxide or nitric oxide reduction to nitrous oxide (Baar *et al*., 2003). The functional genes for denitrification *nirK* and *norB* also occur in several aerobic ammonium oxidizing bacteria (Casciotti and Ward, 2001, 2005).

In the environment, the different physiological groups compete for the substrate nitrate. Depending on the predominating nitrate respiration pathway, nitrogen stays in the habitat or is released into the atmosphere. Formation of dinitrogen and nitrous oxide, a potent greenhouse gas, leads to the removal of nitrogen from a habitat. Anammox also leads to nitrogen removal, and nitrous oxide is sometimes released as a byproduct in considerable amounts. In a full scale anammox bioreactor approximately 1% of the nitrogen load was converted to nitrous oxide (Kampschreur *et al*., 2008). This points out a further intersection in the nitrogen network. In contrast, formation of ammonium by DNRA keeps the nitrogen fixed and available for further biological processes. The nitrogen cycle is also tightly linked to other biogeochemical cycles like the carbon or the sulfur cycle (Lavik *et al*., 2009; Ward *et al*., 2007). For the complete understanding of the nitrogen network and its interactions the environmentally important microbial performers of each process and the conditions that influence the predominance of one or the other pathway have to be identified and studied. To obtain such knowledge, interdisciplinarity including geochemical, biochemical and molecular biological methods is required. To make efficient use of the interconnection of the cycles of matter on local and global scales, for example in agri- and aquaculture and wastewater treatment, a thorough knowledge of the involved processes is necessary. Comprehending the course of niche adaptation in nature enables and improves the directed and precise practical application of microbial activities in the field of biotechnology.

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Chapter 3

3 The binning of metagenomic contigs for microbial physiology of mixed cultures

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BK performed culturing and DNA extraction. HET performed Illumina sequencing. RB performed the assembly. MS developed and implemented the binning algorithm with help of RB. The manuscript was written by MS with input from **BK**, RB and HET..

3.1 Abstract

So far, microbial physiology has dedicated itself mainly to pure cultures. In nature, cross feeding and competition are important aspects of microbial physiology and these can only be addressed by studying complete communities such as enrichment cultures. Metagenomic sequencing is a powerful tool to characterize such mixed cultures. In the analysis of metagenomic data, well established algorithms exist for the assembly of short reads into contigs and for the annotation of predicted genes. However, the binning of the assembled contigs or unassembled reads is still a major bottleneck and required to understand how the overall metabolism is partitioned over different community members. Binning consists of the clustering of contigs or reads that apparently originate from the same source population. In the present study eight metagenomic samples from the same habitat, a laboratory enrichment culture, were sequenced. Each sample contained 13–23 Mb of assembled contigs and up to eight abundant populations. Binning was attempted with existing methods but they were found to produce poor results, were slow, dependent on non-standard platforms or produced errors. A new binning procedure was developed based on multivariate statistics of tetranucleotide frequencies combined with the use of interpolated Markov models. Its performance was evaluated by comparison of the results between samples with BLAST and in comparison to existing algorithms for four publicly available metagenomes and one previously published artificial metagenome. The accuracy of the new approach was comparable or higher than existing methods. Further, it was up to a 100 times faster. It was implemented in Java Swing as a complete open source graphical binning application available for download and further development (http://sourceforge.net/projects/metawatt).

Keywords: metagenomics, binning, tetranucleotide frequencies, interpolated Markov models

3.2 Introduction

Prokaryotes (Bacteria and Archaea) comprise a significant portion of the living biomass on earth and sustain the geochemical element cycles, a vastly complicated, planetary-scale metabolic network. Prokaryotes form complicated ecological communities consisting of a multitude of species and only a small fraction of these species has been cultivated in the laboratory, studied experimentally and has a known genome sequence. More importantly, these species have been studied in isolation, after a pure culture was obtained. To further refine our understanding of geochemical element cycling it is essential to study the physiology of microbes in their natural context, i.e., the microbial community. Microbial communities can be cultivated in the laboratory under meaningful, near-natural conditions by continuous cultivation of microbial enrichment cultures.

Given such a mixed microbial culture, metagenomics (sequencing and analysis of DNA obtained from complete microbial communities) is a powerful approach to determine both the community composition and the potential physiology of the abundant community members. This way, function-identity relationships (e.g., Walsh *et al*., 2009; Ettwig *et al*., 2010) can be resolved in a simple and standard way. "Binning" is an essential step in this analysis. Binning can be performed after assembly of raw sequence reads into contigs and consists of the clustering of those contigs that belong together, constitute a (partial) genome of a single population (or of a group of closely related populations). When the sequencing coverage is sufficiently high and when the "microdiversity" is not too high, the resulting bins can be considered provisional whole-genome-sequences of the source populations. The ecological function of those populations can then be investigated, first by genome annotation and subsequently by experiments. Both for assembly and annotation, well developed algorithms and pipelines are available but the binning is still a bottleneck in metagenomic analysis.

Several approaches have been investigated for the binning problem; they can roughly be divided into similarity-based methods, such as BLAST (Huson *et al*., 2011) and hidden Markov models (Krause *et al*., 2008), and compositional approaches such as tetranucleotide frequencies (Teeling *et al*., 2004a,b; McHardy *et al*., 2006; Chatterji *et al*., 2007; Bohlin *et al*., 2008; Diaz *et al*., 2009; Saeed *et al*., 2011), interpolated Markov models (IMM; Kelley and Salzberg, 2010) and Markov chain Monte Carlo models (Kislyuk *et al*., 2009). The advantage of compositional approaches is that they are able to bin contigs with genes that are not homologous to the reference species. This advantage is essential because even closely

related species share only a relatively small core genome and the detection of non-homologous genes in related species is the essence of unraveling new function-identity relationships. The advantage of similarity-based methods is that these approaches are very robust – given a contig of sufficient length they generally provide a clear indication about the (approximate) taxonomic position of the source population. Therefore, without a similarity-based method, it is impossible to evaluate the binning results obtained by a compositional algorithm, except with artificial datasets (e.g., FAMeS; Mavromatis *et al*., 2007). In practice, a complete binning procedure should therefore consist of a combination of similarity and composition based methods.

Compositional approaches can further be subdivided into supervised (i.e., comparison of the metagenomic contigs to existing genome data) and unsupervised (comparison of the metagenomic contigs only to each other) methods. Given the facts that (a) microbial diversity is vast and (b) relatively few reference genome sequences are available, an unsupervised method is usually essential to prime the binning process; supervised methods do not perform well when no closely related organism is available to train the models. Once bins have been primed with an unsupervised method, models of different types can be trained on the primed bins and the binning can be completed. Such two-step procedures were recently shown to be promising (Kelley and Salzberg, 2010; Saeed *et al*., 2011).

In the present study we analyzed microbial communities growing in continuous culture in the laboratory. These communities were of medium complexity (up to eight “binnable” popula-tions). Our metagenomic samples contained 4–10 million 50– 150 basepairs reads (Illumina) and these were first assembled into contigs. Assembly yielded contigs of reasonable size (longest contigs between 30 and 200 kb). For the binning of these contigs, we developed the new integrated binning procedure that is the topic of this paper. It is similar to the two-step approach described by Kelley and Salzberg (2010) and Saeed *et al*. (2011) but uses a newly developed, ultrafast algorithm based on multivariate statistics of tetranucleotide frequencies for the priming of the bins.

Compared to previous methods the new unsupervised priming algorithm is very fast (seconds) and does not require an estimate for the number of binnable populations. Further, for each of the produced bins, a taxonomic signature is calculated with a similarity-based approach (BLAST). By inspection of this signature in combination with sequencing coverage information, promising bins can be identified and used to train IMM. These models are then used for final binning.

With this procedure, eight populations from our community could be binned with high apparent accuracy (>90% at the genus level, >96% at the family level). The general performance of our procedure was further evaluated by comparison to two existing methods (Kelley and Salzberg, 2010; Saeed *et al.*, 2011) for four publicly available metagenomes and one previously published artificial metagenome (table 3.1). Our evaluation showed that the new approach is much faster and achieves better or comparable accuracy. It was implemented in a stand-alone graphical interactive binning environment, the "Metawatt binner" that is available for download, use, and further development.

Table 3.1: Performance (time,T, minutes, recall, R, percentage, accuracy, A, percentage) of the presented binning approach (Metawatt) for five publicly available metagenomes in comparison to two published two-step compositional binners: SCIMM (Kelley and Salzberg, 2010) and 2Tbinner (Saeed *et al.*, 2011).

Meta genome	Total (Mb)	Contigs	SCIMM			2Tbinner			Metawatt*		
			T	R	A	T	R	A	T	R	A
Acid mine drainage	11.2	1703	22	79.2	72.3	190	**	**	1	80.4	82.8
Olavius symbionts	22.3	868	34	78.2	76.3	371	**	**	1	77.0	88.4
EBPR	24.4	11188	30	83.8	74.5	36	39.3	98.4	3	93.3	81.3
Whalefall bone	28.9	26232	45			24	**	**	4		
SimBG	39	40000	53	77.6	75.6	33	4.5	90	7	91.6	92.6

**Time includes tetranucleotide and IMM training and binning, but not the evaluation by BLAST.*
***The R script produced an error and/or no meaningful bins were generated.*

3.3 Materials and methods

Samples and metagenomic sequencing

Eight samples were taken from a microbial enrichment in continuous culture, inoculated with sediment from the Janssand tidal flat in the German Wadden Sea (N 53.73518; E 07.69912). DNA was extracted. Barcoded Illumina TruSeq libraries were generated and sequencing was performed (together with four further libraries of a different study) on one flow cell lane of

an Illumina Genome Analyser GA IIx instrument, in a 2x150 cycles paired end run. Reads were submitted to the Short Read Archive (SRA, accession number SRP012152) and assembled contigs (see below) to the Whole Genome Shotgun repository (WGS, accession numbers SUB086333, SUB122313, SUB122314, SUB122316, SUB122317, SUB122318, SUB122319. SUB122321).

Assembly

Assembly was performed with MetaVelvet -v0.3[1]. After quality trimming (sliding window approach: window length 15 basepairs, within this window quality value of at least 99%, minimal read length after trimming 25 basepairs) between 4,414,212 and 9,986,877 reads (392,800,534–901,487,996 bases) per sequenced library were assembled. Assembled contigs were submitted to WGS (see above). An overview of the assembly results is presented in table 3.2.

Table 3.2: Assembly results of the eight sequenced metagenomes.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Number of reads (millions)	4.8	7.6	4.4	8.2	5.6	5.7	**10**	**5.2**
Total sequence data in reads (Mb)	474	687	424	751	550	537	**901**	393
Number of contigs (thousands)	16	6.3	40	7.6	8.9	**52.6**	5	19.3
Total sequence data in contigs (Mb)	15.3	13.7	20.4	15.7	13.4	**23.3**	13	13.3
Longest contig (kb)	**182**	**182**	77	167	145	37	177	93
N50 contig length (kb)	2.7	7.7	1	5.2	6.8	0.8	**26.3**	1.8
K-mer size for assembly	51	51	51	61	61	61	61	51

Ultrafast unsupervised binning based on tetranucleotide composition

There exist 256 (4^4) different tetranucleotides. However, when we assume that both DNA strands are sampled equally, the reverse complements of every tetranucleotide become redundant and 136 non-redundant tetranucleotide pairs remain. (The number of actual degrees of freedom is lower, 103, see Kislyuk *et al.*, 2009). Each of these remaining pairs consists of the tetranucleotide itself and its reverse complement. The frequencies of the 136

[1] http://metavelvet.dna.bio.keio.ac.jp/

different non-redundant tetranucleotide pairs were calculated for each contig, normalized to contig length, and the composition of each contig was represented as a 136-dimensional vector. The normalized frequency of tetranucleotide *x* in contig *y* was calculated as follows:

$$\text{frequency}\,(x) = \frac{\text{occurrence}\,(x \text{ in } y)}{\text{total number of tetranucleotides matched in } y^{*}\ 136}$$

After the multiplication by 136 and with a GC content of 50%, average frequencies correspond to a value of 1.

One-hundred artificial contigs of distinct lengths (0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.75, 2, 2.25, 2.5, 2.75, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 18, 20, 25, 30, 40, 50, 60, 70, 80, 100 kb) were sampled from each of the 794 prokaryotic whole-genome-sequences representing the known biodiversity (one genome per genus, table S3.1 in Supplementary Material). Tetranucleotide vectors were calculated for each reference genome as a whole (the mean vector) and for each artificial contig (sample vectors). Next, at each contig length *l*, the standard deviation of the tetranucleotide frequencies observed in the population of sample contigs (*s*) was plotted against the mean frequency observed in the source genome (*m*). Figure 3.1A displays plots for four different values of *l*.

Figure 3.1 shows that independent of the nature of the tetranucleotide and independent of the source genome, *s* can be predicted when *m* and *l* are known.

We attempted to describe the observed empirical relationship *s* D *f (m, l)* as a formal mathematical function, but no satisfying function was found that accurately described the relationship for all relevant values of *m* and *l*. Therefore, the observed empirical relationship *s* D *f (m, l)* was interpolated into a lookup table to be able to estimate the standard deviation based on the mean frequency and the contig length.

With this lookup table [*s* D f (*m, l*)] it was now possible to estimate the multivariate probability (P) that a contig (length *l*) of unknown origin belonged to any source organism, given the observed tetranucleotide frequency vector *v* for the contig and a known or estimated mean tetranucleotide vector *m* of the source organism:

$$P = \prod_{x=1.136} \frac{1}{\sqrt{2\pi}\, s_x} e^{-\frac{1}{2}\frac{(v_x - m_x)^2}{s_x^2}}$$

During binning *m*, the mean vector of the "source organism" is estimated as described below.

Next, the artificial contigs were used to determine empirical threshold values for *P* that could be used by the algorithm to decide whether the unknown contig belonged to the source organism or not (figure 3.1B). Three thresholds were defined, a high confidence threshold that only accepted 90% of the artificial contigs belonging to a given organism, a medium confidence threshold that accepted 95% of the artificial contigs and a low confidence threshold that accepted 98% of the artificial contigs. It was found empirically that the threshold value for *P* depended on the length *l* of the unknown contig, as shown in figure 3.1B. Again, this function was interpolated into a lookup table with *P* threshold D *f*(90, 95, or 98%), *l*).

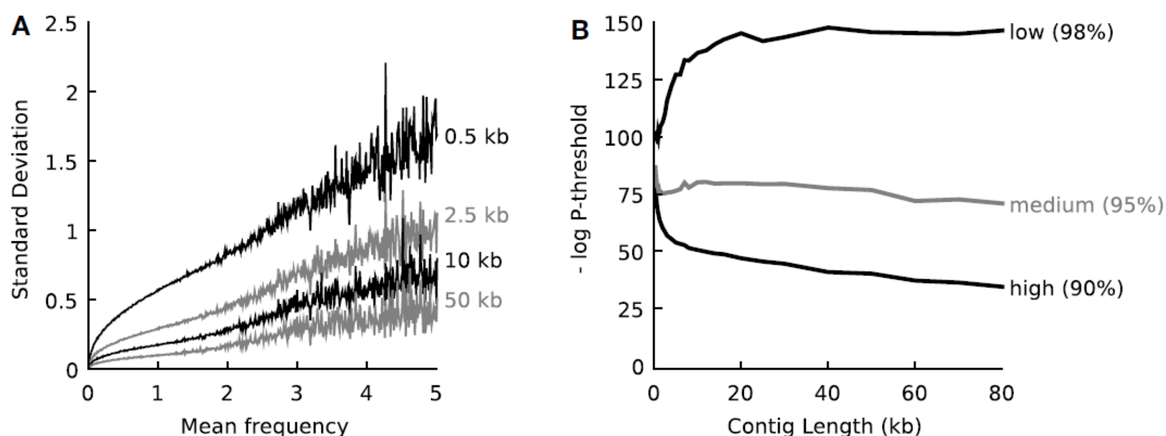


Figure 3.1: **(A)** When populations of DNA fragments of defined length were sampled from a source genome, an empirical relationship was observed between the mean frequency, the frequency of any tetranucleotide in the source genome, and the standard deviation in the frequency of that tetranucleotide observed in the sample populations. The relationship is shown for four different lengths of DNA fragments and at each mean frequency the average standard deviation is shown. For an explanation on the calculation of the frequencies, see main text. **(B)** Empirical relationship that defines the probability threshold as a function of DNA fragment length at high (90% recall), medium (95% recall) and low (98% recall) confidence. The relationships were determined empirically by sampling 794 representative reference genomes (n= 100 for 37 different DNA fragment lengths between 0.3 and 100 kb).

Using the relationships of Figure 3.1, the binning now proceeded as follows

First the contigs were sorted by length and tetranucleotide vectors were calculated for all contigs. The longest contig was processed first and a bin was created for this contig. Next, contigs were processed one by one, from long to short, and for each existing bin, the

probability that the contig belonged to the bin was calculated. Because the probability value *P* only decreases as more dimensions are analyzed, the comparison was aborted once it fell below the threshold. When no bin could be found for the new contig (all *P* values below *P* threshold), it was used to seed a new bin. Otherwise, the contig was joined with the most probable bin (highest *P*) and a new mean tetranucleotide vector was calculated for that bin (vectors were weighed by contig length).

In this comparison, it was necessary to assume that the tetranucleotide vector of any existing bin approximated the mean vector of its source genome. This approximation would be better for longer contigs, and this was the reason why the contigs were first sorted. For short contigs the approximation would not be valid and therefore, no new bins were seeded with contigs less than 1000 basepairs long. The entire procedure was performed three times, once for every confidence level (low, medium, and high, figure 3.1B). Less than 1 min was required to complete each of these calculations with a i5 M430 processor (2.27 GHz) for all datasets (up to 39 Mb of assembled contigs, see table 3.1).

Analysis of bin taxonomic compositions with BLAST

First every contig was fragmented into 500 basepair pieces. BLAST (Camacho *et al*., 2009) was used to compare these pieces to a database with the 794 prokaryotic whole-genome-sequences representing the known biodiversity (one genome per genus, table S3.1 in Supplementary Material). Hits of >200 basepairs length and with at least 25% nucleotide identity were used to create a taxonomic profile for each contig. The profile consisted of five taxonomic ranks (phylum, class, order, family, genus). At each rank the taxon with the most hits was recorded together with the number of hits to this taxon and the median *e*-value of the hits. After binning, the contig profiles were added and averaged to calculate a taxonomic profile for the bin as a whole. This profile was displayed as a pie diagram; see figure 3.3 for examples.

Calculation of sequencing coverage

Next to taxonomic composition, the sequencing coverage constitutes a second, independent criterium to evaluate binning success. Contigs that belong to the same source population should have similar coverage, whereas different source populations can have different coverages (dependent on the relative abundances, chromosome copy number, and DNA extraction efficiency). Coverage was parsed from the header line of the fasta output produced

by the assembler or estimated for each contig from the average read length of the sequencing run and the number of source reads for the contig parsed from the header line of the contig fasta file produced by the assembler. The regular expressions used for the parsing of coverage or number of reads were “cov[a–z]*?[=_]([\\d\\.]+)” and “numreads = (\\d+).”

Interpolated Markov modeling

After inspection of the unsupervised binning results for all samples, good bins were selected for the final binning step. “Good bins” were bins with relatively long contigs, a consistent taxonomic profile and a equally distributed sequencing coverage (decision made by the scientist). IMM were created with the program “build-icm” from the Glimmer package (Delcher *et al*., 2007). The models were used to score all contigs of all samples with the program “simple-score” from the Glimmer package, used with the N option (no negative model). For each contig, the scores were compared for each model and the contig was binned to the model with the highest score.

Evaluation of binning accuracy with publicly available metagenomes

Four publicly available metagenomes were selected for evaluation: a metagenome sampled from acid mine drainage (Tyson *et al*., 2004, accession numbers AADL01000110.1-AADL01001068.1, CH003545.1-CH004435.1,DS995259.1-DS995275.1),one obtained from enhanced biological phospate removing (EBPR) sludge (Martin *et al*., 2006, accession numbers AATN01000001.1-AATN01011188.1), one from an *Olavius algarvensis* microbial symbiont community (Woyke *et al*., 2006, accession numbers AASZ00000000.1, DS021108.1-DS022223.1), and one from an Antarctic whale fall bone (Tringe *et al*., 2005, accession num-bers AAGA01000001.1-AAGA01026232.1). In addition, an artificial metagenome was used (SimBG, Saeed *et al*., 2011). All five metagenomes were also used for evaluation by Saeed *et al*. (2011). Additional information is provided in table 3.1. For evaluation of the real metagenomes, we assumed that the annotations provided by the authors of the original studies were correct. In the EBPR case no annotations were provided, so we used the published genome of *Candidatus* “Accumulibacter phosphatis” as the reference. After binning, a bin was assigned to each population and the accuracy was calculated as the number of correctly binned nucleotides divided by the total number of nucleotides in the bin (100%). Recall was calculated as the number of nucleotides of the source organism assigned to the bin divided by the total number of nucleotides of the source organism present in the

metagenome (100%). For the Whale Fall metagenome, evaluation of accuracy and recall was impossible, as binning was reported to be unsuccessful by the authors. The results (accuracy, recall, and computation time) were compared to two comparable previously published state of the art *de novo* compositional binners (Kelley and Salzberg, 2010; Saeed *et al*., 2011). For SCIMM (Kelley and Salzberg, 2010), bins were seeded with a single trial of Likely Bin and the algorithm was run multiple times with different estimates for the number of populations. In table 3.1 only the results for the optimal choice are shown. 2T binner was run with the default options.

Implementation

The procedure was implemented in java as a Swing application that has been tested on Linux (64 bit). A graphical user inter-face was necessary because our method depends on an important choice by scientist: which tetranucleotide bins should be used to train IMM models? For this reason, visualization of the binning results is important. The application is freely (Academic Free License) available for download and further development at http://sourceforge.net/projects/metawatt. It depends on BLAST (Camacho *et al*., 2009), Glimmer (Delcher *et al*., 2007) and the batik library[2] for exporting structured vector graphics (SVG). For evaluation of binning results a BLAST library of sequenced genomes and a taxonomy of these genomes is necessary. Metawatt can generate these files automatically when it is provided with the genbank files of all reference organisms (downloadable from http://ftp.ncbi.nlm.nih.gov/genomes/Bacteria/all.gbk.tar.gz).

3.4 Results

When we inspected multivariate distributions of tetranucleotide frequencies of artificial DNA fragments sampled from reference genomes we observed that for all organisms these distributions can be approximated by a single Gaussian function characterized by a generally valid empirical relationship between the mean frequency of any tetranucleotide in a genome, the standard deviation of the observed frequency in DNA fragments sampled from this genome and the fragment length. See materials and methods and figure 3.1A for details.

[2] http://xmlgraphics.apache.org/batik

Given a DNA fragment of known length and tetranucleotide composition, the relationship can be used to calculate a probability that the fragment belongs to a source genome with known or estimated mean tetranucleotide composition. Further analysis of calculated probabilities for artificially sampled DNA fragments enabled the definition of a threshold for the probability value that could be used to determine whether a DNA fragment most likely belongs to a source genome or not. figure 3.1B shows the empirical relationship between the DNA fragment length and the threshold probability at 90, 95, and 98% recall. See section “Materials and Methods” for details.

The two empirical relationships shown in figures 3.1 A, B enabled us to rapidly calculate whether a metagenomic contig should be binned together with another contig. Apart from the contig sequences themselves this calculation made use of only a single parameter – the confidence value (90, 95, or 98% recall). It depended on only a single assumption: that the average tetranucleotide composition of the two contigs under consideration approximated the composition of the source genome. The validity of this assumption obviously depended on contig length; the longer the contigs, the better their tetranucleotide composition would approximate that of their source genome. For this reason, contigs were sorted by length before binning.

We first investigated the possibility to use the two empirical relationships of figure 3.1 to classify DNA fragments. The classification accuracy was compared to the accuracy obtained with IMM as follows: first artificial communities were created from reference organisms randomly sampled from 794 available whole-genome-sequences of different genera (table S3.1 in Supplementary Material; 10, 25, 50, and 100 species per community). For each species three artificial long DNA fragments were created (10, 50, or 1000 kb) and also five groups of 100 artificial short DNA fragments (500, 1000, 2000, 4000, and 8000 basepairs length). Next, all short DNA fragments were classified based on comparisons with one of the long fragments: either the tetranucleotide frequencies were compared as explained above or an IMM was trained with the long fragment. The classification accuracies are shown in figure 3.2. The figure shows that when the DNA fragment used for training was longer than 50 kb, IMMs outperformed our algorithm for classification. With shorter fragments, the tetranucleotide classifier outperformed IMMs.

A metagenomic binner was now created that made use of the tetranucleotide classifier. Binning started by seeding the first bin with the longest contig. Next, the remaining contigs were processed one by one in order of decreasing size. Each contig was binned to the bin that

yielded the highest probability value, or when no probability was above the threshold, a new bin was created. When a contig was joined into an existing bin, a new mean frequency vector was calculated for the bin. And so on. With growing bin sizes (and few misbinnings), the mean vectors of the bins would approach those of the source genomes. The two arrows in figure 3.2 provide an indication of the level of accuracy achieved during binning. In the initial stages with small bin sizes, the estimate for the tetranucleotide composition of the source genome is poor, but the binned contigs are long (blue arrow) and the accuracy is around 80%. Toward the end, the estimates for the tetranucleotide com-position of the source genomes are much better (large bin sizes) but the binned contigs are smaller (green arrow) and the accuracy is also around 80%.

The procedure allowed us to bin a metagenome without an *a priori* estimate for the number of populations that might be binnable, as is necessary for some other algorithms (e.g., Kislyuk *et al*., 2009). As expected, the binning algorithm was very fast (seconds for all tested metagenomes, up to 39 Mb, see table 3.1). We found that no general rule existed as to what confidence (90, 95, or 98% recall) was best for the calculation of the threshold values. In all cases, the application of a high confidence threshold (e.g., 90%) led to a larger number of bins. In some cases, this was justified. In other cases it was not and populations that were binned into a single bin at lower confidence were distributed over multiple bins at higher confidence. For this reason, binning was always performed three times, once for each confidence value.

To evaluate the binning results, a taxonomic profile was created for each bin and its sequencing coverage was calculated (as explained in the Materials and Methods section). The taxonomic profiles and coverage distribution of the bins produced at all three levels of confidence were now inspected. Bins with a consistent taxonomic profile and homogeneous sequencing coverage were selected and used to train IMM, one for every selected bin. These models were then used to rebin all contigs in a final binning step. As shown above, IMMs outperformed our tetranucleotide-based algorithm when much sequence information was available.

The performance of the binner was evaluated with four publicly available metagenomes and one artificial metagenome (table 3.1). For comparison the binning was also performed with two other state of the art two-step compositional binners (Kelley and Salzberg, 2010; Saeed *et al*., 2011). It appeared that our binning procedure was up to a 100 times faster and the accuracy was com-parable or better. The overall binning procedure completed in 7 min

for the largest metagenome tested (39 Mb). The binning results presented quantitatively in table 3.1 are briefly described qualitatively below.

The SimBG artificial metagenome contains contigs belonging to eight different bacteria and one artificial “fake” bacterium (Saeed *et al*., 2011). The binner made very few errors, except in case of *B. halodurans*. Its contigs were binned only at 70.2% accuracy because of the misbinning of some *P. marinus* and *D. hafniense* contigs.

The EBPR metagenome (Martin *et al*., 2006) contains contigs belonging to “*Candidatus* Accumulibacter phosphatis*”* and several side populations.

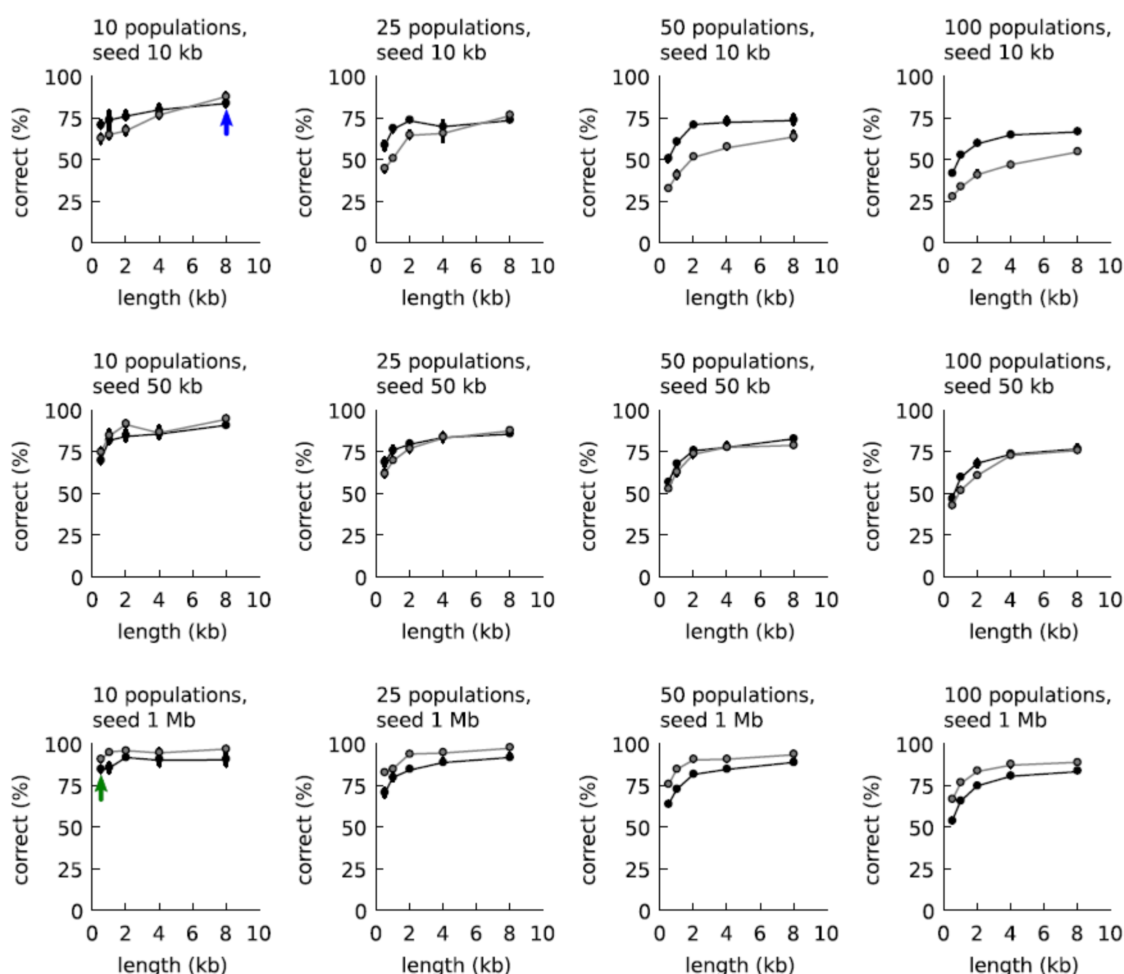


Figure 3.2: Classification accuracy with the empirical relationships of figure 3.1 (black line, closed symbols) and Interpolated Markov models (gray lines and symbols). The accuracy was calculated as function of community complexity (10, 25, 50, and 100 populations), length of the long DNA fragments used for training (10, 50, and 1000 kb, one for each population), and length of the short DNA fragment to be classified (100 fragments, 0.5–8 kb). Each point is the average of three communities randomly sampled from 794 reference genomes. For the explanation of the blue and green arrow, see main text.

After IMM binning, the contigs belonging to *A. phosphatis* were binned at excellent recall but at relatively low accuracy. Tetranucleotide binning actually yielded some bins with very high accuracy (97.4%) but in that case the recall was lower (46.8%), values comparable with the Two-tiered binner of Saeed *et al.* (see table 3.1). Three side populations were recovered as taxonomically consistent bins: the Gammaproteobacterium related to *Thiotrichales* (5.3 Mb), already recovered by Saeed *et al*. (2011), a *Flavobacterium* (3.5 Mb), and a *Xanthomonas* (2.5 Mb).

The *Olavius algarvensis* symbiont metagenome (Woyke *et al*., 2006) consists of contigs of unknown origin and contigs belonging to three symbiotic bacteria: the Gamma-1, Gamma-3, and Delta-1 symbionts. The contigs of the Delta-1 and Gamma-3 symbionts were binned without problems (accuracy 90.6 and 98.1% respectively). The contigs of the Gamma-1 symbiont were difficult to separate from some contigs of unknown origin leading to a lower binning accuracy (76.5%) for this organism.

The contigs obtained from the Antarctic whale fall bone (Tringe *et al*., 2005) were not binned in the original study, so evaluation of the accuracy was impossible. However, as already reported by Saeed *et al*. (2011), they are binnable with modern methods. After tetranucleotide binning, two bins contained contigs of mainly Flavobacterial origin (the first bin was 3.9 Mb at 31.7% GC, the second 7.7 Mb at 39.7% GC). Four additional bins with a con-sistent taxonomic signature were recovered: a Pseudomonad bin (4.3 Mb, 45.5% GC), an Alteromonas bin (2.1 Mb at 40.5% GC), a Rhodobacter bin (3.2 Mb at 55.8% GC) and a Sphingomonas bin (3.8 Mb at 57.6% GC). The uncultured *Actinomycete* sequences previously reported were split over three different bins but were well separated from other organisms.

The acid mine drainage metagenome (Tyson *et al*., 2004) consists of contigs from five different populations: "*Ferroplasma acidarmanus* Type I", "*Ferroplasma sp.* Type II", "*Thermoplasmatales archaeon*," "*Leptospirillum sp.* Group III," and "*Leptospirillum sp.* Group II." The contigs of the two *Ferroplasma*'s were binned together with some contamination of contigs from the *Thermoplasmatales archaeon*. The remainder of the *Thermoplasmatales archaeon* contigs were binned accurately in a separate bin. One bin contained only *Leptospirillum* Group III contigs and the final bin the *Leptospirillum* group II contigs with some contamination of *Leptospirillum* Group III contigs.

Once the benchmarking and testing was complete, the new binner was applied to eight metagenomes sampled from a microbial enrichment in a continuous culture mesocosm. The

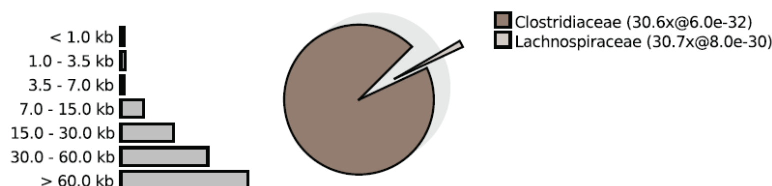
eight samples were tagged and sequenced on a single lane of an Illumina Genome Analyzer GA IIx instrument. Assembly yielded some reasonably long contigs and many short ones (table 3.2), as usual in sequencing projects.

Tetranucleotide binning was performed as described above and again a taxonomic profile was calculated for each bin (figure 3.3). The binner binned >99% of all contigs but the quality of the bins produced varied between samples, populations, and the applied threshold value. figure 3.3A shows that the binning was apparently successful for sample 7, at medium threshold. Each of the bins had a distinct taxonomic signature as well as a distinct sequencing coverage. For example, apparently two different Epsilonproteobacteria (green colors) were present in this sample, one with a GC content of 26.8% and a sequencing coverage of 12 times and one with a slightly higher GC content and a coverage of 40 times. One of the bins may belong to an uncultured *Rhodobacter*, relatively unrelated to reference Alphaproteobacteria with sequenced genomes. This could be inferred from the relatively high BLAST *e*-values, and the scattering of the BLAST hits over different families. One may argue that this bin contains contigs of many different Alphaproteobacteria but this could be ruled out by inspecting other samples where this organism was more dominant and yielded longer contigs. The distribution of BLAST hits for individual contigs was very similar to the distribution of the bin as a whole. In fact, this is a nice example of what happens when supervised binning approaches (based on reference genomes) are applied to organisms only distantly related to those reference organisms – the contigs get scattered and are assigned to many different reference taxa.

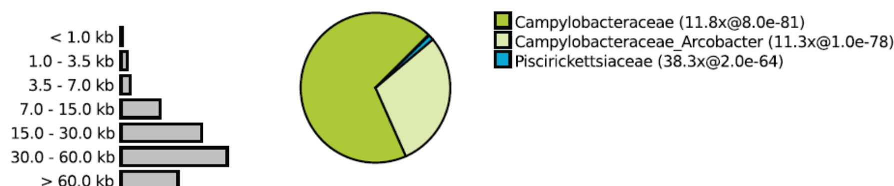
Figure 3.3B shows an example of unsuccessful binning in sample 1 with a low confidence threshold (98% recall). Here, contigs from a Pseudomonas population appear to get mixed up with contigs from a Vibrio population in bin 1. Bin 2 contains sequences from Vibrio and a Clostridum populations. Note that these misbinnings were not observed at the high confidence threshold, but in that case the contigs of the Vibrio and the Clostridium were divided over many bins.

Figure 3.3 (*facing page*): Contig size distribution, sequencing coverage and taxonomic distribution of the four largest bins of sample 7 binned at medium confidence (A) and sample 1 binned at low confidence (B). The exploded pies show the taxonomic distribution of the bins. The distance of each part from the center of the pie is a measure for the median e-value of the associated hits (the larger the e-value the larger the distance from the center). Coverage is shown for the bin as a whole and separately for each pie part.

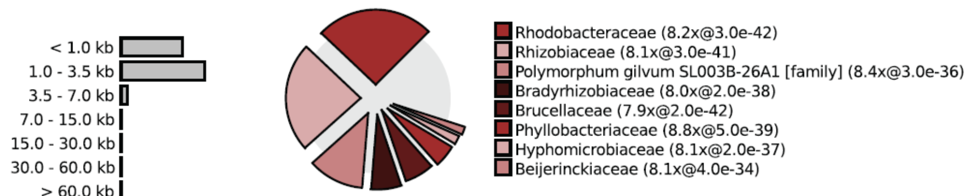
A **bin 1 (3.3 Mb; 37.4%GC; 30.5x; 179 contigs)**



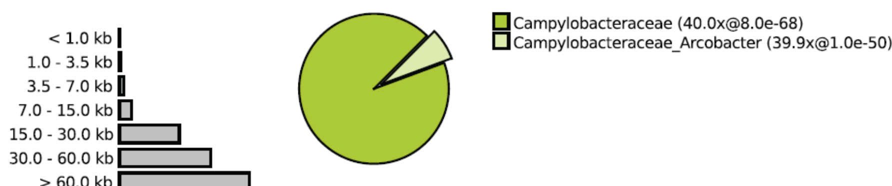
bin 2 (2.9 Mb; 26.8%GC; 12.0x; 167 contigs)



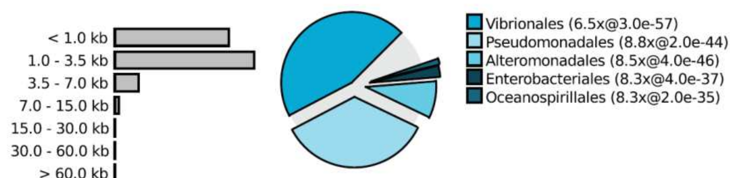
bin 3 (2.5 Mb; 50.5%GC; 8.1x; 2567 contigs)



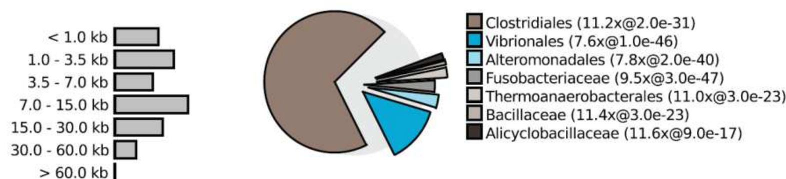
bin 4 (2.5 Mb; 33.5%GC; 40.0x; 81 contigs)



B **bin 1 (6.2 Mb; 45.2%GC; 7.7x; 6481 contigs)**



bin 2 (5.6 Mb; 36.5%GC; 10.1x; 2572 contigs)



Interpolated Markov models yielded better results than tetranucleotide frequencies once sufficient training data was accumulated. Therefore, we again selected the good bins (long contigs, consistent taxonomic profile, as described above) from all samples to train IMM models. Eight distinct bins were identified that apparently defined the binnable part of the microbial community in all samples. All samples were binned *de novo* with these eight models and the resulting bins looked convincing in all cases, both with respect to coverage and taxonomic profile

To further validate the results, BLAST was used to compare every sample with the eight reference bins (the bins used to construct the models, arrows in figure 3.4). Only BLAST hits that were >98% identical to the reference sequence were considered. The IMM binning and the BLAST results were totally consistent, with two exceptions: (1) There was some cross binning of the two Clostridia populations. (2) The model predicted an abundant *Vibrio* population in sample 1 whereas this population could not be validated by BLAST. Apparently, the *Vibrio* population in sample 1 was different from the reference population in sample 3 (its contigs less than 98% identical to the contigs that constituted the defined *Vibrio* bins) and a different IMM model could be created for these contigs.

The community enriched in the continuous culture was provided with a marine medium with organic carbon as the only electron donor and nitrite as the electron acceptor. Organic carbon was present in excess, whereas nitrite was the limiting substrate. The binning results showed that the enriched community consisted of denitrifiers (affiliated with *Arcobacter*, *Pseudomonadales* or *Rhodobacterales*) and fermenters (affiliated with *Vibrionales, Clostridiales* and *Fusobacterales*).

This is in agreement with text book knowledge: one would expect the denitrifiers to consume most of the organic carbon while respiring nitrite, while the fermenters would consume the remainder of the carbon. Some dynamics appear to occur, but because of potential methodological biases between sequencing runs and assembly, this first needs to be confirmed with other methods (FISH). The biology of the experiment will be addressed in detail elsewhere once these data are available. The aim of the present study was to develop a method for metagenomic binning of these and future metagenomes sampled from laboratory enrichment cultures.

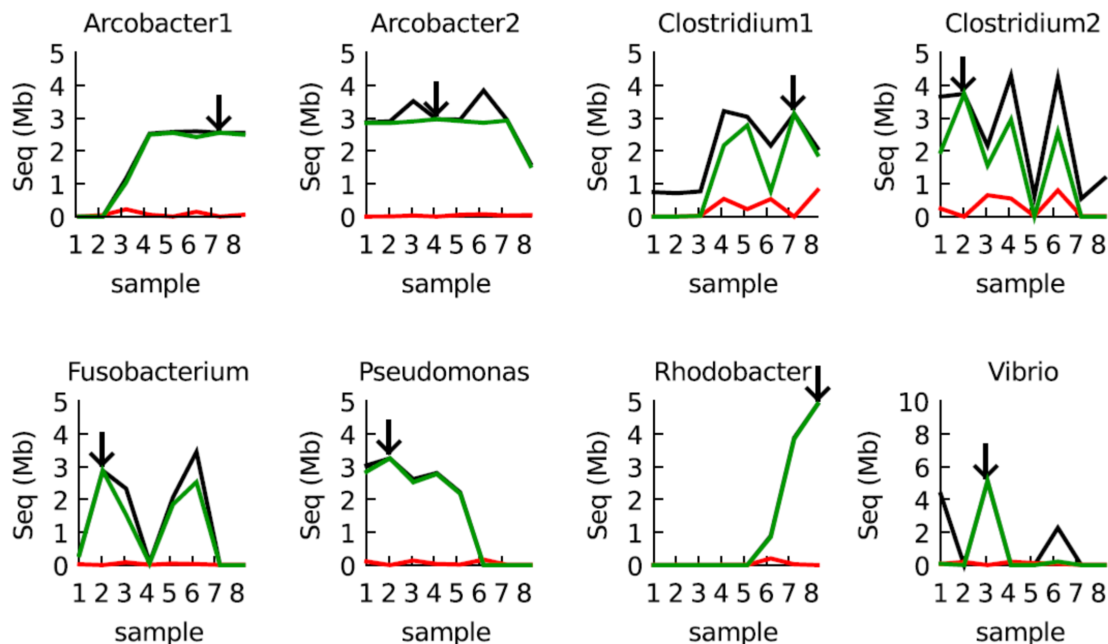


Figure 3.4: Validation of the IMM binning results for the eight samples by BLAST. The black line shows the total amount of assembled sequence information assigned to each of the eight populations by the IMM binner. The green line shows how much of that data was also recovered by BLAST (megaBLAST at 98% identity cutoff). The red line shows how much data was recovered in other bins (errors). Arrows indicate the origin of the reference bin used to train the IMM.

3.5 Discussion

In this study we have shown that it is currently possible to bin metagenomic data obtained from relatively simple microbial com-munities with a modest sequencing effort. The eight samples investigated were tagged and sequenced on a single lane of an Illumina Genome analyzer GA IIx (consumable costs <5 kEuro). Our results also show that it is very important to sequence multiple samples from the same habitat: first, there appears to be quite some dynamics, even though these samples were obtained from the same bioreactor that was running at totally constant environmental conditions. Second, the possibility to assemble and bin a target population depends on the context of the overall community. Third the quality of the sequencing and/or assembly results differed between samples. This probably resulted from (unintended) differences during the manual library preparation and potentially a different degree of microdiversity among populations in different samples. Last but not least, by comparing results between samples with BLAST it was possible to validate the binning results and not depend on *a priori* estimates of binning accuracy.

The first step of the overall binning procedure makes use of a novel algorithm based on an empirical relationship between the mean and standard deviation of tetranucleotide frequencies. It has some advantages compared to existing methods: it does not depend on an estimate for the number of binnable populations, is open source, portable to any platform (that supports BLAST and Glimmer) and is extremely fast. The latter advantage also means that it is more scalable: it will be able to cope with the large amounts of data that will be produced by future sequencing technologies and can be applied to more complicated communities. By always running the binner at three different threshold values, all tested metagenomes could be binned successfully without the need for optimizing additional parameters.

This newly developed algorithm was combined with the use of IMM, already applied previously as a final "polishing" step in binning. Our study confirmed that IMMs outperform tetranucleotide frequencies when sufficient training data is available. However, where Kelley and Salzberg (2010) use a fully automated iterative method to refine the bins, we created the possibility for the scientist to choose which seed bins should be used to build the IMM models. This choice should be based on the characteristics of the bins such as contig length, sequencing coverage, and a taxonomic profile. We present evidence that this human intervention can outperform the fully automated method. This may be caused by difficulties in repairing totally failed bins (e.g., figure 3.3B) by an iterative approach. In our enrichment culture metagenomes, iterations generally reallocated only a small amount of contigs (less than 50).

To facilitate the necessary human decisions, we implemented the complete procedure in Java Swing (the "Metawatt binner") where the binning results are presented to the scientist as a graphical overview like the one displayed in figure 3.3. This enables the selection of promising bins for IMM modeling. We made use of the Batik library to enable the export of these graphics in SVG format which can be directly used for publications. The produced bins can be exported as fasta files for further annotation in standard pipelines.

There is certainly room for improvement. Perhaps the biggest step forward could be achieved by integrating the assembly and the binning. Assembly speeds may increase when the assembler can be provided with compositional information, to more efficiently recruit promising reads for comparisons. "Associations" between contigs (with paired end reads) that are too weak to allow assembly directly may still be used for binning, as was recently shown by Iverson *et al*. (2012). Unfortunately the latter study provided no methodological

details. Finally, next to sequencing coverage information, the frequency of single nucleotide polymorphisms may be used as an additional parameter to evaluate the binning results.

3.6 Conclusion

We have developed and implemented a (partially) new approach for the binning of metagenomic contigs. This approach was born out of need, existing approaches did not produce satisfying results for our metagenomes. Evaluation of the binning accuracy and recall was done with artificial as well as real metagenomes and showed that it was comparable to the best existing approach tested. In addition several key improvements were realized. Most notably, the seeding of the bins does not depend on an estimate of the number of binnable populations and is very fast and scalable. The approach has been implemented in Java SWING as an open source application (the “Metawatt binner”) with an easy to use graphical user interface. Evaluation of the binning results by BLAST, training of models and manual editing of bins is included in the implementation.

Our results show that the metagenomic binning of relatively simple microbial communities is currently feasible even when the sequencing effort is moderate. We also show that it is important to sequence and compare metagenomes for multiple samples of the same habitat. Continuous culture of microbial enrichment combined with metagenomic sequencing is a powerful approach that can carry the study of microbial physiology from pure cultures to simple communities. An accurate and easy to use binning procedure is an essential aspect of this change.

Acknowledgments

Marc Strous, Beate Kraft, and Regina Bisdorf were supported by ERC starting grant MASEM (242635). Halina E. Tegetmeyer was supported by a grant from the German Federal State of North Rhine-Westphalia. The generous support of the Max Planck Society is acknowledged. The help of Ines Kattelmann in DNA sequencing is gratefully acknowledged. We would like to thank Dr. Harald Gruber-Vodicka for the critical reading of the manuscript, Dr. Isaam Saeed for help with running the two-tiered binner scripts and the two reviewers for their critical and constructive comments.

Supplementary material

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Microbial_Physiology_and_Metabolism/10.3389/fmicb.2012.00410/abstract

Table S1 Representative reference genomes used for deriving the empirical relationships shown in figure 3.1. Online available at:
http://journal.frontiersin.org/Journal/10.3389/fmicb.2012.00410

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Chapter 4

4 Rapid succession of uncultured marine bacterial and archaeal populations in a denitrifying continuous culture

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Authors' contributions:

BK conceived and designed research together with MS. BK performed sampling, incubations, chemical analyses, DNA extractions and ARISA with support from JSG and MS. HET performed DNA sequencing and assembly of the metagenomes. MS and BK analyzed the metagenomes. BK performed the phylogenetic analyses. DM designed the archaeal FISH-Probe. The manuscript was written by BK with support from all other authors.

4.1 Summary

Marine denitrification constitutes an important part of the global nitrogen cycle and the diversity, abundance and process rates of denitrifying microorganisms have been the focus of many studies. Still, there is little insight in the ecophysiology of marine denitrifying communities. In this study, a heterotrophic denitrifying community from sediments of a marine intertidal flat active in nitrogen cycling was selected in a chemostat and monitored over a period of 50 days. The chemostat enabled the maintenance of constant and well-defined experimental conditions over the time-course of the experiment. Analysis of the microbial community composition by automated ribosomal intergenic spacer analysis (ARISA), Illumina sequencing and catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH) revealed strong dynamics in community composition over time, while overall denitrification by the enrichment culture was stable. Members of the genera *Arcobacter*, *Pseudomonas*, *Pseudovibrio*, *Rhodobacterales* and of the phylum Bacteroidetes were identified as the dominant denitrifiers. Among the fermenting organisms co-enriched with the denitrifiers was a novel archaeon affiliated with the recently proposed DPANN-superphylum. The pangenome of populations affiliated to *Pseudovibrio* encoded a NirK as well as a NirS nitrite reductase, indicating the rare co-occurrence of both evolutionary unrelated nitrite reductases within coexisting subpopulations.

4.2 Introduction

Currently, anthropogenic nitrogen input to the environment exceeds natural nitrogen fixation (Fowler *et al*., 2013). To understand the consequences of this aspect of global change, it is important to understand the fate of fixed nitrogen compounds such as nitrate and nitrite in the environment. Denitrification in marine coastal sediments contributes significantly to the removal of fixed nitrogen from the marine environment (Rao *et al*., 2007; Gao *et al*., 2012) and may be responsible for up to half of the marine export of fixed nitrogen to the atmosphere (Gruber, 2004). Especially, sandy coastal sediments are characterized by high-potential denitrification rates (Gao *et al*., 2010). Despite the important contribution of denitrifying microorganisms to biogeochemical cycling of nitrogen and carbon, the microbial community responsible for organic carbon turnover coupled to denitrification in coastal sediments remains understudied compared with sulfate reduction in deeper sediment layers (Llobet-Brossa *et al*., 2002; Gittel *et al*., 2008). The community composition of denitrifiers is

often determined via molecular methods targeting the functional genes of denitrification, which encode the nitrite reductases NirS or NirK, nitric oxide reductase NorB and nitrous oxide reductase NosZ (for review, see Kraft *et al*., 2011), but incongruence between the functional genes and 16S rRNA gene phylogenies makes it hard to link functional gene diversity to the microbial community structure (Jones *et al*., 2008).

Next to denitrification and aerobic respiration, fermentation is the main organic carbon degradation pathway in the upper layers of coastal tidal-flat sediments; fermenting organisms may even constitute the largest part of the anaerobic microbial community in marine sediments (Wilms *et al*., 2006, Köpke *et al*., 2005). They take part in the initial degradation of organic carbon substrates and provide a variety of fermentation products, which serve as substrate for denitrification and other terminal degradation processes (Schink, 2002).

Fermenting organisms are mostly facultative anaerobes; they are also able to respire oxygen, when it is available. Therefore, *in situ* detection of active fermenters is difficult and little is known about the ecophysiology of fermentative bacteria *in situ* and their potential interactions with respiratory heterotrophs such as denitrifiers. These potential interactions also play a role in the stability of ecosystem functions such as denitrification. It has been hypothesized that in communities with significant cross feeding, a high (background) diversity along with a dynamic population structure maintains functional stability (Briones and Raskin, 2003). However, it is difficult to directly observe syntrophic interactions between microbes in nature. Indeed, by direct determination of *in situ* microbial community composition, it can already be difficult to link specific phylotypes to specific processes or to correlate the abundance of specific phylotypes to specific environmental factors (Leser *et al*., 2002; Curtis and Sloan, 2004), because changes in microbial activity, abundance and community composition are generally caused by a multitude of interconnected environmental factors (Wallenstein *et al*., 2006).

Cultivation of natural microbial communities in continuous culture (e.g. chemostats) offers the possibility to monitor microbial community composition as well as the stability of specific processes (such as denitrification) under constant, environmentally relevant conditions. Continuous cultivation selects for a simplified microbial community that is optimally adapted to the applied conditions and may thus bridge the gap between direct ecosystem observations and pure culture studies.

In the present study, we cultivated a natural microbial community sampled from a sandy coastal sediment in continuous culture under stable, denitrifying conditions for 50 days. We

observed that while denitrification was stable, rapid microbial community succession occurred as shown by metagenomics and other methods.

4.3 Results

A chemostat was inoculated with biomass from a coastal intertidal-flat sediment and fed with a medium containing nitrite, nitrate and organic carbon in order to select for a nitrate-respiring microbial community. The organic carbon mixture (consisting of glucose, amino acids and acetate) represented the composition (monomers) of decaying biomass, the main carbon and energy source *in situ*. After an initial phase (8 days) in which the concentrations in the inflowing medium were gradually increased, nitrate and nitrite were completely depleted in the culture liquid (figure 1A).

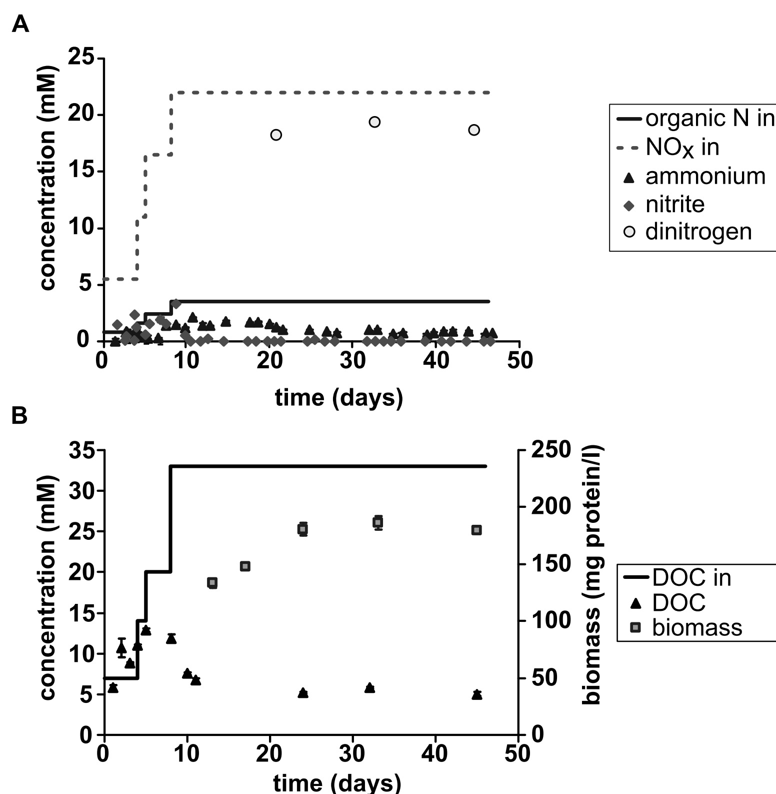

Figure 4.1: Conversion of (A) nitrogen and (B) carbon compounds during continuous culture enrichment. The solid lines represent the concentration of compounds in the medium supplied. The symbols represent the concentrations measured in the culture. DOC, dissolved organic carbon; N, nitrogen. Error bars indicate the standard deviation. For nitrite concentrations, error margins are within the sizes of the symbols.

From this day onwards, the nitrate and nitrite concentrations in the culture remained in the low micromolar range, close to the conditions *in situ* (< 0.5 μM for nitrite, < 10 μM for nitrate). Dinitrogen was identified as the main product and thereby denitrification as the main nitrate-reducing process. The ammonium concentrations were between 0.7 mM and 2 mM. Because a complete degradation of the provided amino acids in the medium would lead to an ammonium concentration of 3.5 mM, the produced ammonium most likely originated from the degradation of amino acids. Even if most of the ammonium produced resulted from the reduction of nitrite to ammonium, this pathway would have been responsible for less than 5% of the nitrite reduction. The concentration of dissolved organic carbon remaining in the culture was constant at 5.3 ± 1 mM (figure 1B). The protein content of cell biomass increased until day 24 and then stabilized at 180 ± 3 mg protein l^{-1}. This corresponded to a biomass yield of 0.36 ± 0.035 C-mol biomass/C-mol substrate. Overall, the continuous culture showed stable conversion of substrates.

Four time points (day 6, 18, 33 and 46) were selected for metagenomic sequencing with an Illumina GA IIx. Between 3.2 and 8.3 million reads per sample were generated (Supporting Information Table S1). Binning of assembled contigs of the four metagenomic samples resulted in the identification of 14 different bins, each associated with a different population. The position of each bin on a coverage versus GC plot of the assembled contigs as well as the taxonomic profiles of the bins are shown in Supporting Information figure S4.1. For almost all bins, corresponding full-length 16S rRNA genes were obtained by *de novo* assembly and iterative read mapping. The phylogenetic affiliation of the 16S rRNA sequences is shown in Supporting Information table S4.2 and figure S4.2. 16S rRNA sequences could be linked to the corresponding bins based on consistent phylogeny between the 16S rRNA gene and the sequence data of each bin and consistent squared Pearson productmoment correlation coefficients between bin coverages and 16S rRNA gene coverages over the four samples (Supporting Information table S4.3). Generally, high correlations between the coverage of a bin and the corresponding 16S rRNA gene were observed. Only bin F and I had low correlation coefficients. Together with the taxonomic profiles of the bins (Supporting Information figure S4.1), this indicated misbinning of contigs from bin E into F [a rather incomplete bin estimated based on the presence of conserved single copy genes (CSCGs), table 4.1] and of bins G and H into I.

The abundance of each of the bins in every sample was estimated by mapping the reads of every sample to the contigs of every bin. Fifty to 85% of the sequence data could be

mapped to the assembled contigs and 35–80% was mapped against contigs that were part of one of the bins. Bin sizes and estimated genome completeness are shown in Table 1. The differences in relative abundances between samples indicated a highly dynamic community with drastic changes from one time point to the next (figure 4.2B).

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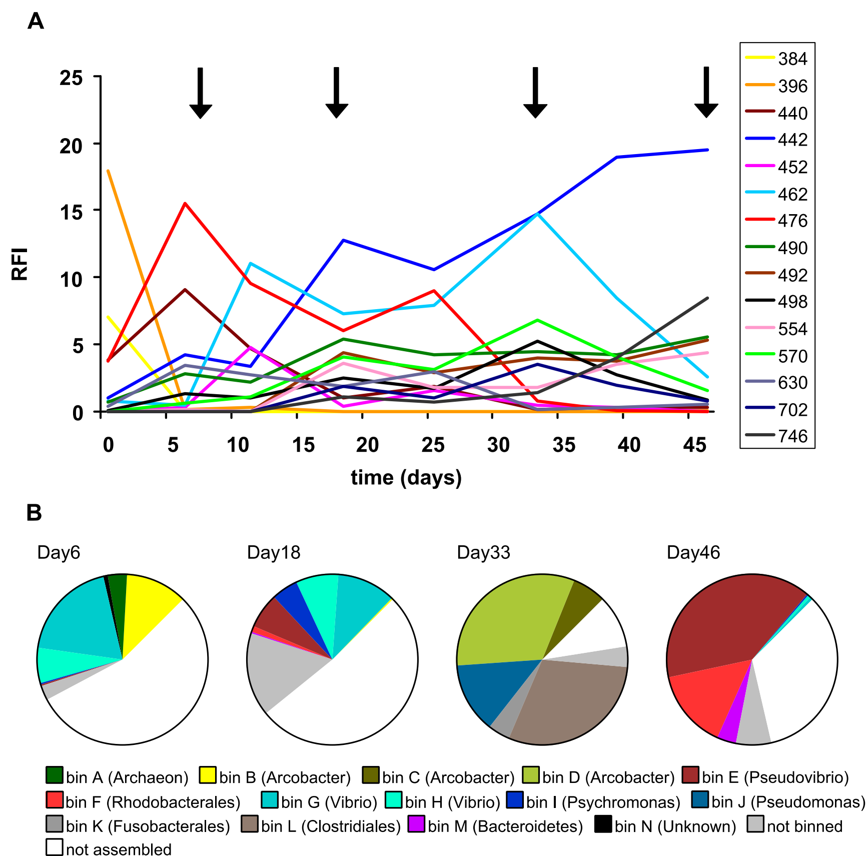

Figure 4.2: Dynamics of the microbial community. A. Automated ribosomal intergenic spacer analysis (ARISA) pattern over time of the 15 most abundant OTUs. OTUs with the highest overall abundance are depicted. Arrows indicate time points of metagenomic sequencing and CARD-FISH. RFI, relative fluorescence intensity. B. Relative abundance estimates of metagenomic bins obtained by read mapping at different time points.

The main bins present in the first sample were affiliated with *Arcobacter* (bin B), *Vibrionales* (bin G and bin H) and Archaea (bin A). The closest relatives based on the 16S rRNA gene are given in Supporting Information Table S2. The bins G and H (*Vibrionales*) were also recovered in the second sample (day 18) together with two bins affiliated with Alphaproteobacteria (bin E and bin F) and a bin affiliated with *Fusobacter* (bin K). On day 33, none of the bins of the previous sample was detected. Bins C and D (*Arcobacter*) predominated, accompanied by bin I (affiliated with *Psychromonas*), bin J (affiliated with

Table 4.1: Characteristics and inferred metabolism of the metagenomic bins.

Bin	Phylogenetic affiliation	CSCGs[1] (duplicates)[2]	Bin size (Mb)	N50 contig length	Denitrification genes					Fermentation genes	Inferred lifestyle
					napA	*narG*	*nirS/ nirK*	*norB*	*nosZ*		
A	**Archaeon**	**43 (0)**	**1.54**	**28074**	-	-	-	-	-	***aadh, dld***	**FERM**
B	**Arcobacter**	**65 (1)**	**3.08**	**5643**	+	-	***nirS***	+	+	***adh, aadh, pta, ackA***	**DEN**
C	**Arcobacter**	**111 (0)**	**2.51**	**51624**	+	+	***nirS***	+	+	***pta, ackA***	**DEN**
D	**Arcobacter**	**85 (1)**	**2.89**	**8992**	+	-	-	-	-	***adh, aadh, pta, ackA***	**FERM**
E	**Pseudovibrio**	**117 (84)**	**5.18**	**719**	+	+	***nirS, nirK***	+	+	***pfl, adh, aadh, ackA, dld***	**DEN**
F	**Rhodobacterales**	**21 (7)**	**1.45**	**543**	+	-	***nirS***	+	+	***aadh, pta, dld***	**DEN**
G	**Vibrio**	**131 (15)**	**4.26**	**1594**	+	-	-	-	-	***pfl, adh, aadh, pta, ackA, dld***	**FERM, NO_3-RED**
H	**Vibrio**	**119 (57)**	**3.20**	**622**	+	-	-	-	-	***pfl, adh, aadh, pta, ackA, dld***	**FERM, NO_3-RED**
I	**Psychromonas**	**39 (3)**	**1.78**	**685**	-	-	-	-	-	***pfl, adh, aadh, ackA, pta, dld***	**FERM**
J	**Pseudomonas**	**125 (3)**	**2.63**	**1171**	-	+	***nirS***	+	+	***adh, aadh, pta, ackA***	**DEN**
K	**Fusobacterales**	**130 (4)**	**2.63**	**4592**	-	-	-	-	-	***pfl, adh, aadh, pta, ackA, dld***	**FERM**
L	**Clostridiales**	**132 (4)**	**3.45**	**37879**	-	-	-	-	-	***pfl, adh, aadh, pta, ackA, dld***	**FERM**
M	**Bacteroidetes**	**72 (6)**	**2.26**	**536**	-	+	***nirS***	-	-	***aadh, pta***	**part. DEN**

Pseudomonas) and bin L (affiliated with *Clostridiales*). On day 46, bins E and F (*Rhodobacterales*) were detected again, accounting together for more than 50% of the sequenced reads. Furthermore, bins affiliated with *Vibrio* and Bacteroidetes (bins H and M) were obtained

To independently validate the composition of the microbial community, catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH) was performed for the most abundant populations (class and genus level) at the same time points as metagenomic sequencing was performed (Table 4.2). An increase in relative abundance of *Rhodobacterales* and a decrease in the fraction of Gammaproteobacteria, particularly *Vibrios*, and Epsilonproteobacteria (*Arcobacter*) during the course of the experiment supported the metagenomic results. Only for day 33, a major difference between the community composition of the CARD-FISH and metagenome data was observed. Generally, the relative abundance of Gammaproteobacteria, particularly *Vibrio* and Epsilonproteobacteria, was overestimated and the relative abundance of Alphaproteobacteria was underestimated by metagenomic abundance estimates based on coverage. However, the overall community dynamics detected by CARD-FISH followed the same trend as the metagenomic data.

Table 4.2: Relative abundance of different phylogenetic groups determined by CARD-FISH with class and genus specific probes.

	day 6	**day 18**	**day 33**	**day 46**
Gammaproteobacteria	33	30	22	12
Vibrio	27	13	10	3
Alphaproteobacteria	46	60	67	87
Roseobacter	49	55	58	84
Epsilonproteobacteria	19	1	12	0
Arcobacter	18	1	1	0

Five bins (B, C, E, F and J) contained the genes necessary for complete denitrification (Table 1). In addition, bin M contained the genes for a part of the denitrification pathway. Interestingly, in bin E (*Pseudovibrio*) genes for the two types of nitrite reductases, *nirK* and *nirS*, were present. The nirS gene had 95% identity to *nirS* from *Pseudovibrio* sp. FO-BEG1. The nirK gene was most closely related to *nirK* genes of other *Rhodobacterales* (63%, *Roseobacter sp*. SK209-2-6; 61%, *Phaeobacter gallaeciensis*). On the contig with the Roseobacter-related nirK gene, we also detected a gene coding for a transposase that had

93% identity to *Pseudovibrio sp*. FO-BEG1. The consistent affiliation of the whole contig with the order Rhodobacterales independently supported that it belonged to bin E. Phylogenetic analysis showed clustering of the nirS sequence together with other alphaproteobacterial *nirS* gene sequences (Supporting Information figure. S4.4). The presence of a gene encoding a transposase suggested that *nirK* might possibly have been acquired by horizontal gene transfer. The phylogenetic analysis of the *nirK* sequence is depicted in Supporting Information figure S4.5.

Two of the three bins affiliated with the genus *Arcobacter* (bin B and C) contained the complete denitrification pathway, whereas bin D did not (table 4.1). Instead, it contained genes for dissimilatory nitrate reduction to ammonium (*napA* and *nrfA*). However, the low concentration of produced ammonium indicated that the activity of this pathway was low compared with denitrification (at most < 5%). Bins B and D both contained a *soxCDXYZAB* operon and thus showed the potential for the complete oxidation of sulfide to sulfate. Bin E (*Pseudovibrio*) also contained genes for sulfide oxidation (*soxB, D, X, Z* and *W*), but these were distributed over different contigs.

In bins D, G, H, I, K and L (affiliating with *Arcobacter*, *Vibrio*, *Psychromonas*, *Fusobacter* and *Clostridiales*), the presence of different sets of genes being associated with a fermentative lifestyle (table 4.1) suggested mixed acid fermentation as the metabolism. Bin K (*Fusobacter*) also contained genes encoding capacity for sulfite reduction (*dsrABC*). The abundance of populations associated with denitrification and fermentation appeared to be relatively constant during the first three time points (days 6, 18 and 33). Only at day 46, denitrifiers seemed to be more abundant.

At the beginning of the experiment, we observed the transient enrichment of a bin related to uncultured Archaea (bin A). Bin A (1.54 Mb, 10× coverage) consisted of 107 contigs and constituted 4% of the assembled sequence data of the first sample. The archaeal 16S rRNA gene shared 90% sequence identity with environmental clones and 74% with members of the Methanobacteria, the most closely related cultured Archaea. Because the phylogeny of the archaeon was not well resolved based on the 16S rRNA gene (Supporting Information figure S4.3), a more in-depth phylogenetic analysis was based on 44 concatenated CSCGs, according to Lloyd and colleagues (2013). This showed that the archaeal population clustered within the deeply branching newly proposed DPANN superphylum (Rinke *et al*., 2013), with Nanoarchaeum equitans being the closest relative

(figure 4.3). The genome size was estimated to be approximately 1.6 Mbp and the genome completeness to be 98% (table 4.11).

The relative abundance of the archaeon estimated by metagenomics was confirmed by CARD-FISH. Cells hybridizing to the newly designed probe specific for this group of Archaea (Supporting Information Table S4) accounted for 5% of all 4,6-diamidino-2-phenylindole counts at day 6. Cells had a coccoid shape with a diameter of 0.5–0.8 μm (figure 4.4).

For bin A, we observed that the genes for RNA polymerase A and B were both split into two parts. This was also observed previously for N. equitans (Spang *et al*., 2010). A split RNA polymerase subunit B has so far been considered to be a characteristic only present in Euryarchaea (Brochier *et al*., 2004; Spang *et al*., 2010). Furthermore, topoisomerase type IIa subunits A and B were present, which also have been assumed to exist within the Archaea exclusively in Euryarchaea (Spang *et al*., 2010).

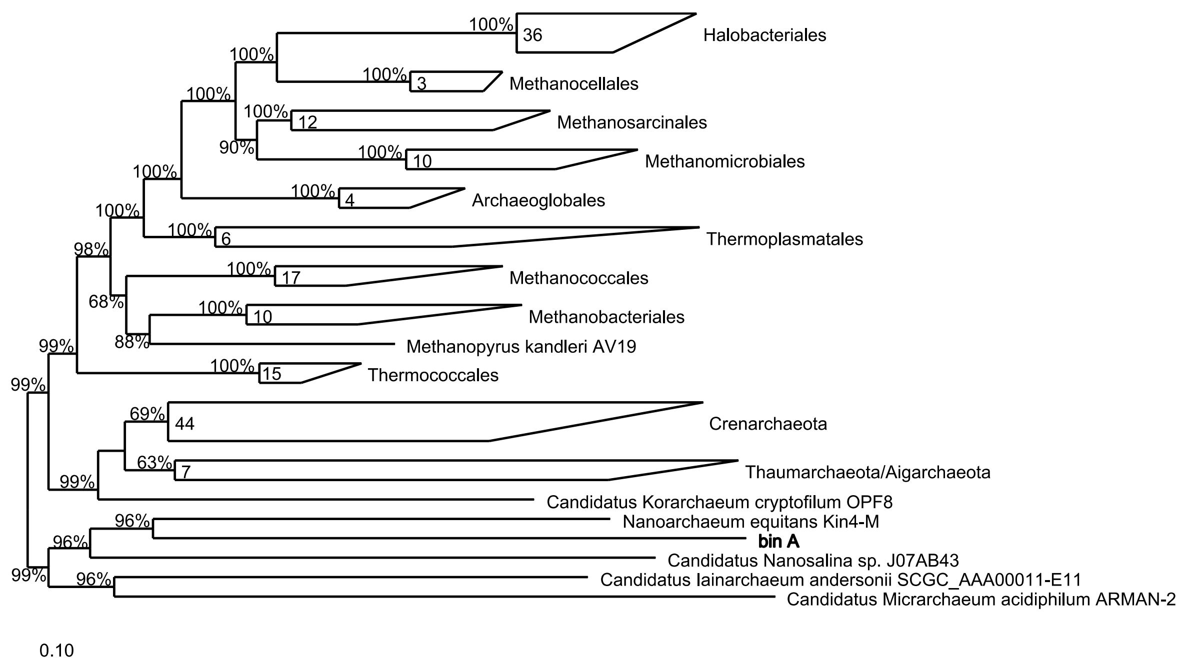

Figure 4.3: Phylogenetic placement of bin A within the archaeal DPANN superphylum. Maximum likelihood tree of concatenated conserved single copy genes (RAxML). Bin A is in bold font. Bootstrap values higher than 50% are given.

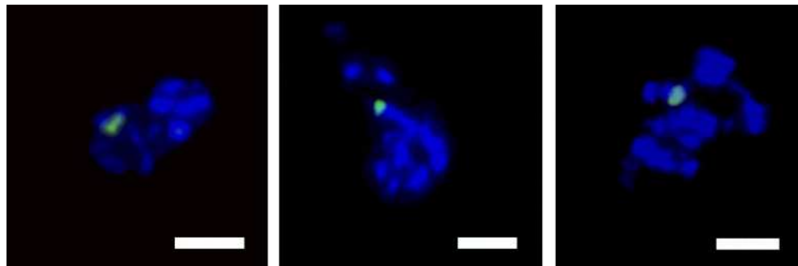

Figure 4.4: Epifluorescence microscopy image (CARD-FISH) of the archaeal population present in the enrichment culture at day 6. Green: archaeal cells hybridized with probe Darch335; blue= DAPI-stained cells. The scale bar corresponds to 2 μm.

In bin A, genes were present that encode a complete glycolysis pathway and a lactate dehydrogenase gene. Bin A also contained an *arcABCD* gene cluster encoding the arginine deiminase pathway. The presence of these genes suggests a fermentative lifestyle by sugar and amino acid fermentation. Consistently, the F-type (proton pumping) ATPase and respiratory complexes were not encoded in this bin and the tricarboxylic acid cycle was incomplete. The *arcABCD* genes were most closely related to homologues previously found in Clostridia and not to archaeal homologues. Also, the gene encoding a putative D-lactate dehydrogenase was most closely related to bacterial genes (*Sphaerobacter thermophilus*). The contigs that contained these genes generally also contained conserved genes that were most closely related to homologues of archaeal origin. Genes encoding the complete glycolysis pathway and an incomplete pentose phosphate pathway were related to homologues of either bacterial or archaeal origin. Because of the large phylogenetic distances, a more detailed phylogenetic analysis would be required to trace the origins of these genes more robustly. Finally, a muramoyltetrapeptide carboxypeptidase, which functions in bacterial cell wall lysis, is present. A RelA- and SpoT-like ppGpp synthetase (signalling molecules involved in bacterial stringent response) was also found in the archaeal bin. This is the third report of an archaeon that possesses complete multi-domain alarmones, with all of them being members of the DPANN superphylum (Rinke *et al*., 2013).

4.4 Discussion

Rapid succession of microbial populations was observed despite the maintenance of stable conditions in the continuous culture. These populations represented populations also observed in situ. For example, *Rhodobacterales* were found to make up 10%, Bacteroidetes

5–25% and *Arcobacter* ~ 1% of the microbial community on the sampled tidal flat (Llobet-Brossa *et al*., 1998; Stevens *et al*., 2008; Lenk *et al*., 2011). Members of the genus *Psychromonas* have previously been identified as important glucose fermenters in sediments from the sampling site by RNA-based stable isotope probing (Graue *et al*., 2011). Based on different sets of genes involved in fermentation that were detected in the different bins, we inferred the potential production of fermentation products such as lactate, hydrogen, formate and acetate. Such compounds would serve as suitable substrates for the part of the community that coupled denitrification to heterotrophic oxidation of monomeric carbon substrates.

The enriched community had the potential for cryptic sulfur cycling with the involvement of Epsilonproteobactria in the sulfide oxidation step. Members of the genus *Arcobacter* have frequently been associated with autotrophic denitrification coupled to sulfide oxidation in marine environments (Lavik *et al*., 2009; Fuchsman *et al*., 2012).

Generally, in marine habitats, the nitrite reductase NirS is considered to be more common than NirK (Abell *et al*., 2010; Jones and Hallin, 2010). This was supported by our study. With our metagenomic approach, we could exclude that the overrepresentation of nirS with regard to nirK results from a bias based on the available primers, which have been shown to fail to detect the nitrite reductases of a great proportion of cultivated denitrifiers (Heylen *et al*., 2006). Interestingly, the bin E (*Pseudovibrio*) not only contained a *nirS* gene but also a *nirK* gene. The presence of genes for both types of nitrite reductases within one organism is very rare (Jones and Hallin, 2010). Bin E (*Pseudovibrio*) contained a relatively large amount of unique sequence data in relatively small contigs and a high number of duplicated CSCGs (Table 1). Together, these observations suggested the presence of multiple subpopulations and that the different nitrite reductase genes might be present in different, closely related populations. The co-enrichment of two closely related subpopulations with different types of nitrite reductases is remarkable as bacteria carrying different Nir types have been suggested to occupy different ecological niches (Jones and Hallin, 2010).

Interestingly, the applied selective pressure did not lead to the enrichment of one single dominant denitrifier but to the co-enrichment of different denitrifying microbes and their succession over time. With different independent methods, we showed that strong shifts in the community composition occurred while the overall performance of the culture was stable. A similar continuous cultivation experiment carried out in parallel (at the same time and with the same inoculum) showed similar ongoing dynamics over an even longer time scale (350

days): strong fluctuations in populations affiliated with *Fusobacter*, *Clostridia* and *Vibrio* and succession of populations affiliated with *Pseudomonas*, *Arcobacter* and *Rhodobacter* (Strous *et al*., 2012). This experiment was performed with a higher ratio of inflowing organic carbon to nitrate and nitrite, which suggests that the observed population dynamics are reproducible even when different conditions are applied. Furthermore, similar dynamics of a functional stable methanogenic community have been described by Fernández and colleagues (1999).

In the present study, functional stability was demonstrated not only in the conversion of substrates, but also in the presence of different populations that possessed the same metabolic potential at all time points. Based on basic kinetic considerations, one could expect the constant build-up of the most successful population under the given conditions (Veldkamp and Jannasch, 1972). Once the fittest population established the highest cell density, it is hard to outcompete and we would expect the stabilization of one dominant population. Because in our experiments we supplied a variety of organic carbon substrates supporting different metabolisms, we potentially created multiple niches for several coexisting populations. Nevertheless, stability of the community composition would still be expected unless other processes controlling population abundance come into play (Waters *et al*., 2013).

Complex mutualism such as cross feeding or antagonistic behaviour such as competition and production of toxins (Huisman and Weissing, 1999; Czárán *et al*., 2002; Dethlefsen *et al*., 2006; Cordero *et al*., 2012) are likely drivers of population dynamics. If the community structures observed at different time points were characterized by specific interactions between specific populations (e.g. obligate cross feeding of specific fermentation products), a change in the abundance of one population would have consequences for other interacting populations. Functional stability could then only be maintained if the community composition would change substantially, as observed in this experiment. In fact, chaotic behaviour of community composition has been hypothesized to be important for maintaining functional stability of complex food webs (Graham *et al*., 2007; Benincà *et al*., 2008). Finally, viruses could also be important drivers causing turnover of dominant populations according to the 'killing the winner' hypothesis (Rodriguez-Brito *et al*., 2010; Shapiro *et al*., 2010).

The strength of the applied selective force can influence the reproducibility of the final community (Pagaling *et al*., 2013). Thus, the nature of the selected metabolism could define the strength of dynamics that occur. For example, specialized aromatic hydrocarbon-

degrading communities have been shown to produce stable communities (Massol-Deya *et al*., 1997). Furthermore, already preadapted communities (communities originating from a habitat that better resembles the conditions in the continuous culture) may show a different community development compared with not adapted communities (Pagaling *et al*., 2013). Thus, we cannot exclude that different source communities might exhibit different dynamics. Multiple repetitions of continuous culture experiments with inocula from multiple sampling sites may help to identify such patterns.

It appears that community composition was governed by niche-based selection as well as neutral processes. Niche-based species sorting led to the selection of denitrifying and fermenting populations, while it seems that neutral processes were responsible for the ongoing dynamics among these populations because stable ecosystem functioning throughout the experiment suggests that the changes in community composition can be regarded as neutral.

Although the general trend in changes over time was consistent for metagenomic and CARD-FISH data, the different approaches sometimes yielded different estimates of community composition. Data obtained by ARISA also showed ongoing dynamics, but it was not possible to link the ARISA results to the other results because the intergenic spacer regions were not assembled properly. However, the results obtained by ARISA and FISH both suggested that some populations only increased in abundance with time whereas the metagenome suggested more complex population dynamics. This might be explained by a variable degree of micro-diversity leading to poor assembly for some samples (Albertsen *et al*., 2012). Furthermore, differences could be explained by different potential biases of the respective methods such as cell lysis and DNA extraction efficiency (Zhou *et al*., 1996) or cell permeabilization efficiency for different microbial populations (Amann and Fuchs, 2008). For ARISA, different rRNA gene copy numbers and polymerase chain reaction (PCR) amplification biases may also lead to artefacts in populationabundance estimates (Crosby and Criddle, 2003).

Among the fermenting organisms was a transiently enriched newly discovered archaeal population. We hypothesize a fermentative lifestyle based on the presence of genes for glycolysis, lactate dehydrogenase and the arginine deiminase pathway, and the absence of genes that could support respiration. This so far unknown archaeon falls into the newly proposed DPANN superphylum (Rinke *et al*., 2013). So far, this superphylum consisted of a few very distantly related extremophiles, which mostly have been recently discovered by

metagenomics or single-cell sequencing. *Nanoarchaeum equitans* is the only cultured member so far (Huber *et al*., 2002) and Archaeal Richmond Mine acidophilic nanoorganisms Archaea have been recently enriched (Ziegler *et al*., 2013). With its origin from coastal sediments of the North Sea, the archaeal population from this study is the only member of this superphylum that does not originate from an extreme habitat. After the addition of the enriched archaeon to a multi-protein tree, the monophyly of the DPANN superphylum was reproduced, even though different sets of CSCGs were used for the phylogenetic analysis (approximately 50% overlap). This finding supported the status of DPANN as a superphylum. The topology within the superphylum was not consistent with Rinke and colleagues (2013). This is probably due to the different selections of CSCG used for the analysis in combination with the very distant relationships between different members and the fact that only few representatives were available for the analysis. All the members of the DPANN superphylum so far are characterized by small genome size and this was proposed to be a defining trait for these Archaea (Baker *et al*., 2010; Narasingarao *et al*., 2012; Rinke *et al*., 2013). With an estimated genome size of 1.6 Mbp, our novel archaeum sets a new upper limit for this archaeal group. A split of polymerase subunit B has so far been assumed to have occurred only once in the archaeal domain, after the divergence of the *Thermococcales* (Brochier *et al*., 2004; Spang *et al*., 2010). The observed split RNA polymerase subunit B and the presence of topoisomerase type IIa subunit A and B show that members of the DPANN superphylum possess characteristics that have so far been believed to be exclusively euryarchaeal. A systematic study of the presence of such features in members of the DPANN superphylum in comparison with other archaeal phyla may give new insights into phylogeny and evolutionary history of Archaea.

In conclusion, continuous cultivation allowed us to select for environmentally relevant denitrifying and fermentative populations, among them a novel member of the archaeal DPANN superphylum. The community was highly dynamic despite stable conditions, indicating that for functional stability; only the presence of the overall metabolic potential is important, independent of the microbial community composition.

4.5 Material and Methods

Sediment collection

Sediment was collected from the back barrier tidal flat Janssand (N53°44.151' and E07°41.945') located in the German Wadden Sea, in January 2011 at low tide. The upper two centimeters of the tidal flat were sampled with a flat trowel.

Continuous cultivation

The sediment was mixed vigorously in a ratio of 1:1 with red sea salt medium (33.4 g/l; Aquaristic.net). After the sediment grains had settled the overlying suspension was used as inoculum for the continuous culture. Cycloheximide (50 mg/l) was added to prevent eukaryotic growth and thus protect bacteria from being grazed. Continuous cultivation was performed in a 3 l glass vessel at controlled temperature (20±1°C). Electron donors and acceptors were separated in an acidic and an alkaline medium, which were pumped into the vessel at a ratio of 1:1 and a pump speed of 1.5 ml/min each (overall dilution rate 1.6 d^{-1}). This dilution rate was chosen according to an estimate of the average growth rate of the microbial community of the sampling site based on codon usage bias in the sampling site's metagenome (Accession number: SRP015924; Marchant *et al.*, submitted) according to Vieira-Silva and Rocha (2010).

The pH of the two media were adjusted in such a way that the resulting pH was 8 ± 0.2 in the culture. Substrate concentrations in the media were gradually increased. The final composition of the acidic medium (pH=2.2) was (g/l): Red Sea Salt (Aquaristic.net) 66.8, $NaH_2PO_4.1H_2O$ 0.276, glucose 0.870, acetate 0.204, glutamic acid 0.207, aspartic acid 0.254, alanine 0.166, serine 0.107, tyrosine 0.118, histidine 0.023, methionine 0.048 and trace element solution 0.5ml/l. The trace element solution contained (g/l): $FeSO_4.7H_20$ 0.21, H_3BO_3 0.03, $MnCl_2.2H_20$ 0.1, $CoCl_2.6H_20$ 0.12, $NiCl_2.6H_20$ 0.024, $ZnCl_2$ 0.07, $Na_2MoO_4.2H_20$ 0.036, $CuSO_4.5H_2O$ 0.015. The alkaline medium (pH= 12.2±0.2) contained (g/l): $NaNO_2$ 2.76 and $NaNO_3$ 0.34. Medium pH was adjusted with HCl or NaOH. The liquid volume in the culture vessel was 2.8 l. The pH and oxidation/reduction potential were monitored by gel-filled electrodes with an Ag/AgCl reference system (Mettler-Toledo, Giessen, Germany). To maintain anaerobiosis the culture was continuously flushed with argon (purity >99,998 %, Air Liquide, Germany) at a flow rate of 10 ml/min dosed by a mass flow controller (Alicat Scientific, USA). The culture was mixed by pumping (1.2 l/min;

pump: Watson Marlow 620S, USA) gas from the headspace through a sintered glass membrane inserted into the bottom of the culture vessel, from where it bubbled through the culture. To prevent growth of photosynthetic organisms, the culture vessel was covered with a neoprene cover.

Analytical procedures

Nitrite, nitrate, ammonium and dissolved organic carbon concentrations and protein content in the enrichment culture as well as dinitrogen production were measured as described in the supplementary information.

Catalyzed Reporter Deposition Fluorescence In Situ hybridization (CARD-FISH)

CARD-FISH was performed as described in the supplementary information.

Automated ribosomal intergenic spacer analysis (ARISA)

DNA for ARISA was extracted using the protocol of Martin-Platero *et al* (2007). The quantity of DNA was set to 10 ng per PCR amplification (quantified by a ND-1000 Nanodrop). The ARISA, including evaluation of the raw fingerprint profiles and binning into operational taxonomic units (OTUs), was performed as described by Ramette (2009). Primers (ITSF/ITSReub) are listed in supplementary table S4.

Sequencing, assembly, binning and annotation

DNA extraction and sequencing was performed as described in the supplementary information. Assembly was performed with MetaVelvet-v0.3 (supplementary table S2). Binning of assembled contigs was performed with the Metawatt binner according to Strous *et al* (2012). More detailed information about the assembly and binning and information about full length 16S rRNA reconstruction and phylogenetic analyses are described in the supplementary information.

Data submission

Illumina sequencing data sets were submitted to the Short Read Archive (http://www.ncbi.nlm.nih.gov/Traces/sra/), and the assembled contigs were submitted to the Whole Genome Shotgun Submission Portal (https://submit.ncbi.nlm.nih.gov/subs/wgs/).

Both reads and contigs are accessible under the BioProject PRJNA226580 and the BioSamples: SAMN02391470 / SRS498469 (day 6), SAMN02391471 / SRS498472 (day 18), SAMN02391472 / SRS498471 (day 33), SAMN02391473 / SRS498488 (day 46). This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accessions AYRE00000000, AYRF00000000, AYRG00000000, AYRH00000000. The version described in this paper is version AYR(E-H)01000000.

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4.7 Supporting Information

Supplementary Methods

Analytical procedures

Nitrite and nitrate concentrations in the enrichment culture were measured daily with Quantofix test strips (0–80 mg/l NO_2^-, 0-500 mg/l NO_3^-; Merck, Germany). Every two to three days the concentrations of nitrite and ammonium in the culture liquid and the medium were measured colorimetrically: Nitrite was measured at 540 nm after a 20 min reaction of 1 ml sample with 0.25 ml of 1% sulfanilic acid and 0.05 % N-naphthylethylenediamine in 2.2 M H_3PO_4 (Van Eck, 1966). Ammonium was measured at 630 nm after a 1h reaction of 1 ml sample with 40 µl of 10% phenol in ethanol (95%), 40 µl of 0.5% sodium nitroprusside in water and 100 µl of 3% hypochlorite in an alkaline trisodium citrate solution (0.6 M) (Solorzano, 1969). Dinitrogen production was measured online with a GAM 400 mass spectrometer (In Process Instruments, Germany) supplied with gas from the headspace at a rate of 4 ml/min. For determination of the protein content, 0.5 ml of culture was centrifuged at 14000x *g* for 5 min. Biomass pellets were suspended in 1 ml 1 M NaOH and incubated at 60°C for 45 min. After cooling, the protein content was determined colorimetrically at 750 nm according to Lowry *et al*. (1951). The dissolved organic carbon concentration in the inflowing medium was obtained by measuring total organic carbon (TOC). The dissolved organic carbon (DOC) of the culture liquid was calculated from the chemical oxygen demand (COD). TOC and COD were measured with cuvette-tests (Hach Lange GmbH, Düsseldorf, Germany) using a Thermostat LT200 and a DR3900 photometer (Hach Lange GmbH, Düsseldorf, Germany).

Catalyzed Reporter Deposition Fluorescence In Situ hybridization (CARD-FISH)

Cells were fixed in 1 % formaldehyde overnight at 4°C and subsequently washed three times with PBS (pH 7.4). Cells were stored in a 1:1 PBS:ethanol mixture at -20°C. CARD-FISH was performed on 0.2 µm pore-sized polycarbonate filters followed by fluorescently labeled tyramide signal amplification as described by Pernthaler *et al*. (2002). Horseradish peroxidase labelled oligonucleotide probes used and hybridization conditions are listed in supplementary table S4. Filters were counter-stained with DAPI (4′,6-diamidino-2-

phenylindole) and counted manually with an epifluorescence microscope (Axioplan 2, Carl Zeiss, Jena, Germany). Probe MDarch335 (Marine DPANN archaeon 335) was designed using ARB (Ludwig *et al.*, 2004) and the SILVA 16S rRNA SSU Ref 115 and checked for specificity against the ARB-SILVA SSU Reference database (release 115) (http://www.arbsilva.de) using 'TestProbe'.

DNA extraction and Sequencing

Samples of four different time points (day 6, 18, 33 and 46) with a volume of 14 ml were centrifuged for 20 min at 4700x *g* and pellets were stored at –80°C until further processing. DNA was extracted according to Zhou *et al.* (1996) after incubation for 30 min at 37°C with 2.5 mg/ml lysozyme, 0.1mg/ml RNAse and 100U/ml mutanolysin. Barcoded Illumina TruSeq libraries were generated and sequencing was performed (together with 8 further libraries not part of the present study) on one flowcell lane of an Illumina Genome Analyzer GA IIx instrument, in a 2x150 cycles paired-end run.

Assembly, binning and annotation

Assembly was performed with MetaVelvet-v0.3 (supplementary table S1). After quality trimming (sliding window approach: window length 15 bp, within this window quality value of at least 99 %, minimal read length after trimming: 25 bp), between 9.7 and 23.4 Mb sequenced library were assembled.

Binning of assembled contigs was performed with the Metawatt binner based on multivariate statistics of tetranucleotide frequencies combined with Interpolated Markov Models according to Strous *et al.* (2012). Briefly, 14 bins were created based on Interpolated Markov models trained with bins based on tetranucleotide composition. Per contig sequencing coverage was estimated by mapping the reads to the assembled contigs with bowtie2 (Langmead & Salzberg, 2012) and coverage and bin size were used to estimate the abundance of each binned population. Genome completeness was estimated for each bin as described by Campbell *et al.* (2013). The contigs of each bin were annotated separately with Prokka (http://vicbioinformatics.com), RAST (Aziz *et al.*, 2008) and KAAS (Moriya *et al.*, 2007). The archaeal bin was additionally annotated using GenDB. Full length 16S rRNA gene sequences were obtained by searching the assembled contigs with a custom hidden Markov Model (Eddy, 2011) trained with representative 16S rRNA gene sequences from the SILVA database (Quast *et al.,* 2013) and, independently, by iterative read mapping with

Emirge (Miller *et al.*, 2011). Phylogenetic trees of 16S rRNA sequences were calculated by maximum likelihood (RAxML). Bootstrap values were generated from 100 replicates.

Phylogenetic analysis

For the archaeal ribosomal protein tree we used the alignment of 44 conserved single copy gene amino acid sequences of 171 completely finished archaeal genomes from Lloyd *et al.* (2012). The corresponding sets of sequences from our archaeal bin and 4 representatives of the DPANN superphylum were added to the alignment with MAFFT (Katoh *et al.*, 2002). A 30% positional conservatory filter was applied and a Maximum likelihood tree was calculated using RAxML-HPC2 (Stamatakis *et al.*, 2008) with the JTT protein evolution model (Whelan and Goldman, 2001) as provided by the CIPRES cluster at the San Diego Supercomputing Center (http://www.phylo.org/; Miller *et al.*, 2010) according to Lloyd *et al.* (2012). The Maximum likelihood tree for nirS and nirK amino acid sequences were calculated using RAxML with the JTT protein evolution mode.

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Table S4.1 Assembly results of the four sequenced metagenomes.

Sampling day	**day 6**	**day 18**	**day 33**	**day 46**
Number of reads (millions)	8.1	3.2	8.4	4.1
Total sequence data in reads (Mb)	689	245	783	288
Number of contigs (thousands)	10.0	123.0	6.5	54.0
Total sequence data in contigs (Mb)	9.7	23.4	14.6	15.1
Longest contig (kb)	82	13	143	11
N50 contig length (kb)	2.8	0.2	9.6	0.3
K-mer size for assembly	51	41	61	51

Table S4.2 Closest relatives (based on BLAST) to reconstructed 16S rRNA gene sequences.

Bin	Closest related sequence	Accession code	Isolation source	Sequence Identity (%)
A	Uncultured euryarchaeote clone	EU731037	Hypersaline microbial mat	90
	Methanobacterium sp. AL-21	NR102889		74
B	*Arcobacter* sp. MA5	AB542077	Gut of abalone	99
C	Uncultured bacterium	AJ853504	Leachate of municipal solid waste landfill	99
	Arcobacter sp. CpA_a5	FN397893	Marine sub-surface mud volcano sediments	99
D	*Arcobacter marinus* strain CL-S1	EU512920	Marine	98
E	*Pseudovibrio japonicus* strain WSF2	NR_041391	Coastal seawater	99
F	Uncultured bacterium clone	KC631560	Sediment of finfish aquaculture farm	100
G	*Vibrio splendidus* strain W676	JF836194	Marine sponge	100
H	*Vibrio splendidus* strain W676	JF836194	Marine sponge	100
I	*Alteromonas sp*. KT1114	AF235108	North Sea	98
J	*Pseudomonas sp*. C27	GQ241351	Wastewater	99
K	Fusobacteria bacterium Ko711	AF550592	Extinct smoker pipe, Iceland	99
L	Clostridia bacterium S710(0)-1	GU136592	Marine sediment	99
M	Uncultured bacterium clone	HQ190478	Oil field	98

Table S4.3 Squared Pearson product-moment correlation coefficients between bin coverages and 16S rRNA gene coverages over four samples. Rows: Bin coverages; Columns: 16S rRNA coverages.

	#copies[1]	A	B	C	D	E	F	G	H	I	J	K	L	M
A Archaeon	3.6	**1.00**	1.00	0.12	0.12	0.15	0.14	0.97	0.82	0.04	0.12	0.12	0.12	0.14
B Arcobacter	14.3	1.00	**1.00**	0.09	0.09	0.16	0.14	0.94	0.78	0.06	0.09	0.09	0.09	0.14
C Arcobacter	2.6	0.11	0.11	**1.00**	1.00	0.16	0.13	0.19	0.28	0.18	1.00	1.00	1.00	0.13
D Arcobacter	3.8	0.10	0.10	1.00	**1.00**	0.17	0.13	0.17	0.26	0.18	1.00	1.00	1.00	0.13
E Pseudovibrio	6.0	0.18	0.18	0.18	0.18	**0.99**	0.98	0.22	0.24	0.02	0.18	0.18	0.18	0.98
F Rhodobacterales*	0.4	0.35	0.36	0.28	0.28	0.66	**0.59**	0.28	0.18	0.14	0.29	0.28	0.28	0.59
G Vibrio	10.3	0.94	0.94	0.22	0.22	0.17	0.17	**1.00**	0.95	0.00	0.22	0.22	0.22	0.17
H Vibrio	25.7	0.99	0.99	0.17	0.17	0.15	0.14	0.99	**0.88**	0.01	0.16	0.17	0.17	0.15
I Psychromonas*	6.3	0.74	0.73	0.34	0.34	0.20	0.22	0.89	0.99	**0.10**	0.34	0.34	0.34	0.22
J Pseudomonas	2.4	0.01	0.01	0.81	0.81	0.38	0.31	0.00	0.02	0.28	**0.82**	0.81	0.81	0.31
K Fusobacter	5.1	0.11	0.11	1.00	1.00	0.16	0.13	0.19	0.28	0.18	1.00	**1.00**	1.00	0.13
L Clostridiales	2.8	0.11	0.11	1.00	1.00	0.16	0.13	0.19	0.28	0.18	1.00	1.00	**1.00**	0.13
M Bacteroidetes	1.3	0.31	0.31	0.04	0.04	0.95	0.95	0.39	0.44	0.06	0.04	0.04	0.04	**0.95**

* Low correlation coefficients may be explained by misbinning,

[1]#copies is an estimate for the 16S rRNA gene copy numbers based on the slope of the correlation.

Table S4.4 List of oligonucleotide probes and primers used

Probe	Sequence (5′→3′)	Target organisms	FA[1] (%)	E. coli position	Ref erence
EUB338 I[2]	GCTGCCTCCCGTAGGAGT	Most Bacteria	35	338–355	Amann *et al*, 1990
EUB338 II[2]	GCAGCCACCCGTAGGTGT	*Plancto mycetales*	35	338–355	Daims *et al*, 1999
EUB338 III[2]	GCTGCCACCCGTAGGTGT	*Verrucomicr obiales*	35	338–355	Daims *et al*, 1999
NON338	ACTCCTACGGGAGGCAGC	Control	35	338–355	Wallner *et al*, 1993
Alf968	GGTAAGGTTCTGCGCGTT	*Alphaproteo bacteria*	25	968–985	Neef, 1997
Gam42a[3,4] (& com petitor)	GCCTTCCCACATCGTTT	*Gammaprote obacteria*	35	1027–1043	Manz *et al*, 1992
Epsy914	GGTCCCCGTCTATTCCTT	*Epsilonprote obacteria*	30	914–931	Loy, 2003
GRb	GTCAGTATCGAGCCAGTGAG	*Rhodobacter Roseobacter*	25	645–664	Eilers *et al*, 2000
GV	AGGCCACAACCTCCAAGTAG	*Vibrio*	30	841–860	Eilers *et al*, 2000
ARC94[4]	TGCGCCACTTAGCTGACA	*Arcobacter*	25	94–111	Snaidr *et al*, 1997
ARC1430[4]	TTAGCATCCCCGCTTCGA	*Arcobacter*	25	1430–1447	Snaidr *et al*, 1997
MDarch335	GCACCCCUUAGGGCUAGG	Certain marine DPANN-archaea	45	335–353	This study
ITSF	GTCGTAACAAGGTAGCCGTA	universal	-	1423–1443	Cardinale *et al*, 2004
ITSReub[5]	GCCAAGGCATCCACC	Bacteria	-	23–38	Cardinale *et al*, 2004

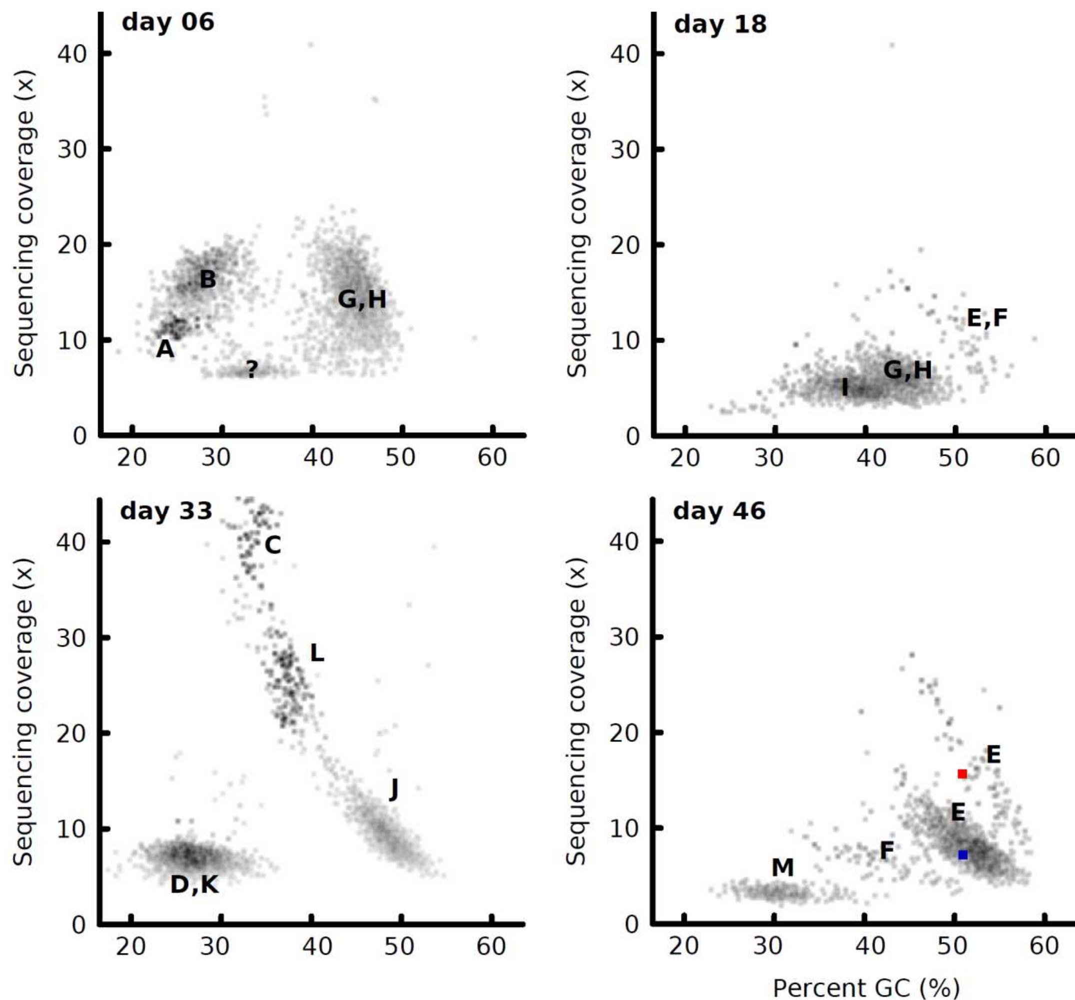
[1]Formamide concentration in the CARD FISH hybridization buffer

[2]Used in the mix of EUB I to III

[3]Used with the unlabeled competitor BETA42a (GCCTTCCCACTTCGTTT)

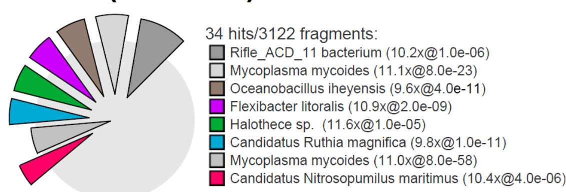
[4]Used in a mix

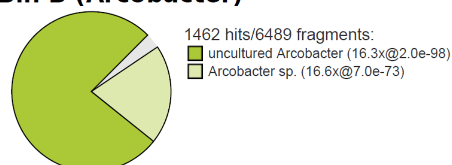
[5]binds to 23S rRNA

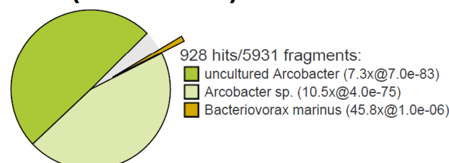


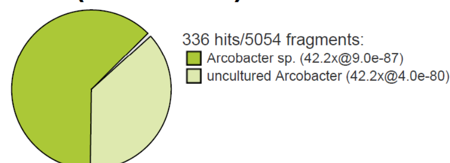
Supplementary figure S4.1 Separation of contigs belonging to the most abundant populations in the samples of four different time points by combined tetranucleotide and interpolated Markov Model binning. **Upper panel**: The distribution of the contigs on a sequencing coverage versus GC plot shows clouds each corresponding to a different bin and population. The contigs of the different populations were clearly separated from each other except for bin G and H, two *Vibrionales* subpopulations, and bin I, as well as bin D and K. They only show a small difference in GC content. The two separate clouds differing in coverage for bin E at day 46 may result from different subpopulations. The squares indicate the contigs, that encode the nitrite reductases of bin E (blue: *nirS*; red: *nirK*). **Lower panel**: The pie charts show the taxonomic distribution of blast hits of fragmented contigs to reference genomes. The distance of each slice from the center of the pie is a measure for the median *e*-value of the associated hits (larger e-value correspond to larger distances from the center).The low number, relatively high *e*-values and scattering of blast hits obtained for bin A (Archaeon) is caused by the distant relationship of the bin to any available reference.

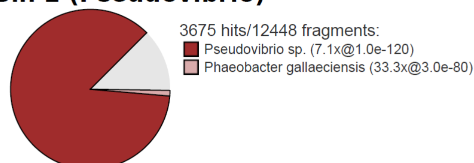
Supplementary figure S4.1 is continued on the next page

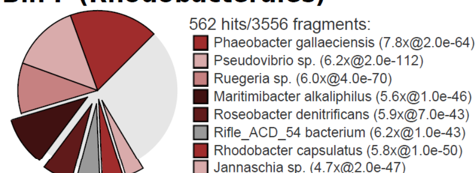

Bin A (Archaeon)
34 hits/3122 fragments:
Rifle_ACD_11 bacterium (10.2x@1.0e-06)
Mycoplasma mycoides (11.1x@8.0e-23)
Oceanobacillus iheyensis (9.6x@4.0e-11)
Flexibacter litoralis (10.9x@2.0e-09)
Halothece sp. (11.6x@1.0e-05)
Candidatus Ruthia magnifica (9.8x@1.0e-11)
Mycoplasma mycoides (11.0x@8.0e-58)
Candidatus Nitrosopumilus maritimus (10.4x@4.0e-06)

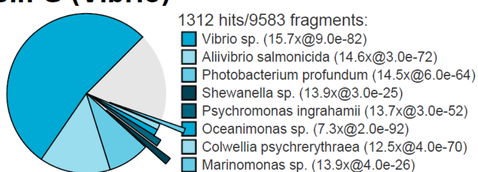

Bin B (Arcobacter)
1462 hits/6489 fragments:
uncultured Arcobacter (16.3x@2.0e-98)
Arcobacter sp. (16.6x@7.0e-73)

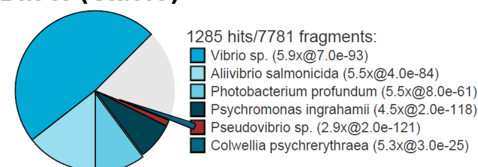

Bin C (Arcobacter)
928 hits/5931 fragments:
uncultured Arcobacter (7.3x@7.0e-83)
Arcobacter sp. (10.5x@4.0e-75)
Bacteriovorax marinus (45.8x@1.0e-06)

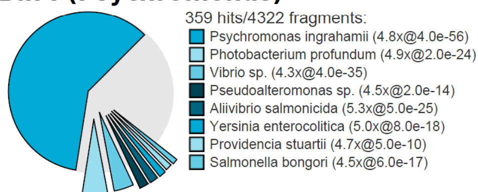

Bin D (Arcobacter)
336 hits/5054 fragments:
Arcobacter sp. (42.2x@9.0e-87)
uncultured Arcobacter (42.2x@4.0e-80)

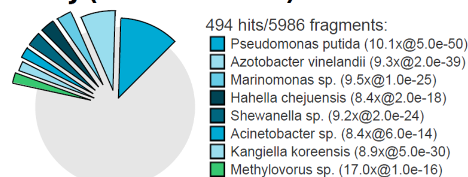

Bin E (Pseudovibrio)
3675 hits/12448 fragments:
Pseudovibrio sp. (7.1x@1.0e-120)
Phaeobacter gallaeciensis (33.3x@3.0e-80)

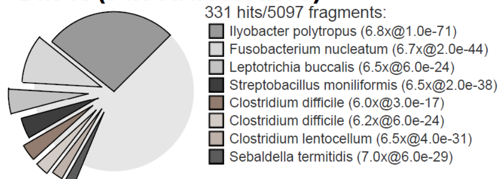

Bin F (Rhodobacterales)
562 hits/3556 fragments:
Phaeobacter gallaeciensis (7.8x@2.0e-64)
Pseudovibrio sp. (6.2x@2.0e-112)
Ruegeria sp. (6.0x@4.0e-70)
Maritimibacter alkaliphilus (5.6x@1.0e-46)
Roseobacter denitrificans (5.9x@7.0e-43)
Rifle_ACD_54 bacterium (6.2x@1.0e-43)
Rhodobacter capsulatus (5.8x@1.0e-50)
Jannaschia sp. (4.7x@2.0e-47)

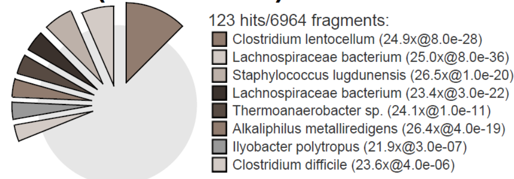

Bin G (Vibrio)
1312 hits/9583 fragments:
Vibrio sp. (15.7x@9.0e-82)
Aliivibrio salmonicida (14.6x@3.0e-72)
Photobacterium profundum (14.5x@6.0e-64)
Shewanella sp. (13.9x@3.0e-25)
Psychromonas ingrahamii (13.7x@3.0e-52)
Oceanimonas sp. (7.3x@2.0e-92)
Colwellia psychrerythraea (12.5x@4.0e-70)
Marinomonas sp. (13.9x@4.0e-26)

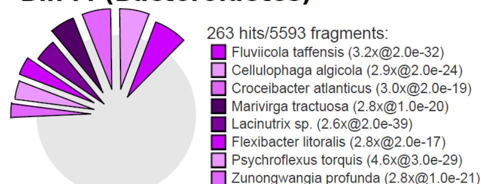

Bin H (Vibrio)
1285 hits/7781 fragments:
Vibrio sp. (5.9x@7.0e-93)
Aliivibrio salmonicida (5.5x@4.0e-84)
Photobacterium profundum (5.5x@8.0e-61)
Psychromonas ingrahamii (4.5x@2.0e-118)
Pseudovibrio sp. (2.9x@2.0e-121)
Colwellia psychrerythraea (5.3x@3.0e-25)

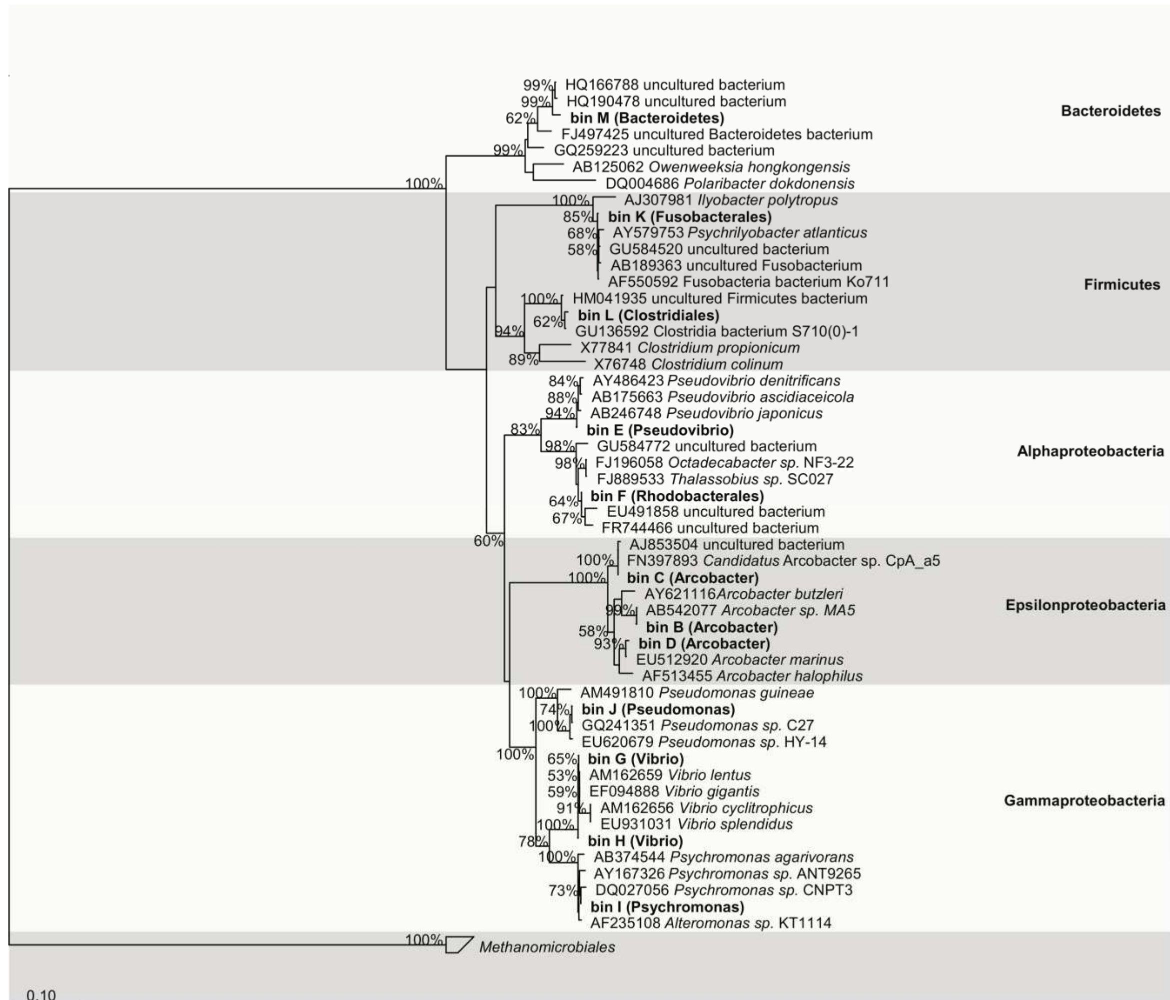

Bin I (Psychromonas)
359 hits/4322 fragments:
Psychromonas ingrahamii (4.8x@4.0e-56)
Photobacterium profundum (4.9x@2.0e-24)
Vibrio sp. (4.3x@4.0e-35)
Pseudoalteromonas sp. (4.5x@2.0e-14)
Aliivibrio salmonicida (5.3x@5.0e-25)
Yersinia enterocolitica (5.0x@8.0e-18)
Providencia stuartii (4.7x@5.0e-10)
Salmonella bongori (4.5x@6.0e-17)


Bin J (Pseudomonas)
494 hits/5986 fragments:
Pseudomonas putida (10.1x@5.0e-50)
Azotobacter vinelandii (9.3x@2.0e-39)
Marinomonas sp. (9.5x@1.0e-25)
Hahella chejuensis (8.4x@2.0e-18)
Shewanella sp. (9.2x@2.0e-24)
Acinetobacter sp. (8.4x@6.0e-14)
Kangiella koreensis (8.9x@5.0e-30)
Methylovorus sp. (17.0x@1.0e-16)

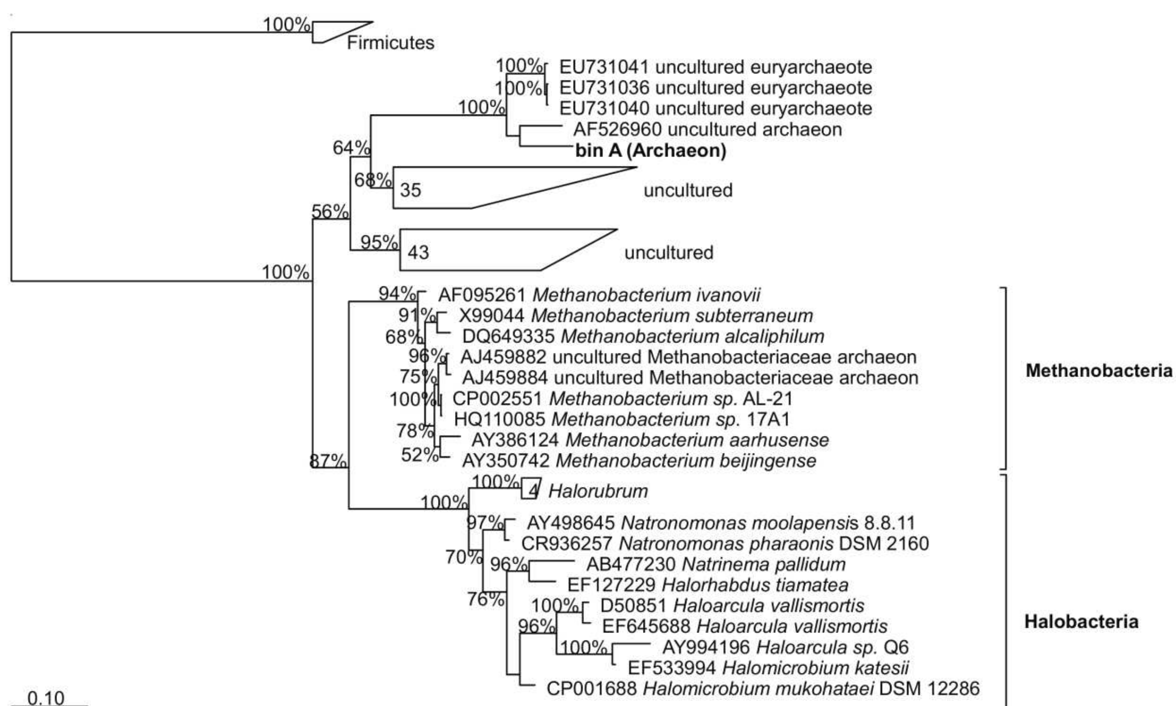

Bin K (Fusobacterales)
331 hits/5097 fragments:
Ilyobacter polytropus (6.8x@1.0e-71)
Fusobacterium nucleatum (6.7x@2.0e-44)
Leptotrichia buccalis (6.5x@6.0e-24)
Streptobacillus moniliformis (6.5x@2.0e-38)
Clostridium difficile (6.0x@3.0e-17)
Clostridium difficile (6.2x@6.0e-24)
Clostridium lentocellum (6.5x@4.0e-31)
Sebaldella termitidis (7.0x@6.0e-29)


Bin L (Clostridiales)
123 hits/6964 fragments:
Clostridium lentocellum (24.9x@8.0e-28)
Lachnospiraceae bacterium (25.0x@8.0e-36)
Staphylococcus lugdunensis (26.5x@1.0e-20)
Lachnospiraceae bacterium (23.4x@3.0e-22)
Thermoanaerobacter sp. (24.1x@1.0e-11)
Alkaliphilus metalliredigens (26.4x@4.0e-19)
Ilyobacter polytropus (21.9x@3.0e-07)
Clostridium difficile (23.6x@4.0e-06)

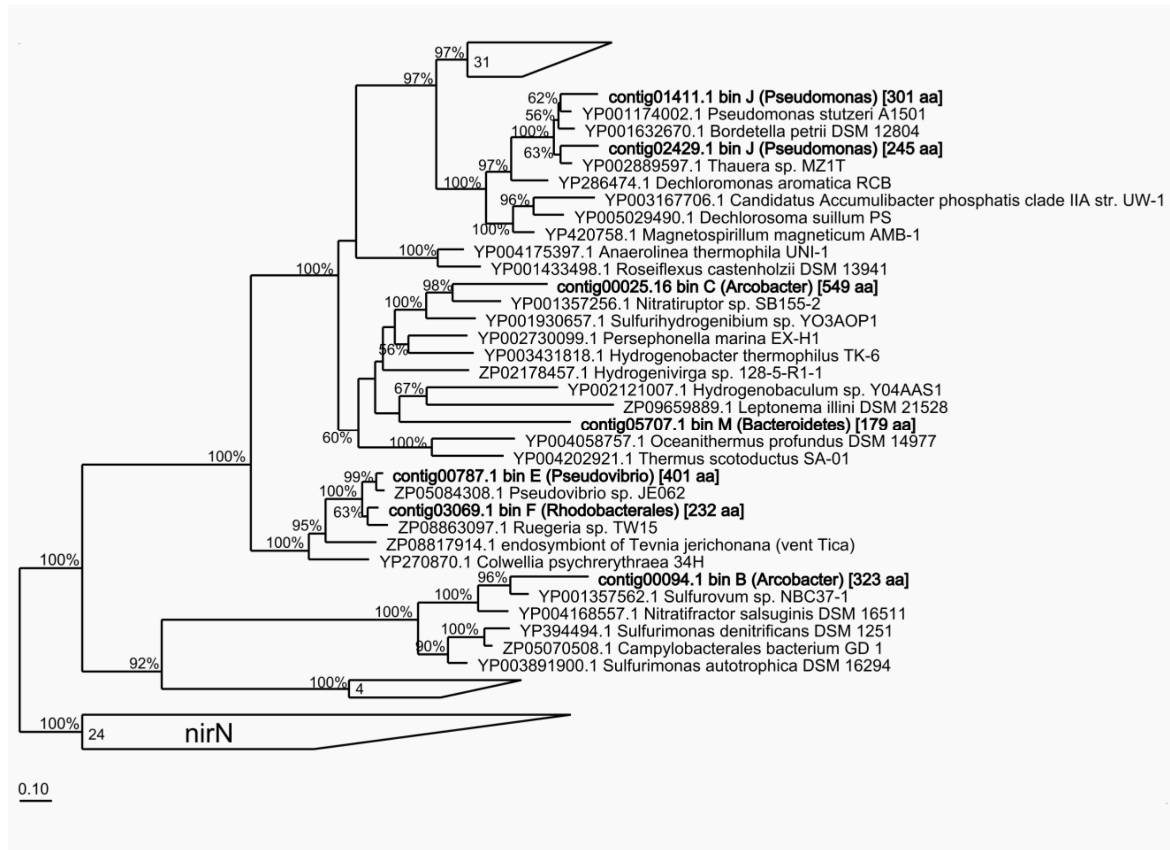

Bin M (Bacteroidetes)
263 hits/5593 fragments:
Fluviicola taffensis (3.2x@2.0e-32)
Cellulophaga algicola (2.9x@2.0e-24)
Croceibacter atlanticus (3.0x@2.0e-19)
Marivirga tractuosa (2.8x@1.0e-20)
Lacinutrix sp. (2.6x@2.0e-39)
Flexibacter litoralis (2.8x@2.0e-17)
Psychroflexus torquis (4.6x@3.0e-29)
Zunongwangia profunda (2.8x@1.0e-21)



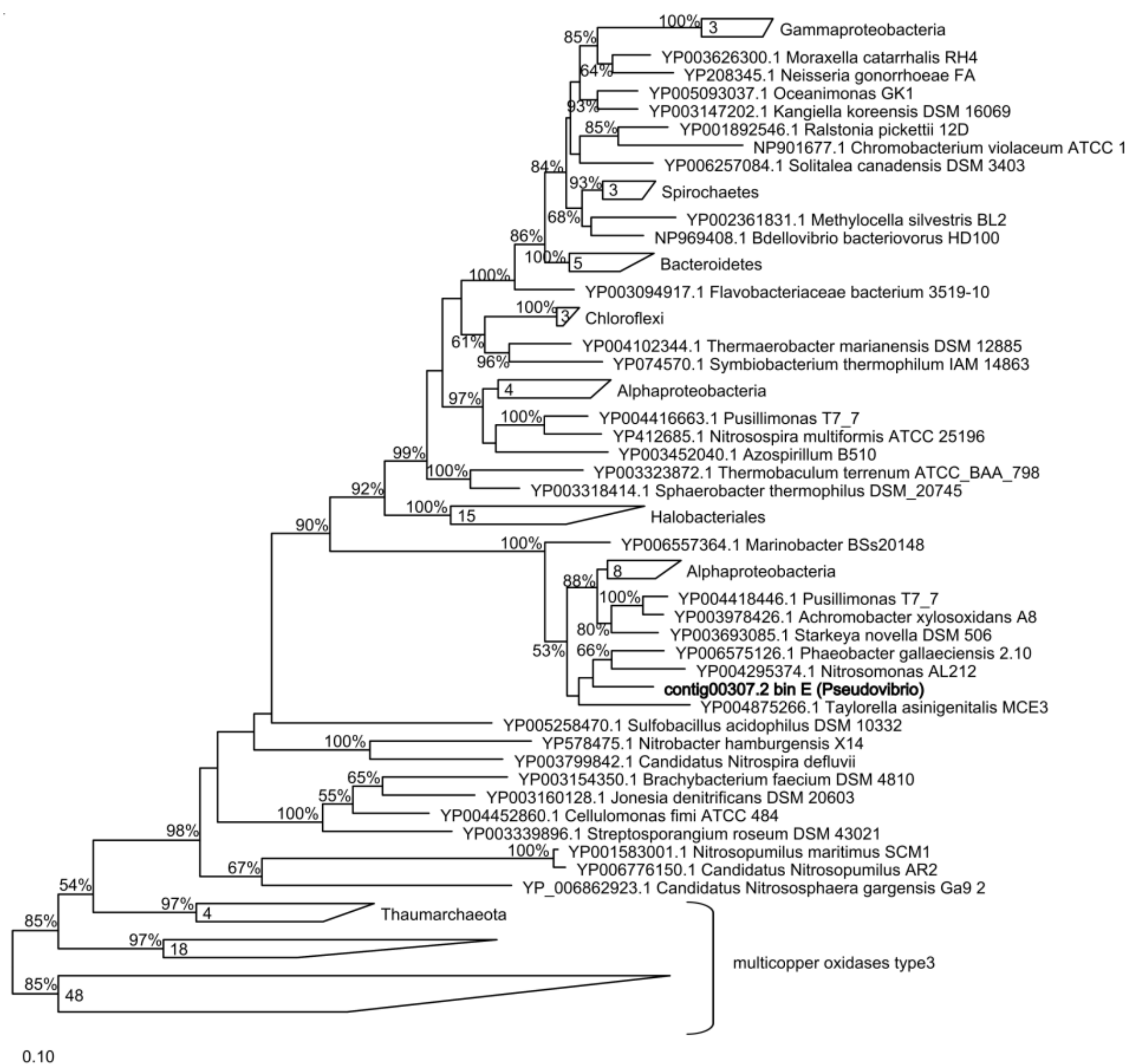
Supplementary figure S4.2: Phylogenetic tree of bacterial 16S rRNA sequences. Phylogenetic affiliations were determined by maximum likelihood (RAxML). Bootstrap values were generated from 100 replicates. Bootstrap values higher than 50% are given. Sequences from this study are written in bold.



Supplementary figure S4.3: Phylogenetic analysis of archaeal 16S rRNA sequences. Phylogenetic affiliations were determined by maximum likelihood (RAxML). Bootstrap values were generated from 100 replicates. Bootstrap values higher than 50% are given. The sequence from this study is written in bold.



Supplementary figure S4.4: Phylogenetic analysis of nirS sequences (aa). Phylogenetic affiliations were determined by maximum likelihood (RAxML). Bootstrap values were generated from 100 replicates. NirN was used as outgroup. Bootstrap values higher than 50% are given. Sequences from this study are written in bold.



Supplementary figure S4.5: Phylogenetic analysis of nirK sequences (aa). Phylogenetic affiliations were determined by maximum likelihood (RAxML). Bootstrap values were generated from 100 replicates. Bootstrap values higher than 50% are given. The sequence from this study is written in bol

Chapter 5

5 The environmental controls that govern the end product of bacterial nitrate respiration

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Authors' contributions:

BK and MS conceived and designed the study. BK performed sampling, incubations, rate measurements and chemical analysis with support from JSG and MS. BK and TGF performed the sulfate reduction rate measurements. HET performed DNA and RNA sequencing and assembly of the metagenomes. MS performed the binning of the metagenomes, and determined the *in situ* abundances. MS and BK analyzed the metagenomes and transcriptomes. MGK analyzed cytochrome *c* sequences. RS and RH performed proteomic analyses. The manuscript was written by BK and MS with support from all other authors.

5.1 Summary

In the biogeochemical nitrogen cycle, microbial respiration processes compete for nitrate as an electron acceptor. Denitrification converts nitrate into nitrogenous gas and thus removes fixed nitrogen from the biosphere, whereas ammonification converts nitrate into ammonium, which is directly reusable by primary producers. We combined multiple parallel long-term incubations of marine microbial nitrate-respiring communities with isotope labeling and metagenomics to unravel how specific environmental conditions select for either process. Microbial generation time, supply of nitrite relative to nitrate, and the carbon/nitrogen ratio were identified as key environmental controls that determine whether nitrite will be reduced to nitrogenous gas or ammonium. Our results define the microbial ecophysiology of a biogeochemical feedback loop that is key to global change, eutrophication, and wastewater treatment.

5.2 Main text

Currently, most fixed nitrogen in the biosphere originates from anthropogenic sources such as the industrial production of fertilizer ammonium. Uptake of fertilizer by crops is only 17% efficient, and 1 to 5% of fertilizer ammonium is converted biologically into nitrous oxide, a long-lived and powerful greenhouse gas (1). Microbial nitrification also converts a large portion of the fertilizer ammonium to nitrate in soil, where it subsequently runs off into surface waters and contributes to eutrophication in coastal zones. Nitrate emissions are partially remediated by denitrification in engineered environments such as wastewater treatment plants. If the end product of microbial nitrate reduction could be influenced by tuning environmental conditions, this would yield substantial ecological and economic benefits for both natural and engineered systems.

Two microbial processes compete for nitrate as an electron acceptor: denitrification (including anammox) and ammonification (including dissimilatory nitrate reduction to ammonium). The carbon/nitrogen ratio (2–5), pH (5), nitrite versus nitrate concentration (5–7), soil sand content (5), availability of fermentable carbon compounds (4, 8), temperature (7, 9), and sulfide concentration (10–12) are potentially important environmental controls on this competition. The lack of consensus regarding which factors are most important, and in which environments, is likely due to the complex and highly variable structure of natural microbial communities.

To unravel the selective forces behind the ecological success of denitrification or ammonification, we subjected natural communities to specific environmental conditions in

multiple parallel long-term incubations (13). The source community was from coastal, sandy tidal flat sediments that make substantial contributions to global denitrification and perform both denitrification and ammonification (12, 14). We did not aim to reproduce the sediment community in the laboratory; we simply used the sediment as a highly microbially diverse inoculum to enable the selection of optimally adapted nitrate-reducing communities. We performed 15 parallel anoxic incubations (figure 5.1 and table S5.1) with continuous substrate supply, which made it possible to maintain the nitrate and/or nitrite concentrations in the in situ micromolar range (<0.5 mM for nitrite, <10 mM for nitrate).

Denitrification and ammonification have two electron acceptors in common: nitrate and nitrite. In theory, the outcome of the competition could be most easily explained by which of these two compounds is supplied. In natural ecosystems, the relative supply of nitrate and nitrite is controlled by nitrification, a two-step process that can yield either compound as the end product. In our experiments with nitrate as the terminal electron acceptor, ammonification emerged as the prevalent pathway, whereas supply of nitrite resulted in denitrification prevalence (figure 5.1). Denitrification was always observed as the prevalent respiratory pathway when nitrite was supplied, even in the presence of fermentable substrates and sulfide, at low pH or at a reduced copper concentration. Thus, the supply of nitrite or nitrate was a key factor in the outcome of the competition between denitrification and ammonification. If elevated sulfide concentrations or changes in pH decrease the rate of nitrite production relative to the rate of nitrate production (15, 16), this would therefore favor ammonification over denitrification, as was observed in some previous studies (5, 12).

We further investigated the apparent success of ammonification with nitrate as the electron acceptor in a 400-day chemostat incubation. In a chemostat, the growth rate (or generation time) of the cultivated bacteria is controlled by the applied dilution rate, enabling us to test whether this factor affected the outcome of the competition in any way. The average in situ generation time of the sampled community was estimated at ~0.4 days, a value derived from a metagenome of the tidal flat community (17). During the 400-day incubation, the generation time was varied between 1.0 and 3.4 days and the nitrite and nitrate concentrations always remained in the low micromolar range (<0.5 mM for nitrite, <10 mM for nitrate). The generation time strongly affected the outcome of the competition for nitrate (figure 5.2).

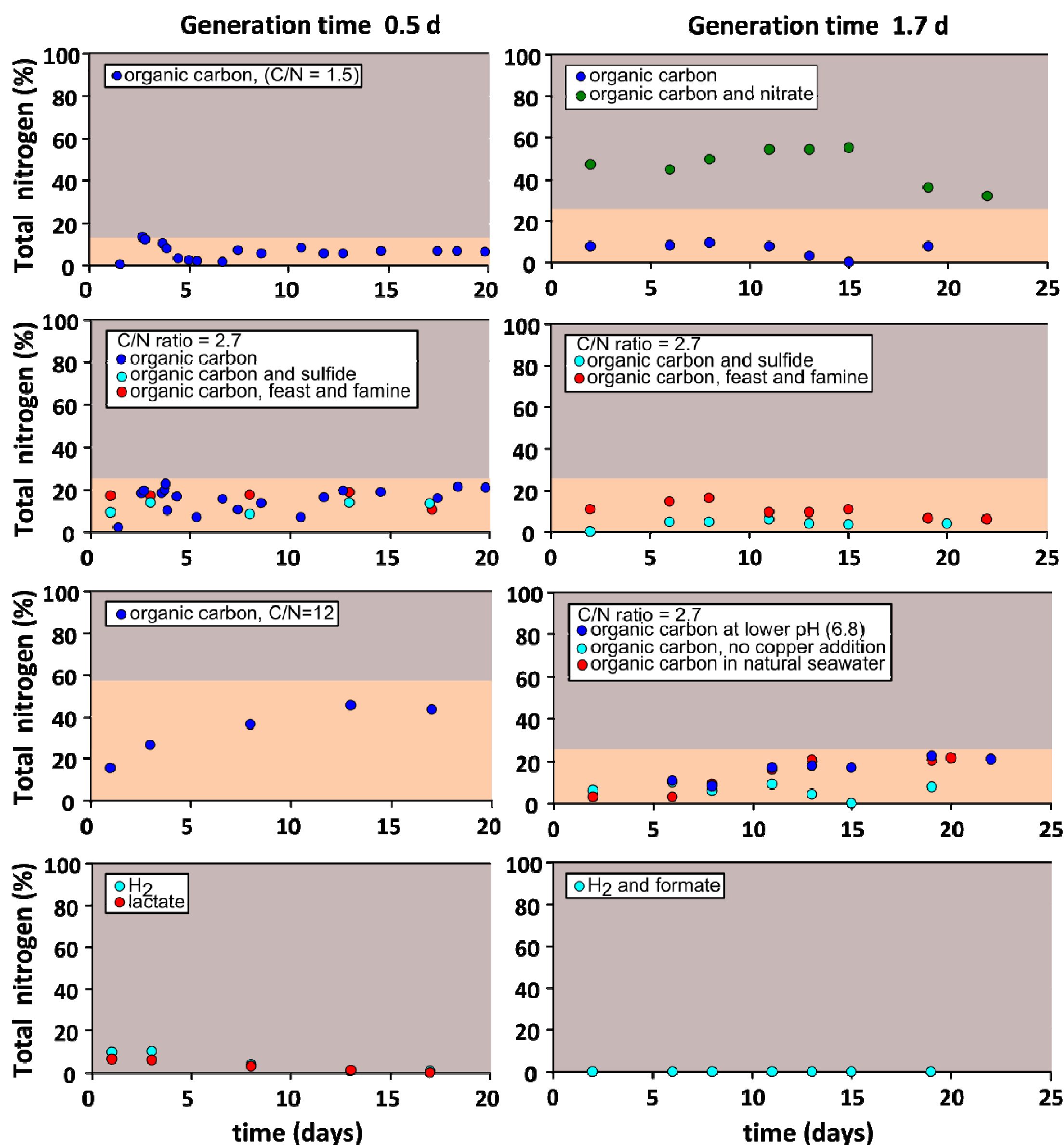

Figure 5.1: Fate of nitrite or nitrate in 15 anoxic incubations with continuous substrate supply. All incubations (except one) received nitrite as the main electron acceptor and exhibited denitrification. Only a single incubation (green circles, exhibited denitrification. Only a single incubation (green circles, top right panel) received nitrate and exhibited ammonification. For medium composition, see table S1. Shaded areas indicate the composition of nitrogen compounds in the inflowing medium (bronze denotes nitrate or nitrite; apricot denotes organic nitrogen from the amino acids provided as organic carbon substrates). Circles indicate the percentage of supplied nitrogen converted to ammonium. If the amount of ammonium produced did not exceed the amount of organic nitrogen provided, it most likely originated from the degradation of organic nitrogen supplied in the medium. This was confirmed by the detection of nitrogen (N2) as the main product. Nitrite and nitrate concentrations were <0.5 mM and <10 mM, respectively (except in the "feast and famine" incubations) and electron donors were always present in a slight excess. SD (n = 3) values did not exceed symbol diameters.

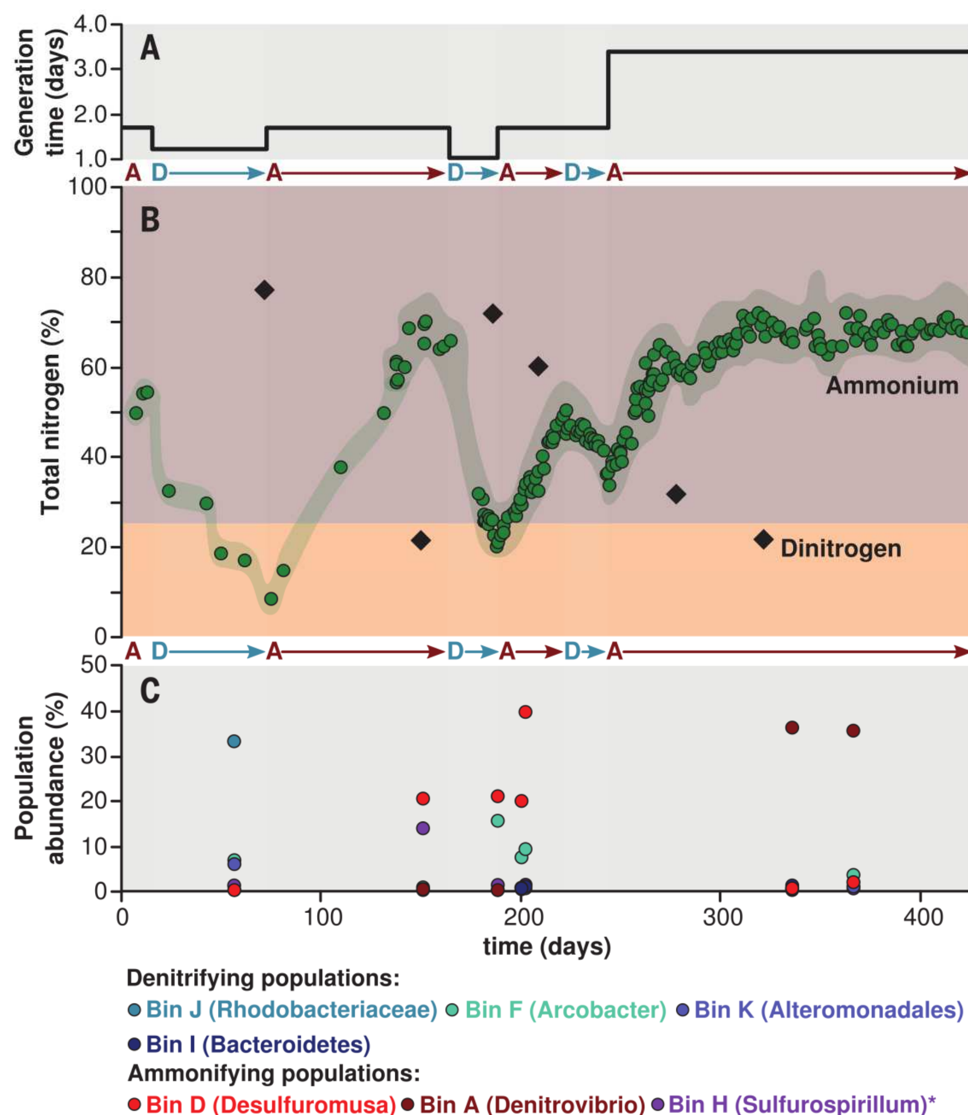

Figure 5.2: Prevalence of ammonification and denitrification as a function of generation time. (A) Generation time applied. (B) Prevalence of ammonification (A) and denitrification (D) as shown by conversion of nitrogen supplied in the form of nitrate (shaded bronze area) and amino acids (shaded apricot) into ammonium (green circles; SD, n = 3, plotted as shaded green area) and N_2 (brown diamonds). (C) Relative abundance estimates of nitrate-respiring populations in metagenomes. Nitrite and nitrate concentrations remained below 0.5 mM and 10 mM, respectively. *Bin H only participated in nitrate reduction to nitrite.

As shown by mass balancing, denitrification prevailed at short generation times, whereas ammonification was most successful at long generation times, with a tipping point detected at a generation time of ~1.7 days (figure 5.2). After 185 days, we increased the generation time in the chemostat from 1.0 to 1.7 days and ammonification slowly became dominant; however, after day 230, denitrification regained prevalence. Ammonification only recovered after the generation time

was further increased to 3.4 days after day 240. To determine the mechanisms responsible for the observed selective effect of microbial generation time, we characterized the selected communities with metagenomic sequencing at different time points (figure 5.2). Metagenomic reads were assembled into contigs, which were binned as described (18). We obtained 11 different bins, each bin constituting a provisional whole-genome sequence of a single population (figure. S5.1 and table S5.2). Each bin could also be linked to a full-length 16S ribosomal RNA gene (figure S5.2); together the bins accounted for 85% of all sequenced reads in all samples. Population abundances estimated from transcriptomes, proteomes, and catalyzed reporter deposition–fluorescence in situ hybridization (CARD-FISH) cell counts agreed well with the estimates based on the sequencing coverage of the bins (figure S5.3 and table S5.2). The metabolic interactions of the different populations can be inferred from the metagenomic, transcriptomic, and proteomic data (figure 5.3 and table S5.3).

The results suggested that denitrification was mainly performed by a population affiliated to Rhodobacteraceae (bin J) on or around day 54, and by a population related to *Arcobacter* (bin F) on or around day 189. Ammonification appeared to be mainly performed by a population related to *Desulfuromusa* (bin D) on or around day 152, and by a population related to *Denitrovibrio* (bin A) on or around day 336. The Desulfuromusa population showed high transcriptional activity of the *nrfAH* operon (encoding the pentaheme cytochrome *c* nitrite reductase complex that facilitates respiratory nitrite reduction to ammonium), whereas these genes were not detected for the Denitrovibrio population (table S5.14). Instead, this function was presumably performed by a transcriptionally highly active octaheme cytochrome *c* protein [OCC (19)]. The protein (contig00200_04753) clustered together with related proteins from Deferribacterales within a group of sequences from bacteria that thrive in anoxic habitats, several of which are capable of performing nitrite reductionto ammonium (figure S5.4). Elevated gene transcripts in the transcriptomic and metaproteomic data suggest that both the denitrifiers and the ammonifiers reduced nitrate to nitrite with a periplasmic nitrate reductase (NapAB) (table S5.14).

Proteomics and transcriptomic analyses suggested that at all time points sampled, specialized populations fermented sugars and amino acids, as shown by the presence and activity of genes involved in mixed acid fermentation and sugar and amino acid transport in bins affiliating with different Clostridiales (bins B, C, and E) and Spirochaetales (bin G) species (figure 5.3 and table S5.3). All these populations were active in both the denitrifying and ammonifying stages of the

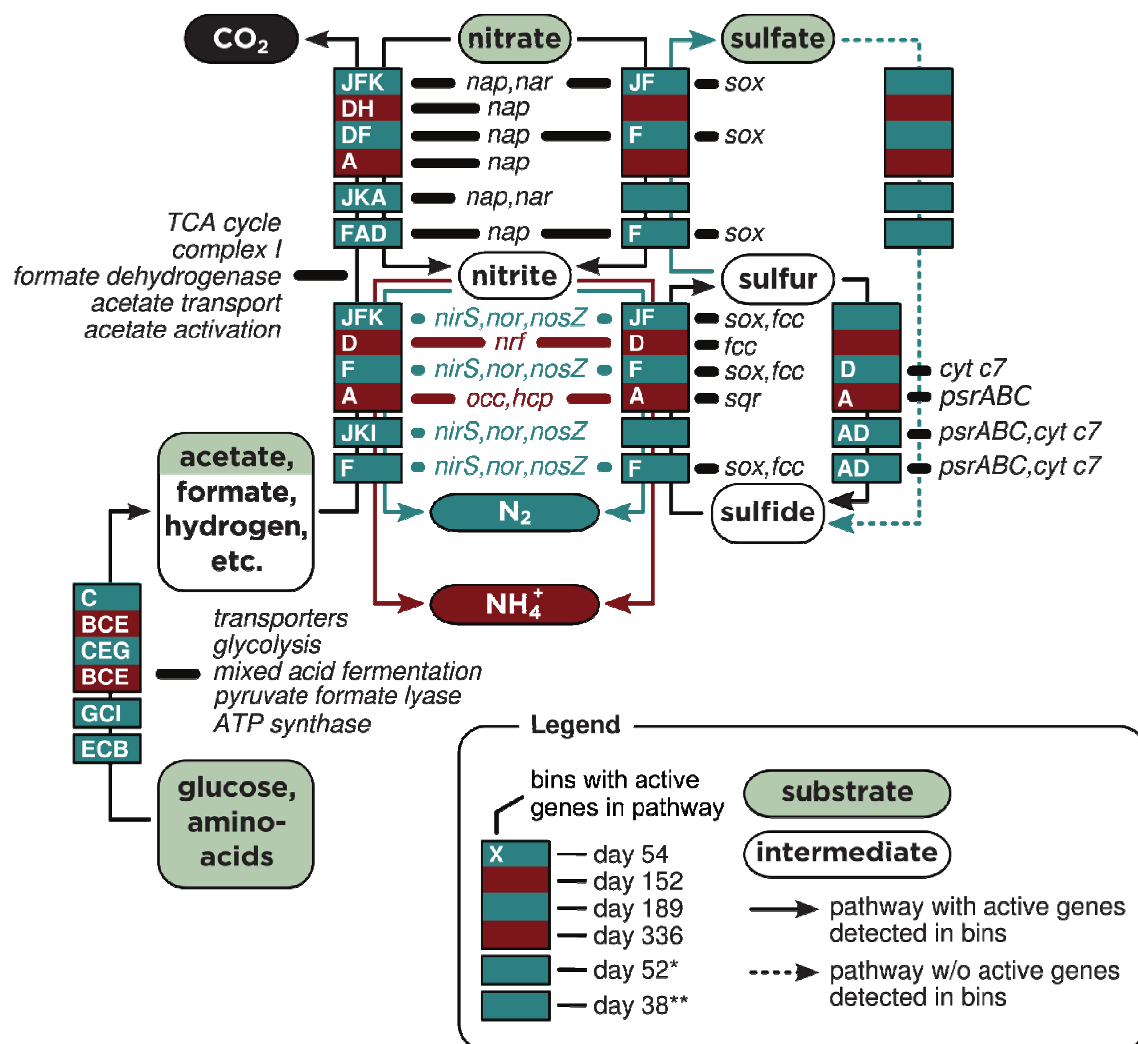

Figure 5.3: Schematic overview of metabolic interactions based on metagenomic, transcriptomic, and proteomic inferences. Days refer to figure 5.2, except for *day 52 (figure S5.7A) and **day 38 (figure. S5.7B).

experiment. These populations apparently did not participate in respiration but simply provided substrates to the nitrate-reducing populations in the form of fermentation products (e.g., acetate, formate, hydrogen). The transcriptomes and proteomes also suggested that sulfide was an additional electron donor driving nitrate respiration by the Rhodobacteraceae, Arcobacter, Sulfurospirillum (bin H), and Denitrovibrio populations (figure. 5.3 and table S5.3), and this was also confirmed experimentally (table 5.1). Although no contigs of sulfate-reducing bacteria were detected in the metagenomes, incubations with [35S]sulfate exhibited active sulfate reduction (1.1 nmol min^{-1} mg $protein^{-1}$; figure S5.5). The experimental results showed that ~25% of the nitrate was respired via a so-called “cryptic” sulfur cycle (20).

Electron acceptor	Electron donor	Denitrification (nmol min^{-1} mg $protein^{-1}$)	Ammonification (nmol min^{-1} mg $protein^{-1}$)
Nitrate	Organic Carbon	61±5	48±1
Nitrate	Sulfide	76 ±5	65 ±1
Nitrite	Organic Carbon	54±5	26±1
Nitrite	Sulfide	36±1	30±1
Nitrite	Acetate	25±5	24±2
Nitrite	Acetate + Sulfide	71±3	33±2
Ea (nitrate, kJ/mol)		23	28
Ea (nitrite, kJ/mol)		(n.d.)	34
Q10 (nitrate)		1.4	1.5
Q10 (nitrite)		(n.d.)	1.6

Table 5.1 Potential rates for nitrate and nitrite reduction coupled to the oxidation of different electron donors, and apparent activation energies Ea and Q10 coefficients for denitrification and ammonification. n.d., not determined

Transcriptomics and proteomics together revealed that the metabolic interactions between the populations were stable and essentially independent of generation time (figure 5.3). Denitrifiers and ammonifiers competed for the same substrates and used the same enzyme for nitrate reduction (NapAB) and therefore should have similar affinities for nitrate. This means that the observed selective force of microbial generation time most likely acted on a slight difference in substrate affinity for nitrite, the branching point of the two pathways. Even though specific enzyme affinities have been determined for isolated nitrite reductases, their *in vivo* affinities are unknown. Apparent substrate affinities are directly proportional to potential rates, and we observed large differences between the potential rates of nitrite reduction of the two pathways, especially when multiple electron donors were provided (table 5.1). For ammonification, the rates were lower than for denitrification, even leading to the transient accumulation of nitrite during ammonification of nitrate, which was not observed during denitrification (figure S5.6). Lower rates with multiple electron donors might be explained by a bottleneck in electron supply to the nitrite reductases of ammonification. These require six electrons per nitrite, versus only a single electron for denitrification.

In all experiments reported so far, nitrate or nitrite were in limited supply in the presence of a slight excess of carbon substrates. According to previous studies (2–5), carbon limitation in the presence of excess nitrate should favor denitrification. To investigate this idea in the present experimental context, we performed a continuous culture incubation under carbon limitation with

excess nitrate. A shorter generation time and a supply of nitrite instead of nitrate were tested in two parallel control experiments. In all three cases, denitrification became the prevalent pathway (figure S5.7), confirming the importance of carbon/ nitrogen ratio in addition to generation time and supply of nitrite relative to nitrate. Finally, we investigated the potential effect of temperature on the competition by determining the apparent activation energy (E_a) and the Q10 temperature coefficient for both processes (table 5.1 and figure S5.8). The calculated coefficients were not significantly different ($F_{2,18} = 1.93$, $P = 0.174$); thus, we were not able to reproduce a previously observed effect of temperature on the competition between two bacterial isolates (9).

Generation time, nitrate versus nitrite supply, and carbon/nitrogen ratio completely and reproducibly explained the fate of nitrate in 21 experimental trials with a microbial community sampled from a marine tidal flat. For these factors to hold such strong selective pressure, it is conceivable that the conditions favored the selected populations for reasons that are unrelated to nitrate respiration and cannot be extrapolated to other habitats. However, the combined results show that denitrifying and ammonifying populations were competing for the same electron donors (mainly organic acids, formate, hydrogen, and sulfide; figure 5.3) provided by the same fermentative populations. Further, selective pressure of pH, copper, presence of sulfide, supply of fermentation products, natural seawater and temperature could be ruled out on the basis of independent experiments (figure 5.1 and figure S5.8). Instead, the results suggest that the selective force acted directly on the nitrite reductases. A slightly higher apparent affinity for nitrite of the cytochrome cd1 nitrite reductases of denitrification would explain the observed higher fitness of denitrification with nitrite as the electron acceptor and at shorter generation times. With nitrate, when the generation time is short, NrfA/OCC cannot keep up with the nitrate reductase (figure S5.6) and denitrification prevails. At longer generation times, NrfA/OCC keeps up kinetically, its stoichiometric advantage with excess electrons (2) pays off, and ammonification outcompetes denitrification.

5.3 References

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5.4 Supporting Information

www.sciencemag.org/content/345/6197/676/suppl/DC1 Materials and Methods
Figures. S1 to S8 Tables S1 to S14 References (19–30)

5.4.1 Materials and Methods

Sediment collection

Sediment was collected from the back barrier tidal flat Janssand (N53°44’151 and E07°41’945) located in the German Wadden Sea, in January, August and November 2011 at low tide. The upper two centimeters of the tidal flat were sampled with a flat trowel.

Continuous culture incubations

The sediment was mixed vigorously with Red Sea salt medium (33.4 g/l; Aquaristic.net) at a ratio of 1:1. After the sediment grains had settled, the overlying suspension was used as inoculum for the continuous culture incubations. Cycloheximide (80 mg/l) was added to prevent growth of eukaryotes and thus grazing of bacteria. For the 15 parallel incubations, 400 ml of inoculum was transferred into 500 ml glass bottles closed with a lid with three gas tight connections, one for the supply of fresh medium, one for the removal of gas and spent medium (including bacteria) and one for the supply of Argon gas. The incubations were mixed with a magnetic stirrer. The pH was

monitored either by gel-filled electrodes with an Ag/AgCl reference system (Mettler-Toledo, Giessen, Germany) or by taking daily samples and measuring with an external pH meter. Medium was supplied continuously, drop by drop, with a peristaltic pump via one of the gas tight connections. To prevent contamination of the fresh medium by bacteria from the culture, the connection was shielded with a 10 ml static gas reservoir. Argon was bubbled through the continuous culture at a flow rate of 3 ml/min. The spent medium (including bacteria) was continuously removed by passive overflow. The filter-sterilized (0.2 µm, Sartopore MidiCaps, Satorius, Göttingen, Germany) medium was buffered with 15 mM HEPES at a pH of 8.0. After inoculation, during the first days of the incubations, the concentrations of the electron donor and acceptor in the inflowing medium were gradually increased, while keeping the ratios constant, to prevent high substrate concentrations in the cultures during adaptation of the inoculated bacteria to the culture conditions. After 2-5 days, these concentrations were kept constant for the remainder of the incubations. Table S1 specifies the substrate supply and dilution rates for all incubations. The organic carbon mixture added to the influent medium was made up in such a way that it represented the monomer composition of decaying biomass, the main source of carbon and energy in the sediment. It contained (% carbon): glucose 44.1, acetate 7.6, glutamic acid 10.7, aspartic acid 11.7, alanine 8.5, serine 4.6, tyrosine 8.9, histidine 1.4, methionine 2.4. The trace element solution contained (g/l): $FeSO_4.7H_20$ (0.21), H_3BO_3 (0.03), $MnCl_2.2H_20$ (0.1), $CoCl_2.6H_20$ (0.12), $NiCl_2.6H_20$ (0.024), $ZnCl_2$ (0.07), $Na_2MoO_4.2H_20$ (0.036), $CuSO_4.5H_2O$ (0.015). 0.5ml of trace element solution was added to one liter of fresh medium. Incubations with natural seawater and no copper in the added trace element solution were carried out as well to ensure that our standard incubation conditions were not selective for pathways dependent on copper enzymes. For example, higher copper availability compared to *in situ* conditions could favor the synthesis of copper-containing enzymes such as NirK (associated with denitrification).

For the 400-day continuous culture incubation, the 400-ml incubation receiving nitrate was transferred into a 3 l vessel and all substrate concentrations in the inflowing medium were doubled. In this experiment electron donors and acceptors were provided separately in an acidic and an alkaline medium, respectively, which were pumped into the vessel at a ratio of 1:1. This was done to prevent artefacts resulting from the dropwise addition of the medium that might otherwise lead to a transient localized competitive advantage of R-strategists. The pH values of the two media were adjusted in such a way that the resulting pH in the incubation vessel was 8±0.2. The composition of the acidic medium (pH= 2.0±0.2) was: Red Sea salt, Aquaristic.net (66.8 g/l), $NaH_2PO_4.1H_2O$ (0.5 mM), organic carbon mixture (see above and table S1), trace element

solution (0.5 ml/l). The alkaline medium (pH= 12.2±0.2) contained 20 mM $NaNO_3$. The medium pH was adjusted with HCl or NaOH. The liquid volume of the culture was 2.8 l and the inflowing medium was supplied dropwise to the culture with peristaltic pumps to keep the substrate concentrations in the micromolar range. The culture was mixed vigorously by recycling gas from the headspace above the culture to the bottom via a sintered glass filter. Spent culture medium including bacteria was continuously removed to keep the liquid volume constant at 2.8 l. Argon gas was supplied continuously (10 ml/min) to maintain anoxic conditions. The dilution rate (proportional to the inverse of the bacterial generation time) was altered at different time points during the experiment by changing the rate at which fresh medium was supplied and spent medium was removed (yielding generation times between 1.0 and 3.4 days, see figure 2).

After 352 and 365 days, bacteria from this long-term incubation were transferred to three further continuous culture incubations in three independent transfers. In the first transfer (after 352 days), the change in generation time was reproduced. After the second transfer (after 365 days), the ratio of electron donor and acceptor was altered so that organic carbon became limiting and after the third transfer (after 365 days), nitrite instead of nitrate was supplied as electron acceptor (table S1).

Analytical procedures

Nitrite, nitrate and ammonium concentrations were measured as previously described *(18)*. For measurement of nitrogen (N_2) gas production rates, the alkaline medium was replaced with an alkaline medium that contained ^{15}N-nitrate for several hours and production of $^{30}N_2$ was measured online with a GAM 400 mass spectrometer (In Process Instruments, Germany) supplied with gas from the headspace at a rate of 4 ml/min. The dissolved organic carbon concentration (DOC) was determined by measuring the chemical oxygen demand (COD) with cuvette-tests (Hach Lange GmbH, Düsseldorf, Germany) using a Thermostat LT200 and a DR3900 photometer (Hach Lange GmbH, Düsseldorf, Germany).

For measurements of sulfide concentrations liquid samples were fixed with zink acetate (5 % w/v) and sulfide was determined as follows: 900µl of fixed sample were mixed simultaneously with 100 µL of a dimethylparafenyldiamine (oxalate salt) solution (0.2 % in 20% H_2SO_4) and 6 µl of a $Fe(NH_4)(SO_4)_2.12H_2O$ solution (10% in 2% H_2SO_4). After 20 min of incubation, the absorbance was measured at 660 nm.

Elemental sulfur was analyzed by centrifuging 2 ml of cultureat 15,000 x g for 5 min and extracting the pellet with 1 ml acetone. Cyanolysis was carried out by adding 0.1 ml KCN solution

(0.125 M). The amount of thiocyanate produced was immediately determined colorimetrically at 460 nm after the addition of 0.1 ml ferric nitrate solution (10% $Fe(NO^3)_3.9H_2O$ in 13% HNO_3).

Batch incubations

The importance of different electron donors (sulfide, acetate, acetate plus sulfide; carbon mixture, each identical to the one used in the long-term incubations) for nitrate or nitrite reduction during denitrification and ammonification were determined in 120 ml serum bottles with gas tight rubber stoppers. The bottles were flushed with Argon (purity >99,998%, 5 cycles of flushing and evacuating) and filled with 40 ml of anoxic medium (Red Sea salt, 15 mM HEPES, phosphate and trace elements as in the medium of the long-term incubations,). The bacterial community was harvested from the continuous incubations, concentrated by centrifugation, washed and added to the incubation bottles as follows: 30 ml bacterial suspension was centrifuged at 4700 x g for 20 min, the supernatant was replaced by 20 ml of anoxic medium and then added to the incubation bottles. Energy and carbon substrates as well as nitrate or nitrite were added to a final concentration of 1 mM each. During batch incubations, bottle contents were regularly mixed by manual shaking. Rates of nitrate and nitrite reduction were analyzed colorimetrically measuring the changes in reactant (nitrate and nitrite) and product (ammonium) concentrations. For incubations with sulfide, a negative control experiment (sterile medium with substrates) was performed to exclude chemical reactions between sulfide and nitrite or nitrate.

Batch incubations at different temperatures (10, 15, 20, 25 and 30°C) were carried out with nitrate and for ammonification also with nitrite as the electron acceptor as already described except for the following modifications: glass bottles with a volume of 60 ml were prefilled with 20 ml of medium containing the organic carbon mixture and 20 ml of bacterial suspension harvested from the culture was washed and added to the bottles without prior concentration. An overpressure was applied to the headspace (2 bar, Argon). The production of nitrogen gas was measured by analyzing the conversion of ^{15}N-nitrate or ^{15}N-nitrite to $^{30}N_2$ using mass spectrometry as described. The activation energies for the ammonification and denitrification pathways were obtained by plotting the rate of the respective processes at logarithmic scale against the reciprocal of the incubation temperatures. Regression lines were compared by analysis of covariance (ANCOVA). Statistical significance was accepted at $P < 0.05$.

Sulfate reduction rates

The incubation set up consisted of a 100-ml glass bottle filled with 50 ml of the nitrate reducing long-term incubation with added HEPES (15 mM final concentration) and argon in the headspace. Anoxic medium identical to the medium of the long-term incubation (35 mM organic carbon mixture and 10 mM nitrate) was supplied through a needle that pierced the rubber stopper of the glass bottle with a peristaltic pump at 3.6 µl/min (=generation time of 3.4 days) for the incubation at the ammonifying stage and 20 µl/min (=generation time of 1.3 days) at the denitrifying stage of the long term incubation. Sulfate reduction rates were determined by measuring the conversion of $^{35}SO_4^{2-}$ to reduced ^{35}S compounds. 2.5 MBq of $^{35}SO_4^{2-}$ was added to the bottle at the beginning of the incubation. For the denitrifying stage, 100 µM of unlabelled sulfide was added to ensure the trapping of produced $^{35}S^{2-}$ in the unlabeled total reduced sulfur pool. Sulfate reducing activity was stopped by fixing 1 ml of sample in 4 ml of 5% $ZnCl_2$. The produced total inorganic ^{35}S compounds were quantified according to (*23*).

Catalyzed Reporter Deposition Fluorescence in situ hybridization (CARD-FISH)

Cells were fixed in 1% formaldehyde over night at 4°C and subsequently washed three times with phosphate buffered saline (PBS, pH 7.4). Fixed cells were stored in a 1:1 PBS:ethanol mixture at -20°C. CARD-FISH was performed on 0.2 µm pore-sized polycarbonate filters followed by fluorescently labeled tyramide signal amplification as reported (1). Horseradish peroxidase labeled oligonucleotide probes used and hybridization conditions are listed in Table S4.. Filters were counter-stained with DAPI (4′,6-diamidino-2-phenylindole) and counted manually with an epifluorescence microscope (Axioplan 2, Carl Zeiss, Jena, Germany).

DNA extraction and DNA library generation, Ion Torrent Personal Genome Machine sequencing and assembly

Samples at four different time points (day 54, 152, 189, 336) during the nitrate-reducing incubation and one time point for each of the transfers with a change in generation time (day 52) and with supply of nitrite instead of nitrate (day 38) with a volume of 14 ml were centrifuged for

20 min at 4,700 x g and pellets were stored at -80°C until further processing. DNA was extracted as previously described (18, 24).

For metagenome shotgun sequencing, 2.5 µg of the extracted DNA per sample were mechanically fragmentend using Nebulizers (Roche) with 32 psi applied for 6 min, in 500µl nebulization buffer (Roche). The fragmented DNA was purified using MinElute PCR purification columns (Qiagen) and eluted in 50µl low TE (Life Technologies). The entire eluate was used for the preparation of barcoded PGM sequencing libraries with the Ion XpressTM Plus gDNA Fragment Library Preparation kit (manual Pub. No 4471989, Rev. M, May 2013; Life Technologies). Library insert sizes were approximately 200 bp. Libraries were sequenced with the Personal Genome Sequencer (PGM) on 318 Chips (pooled with other samples), using the chemistry for 200 bp libraries. Base calling was performed with the Torrent Suite v3.2 software, with default settings. Reads of the four sequenced DNA samples I-IV were assembled in one combined assembly with the Newbler assembler (v. 2.6) with default settings for genomic DNA assembly for non-paired reads. The samples V and VI were assembled separately. The reads were submitted to the assembly in sff format from the Torrent Suite output, for the V and VI assemblies reads were submitted as fastq files. Sequence data were submitted to the short read archive (http://trace.ncbi.nlm.nih.gov/Traces/sra/), and assembled contigs were submitted as whole genome shotgun sequencing projects at DDBJ/EMBL/GenBank, under bioproject identifier PRJNA231836 (http://www.ncbi.nlm.nih.gov/bioproject/231836).

In silico procedures for metagenomics

Binning and annotation of assembled contigs was performed with the Metawatt binner according to (18). Full length 16S rRNA gene sequences were obtained as previously described (18). 16S rRNA gene sequences cannot be binned with high confidence based on tetranucleotide frequencies because of the atypical base composition of these genes. For this reason, the 16S rRNA gene sequences were linked to bins based on phylogeny (consistent phylogeny between the 16S rRNA gene and the phylogenetic signature of the sequence data of each bin; see table S2, Figure. S1), and high squared Pearson product-moment correlation coefficients between bin sequencing coverage and 16S rRNA gene sequencing coverage for the four samples (table S5).

Phylogenetic analysis of the 16S rRNA gene sequences was carried out with the ARB software package (25) using the SILVA 16S rRNA SSU Reference database, release 115. A phylogenetic tree was constructed by the maximum-likelihood method (RAxML, JTT substitution

matrix) (26). Bootstrap values were generated from 100 replicates. The average growth rate of the microbial community of the sampling site (18) was estimated based on codon usage bias in the sampling site's metagenome according to Viera and Rocha (17).

RNA extraction, cDNA library generation and Ion Torrent Personal Genome Machine sequencing

For transcriptome analysis, 2 ml of sample were pelleted and the pellets were stored in RNA later solution at -20°C. Total RNA was extracted from pellets as follows: The pellet was re-suspended in 1 ml of TRI Reagent® solution (Applied Biosystems). The suspension was transferred to a bead beater tube containing 0.25 ml sterile glass beads (0.1 mm diameter) for bead beating at 6.5 m/s for 45 sec. After incubation at room temperature (RT), the tube was centrifuged for 5 min at 12,000 x g and 4 °C, and the supernatant was transferred to a fresh tube. 200 µl of chloroform was added followed by vigorous shaking by hand for 15 sec, incubation at RT for 10 min, and centrifugation at 12,000 x g and 4 °C for 15 min. The upper phase was transferred to a fresh tube, 500 µl of ice-cold isopropanol was added and the tube was inverted several times, followed by incubation on ice for at least 30 min for RNA precipitation. After centrifugation at 20,000 x g and 4 °C for 25 min, the pellet was washed with 1 ml ice-cold ethanol three times (10 min centrifugation at 20,000 x g, 4 °C, between washing steps) and air dried at RT for approximately 10 min. The pellet was re-suspended in sterile TE buffer (pH 8.0) and incubated on ice for approximately 30 min for complete dissolving. The extracted RNA was treated with DNase (Promega) and purified using RNeasy MinElute spin columns (Qiagen).

Prior to library preparation for ion torrent sequencing, rRNA was depleted from 5 µg total purified RNA of each sample using the Ribo-Zero™ rRNA Removal Kit (Bacteria) (epicentre).

The rRNA-depleted sample was then used for library preparation with the Ion total RNA-Seq Kit v2 (Life Technologies) following the protocol for whole transcriptome library preparation.

Generated cDNA libraries were sequenced with the Personal Genome Sequencer (PGM) on 314 and 318 Chips (pooled with other samples), using the chemistry for 200-bp libraries. Base calling was performed with the Torrent Suite v3.2 (R-Fast und R-NO2: v3.6) software, with default settings. Gene transcriptional activities were normalized for length for each bin. This way in table S14 a value of 1.0 corresponds to the length-normalized average transcriptional activity for the bin. To calculate the activities in Table S3, the average transcriptional activities (n=5) and average peptide coverages for the translated proteins (n=6) were averaged for the complete

pathway or enzyme complex and normalized to 100% for the pathways and enzyme complexes investigated. Because the assembled contigs contained many sequence frameshifts that were presumably caused by artifacts related to homopolymers, a known issue of the applied sequencing technology (Ion Torrent), the analysis presented here is based on the presence/absence of multiple genes that form complete pathways or enzyme complexes and also the abundance of the transcripts/detection of peptides were integrated for the complete pathway or enzyme complex.

Phylogenetic analysis of multiheme cytochrome c protein sequences

Sequence-related pentaheme and octaheme (OCC) cytochrome c protein sequences, retrieved from public databases and embargo genome projects with BLAST, were aligned using MUSCLE provided at the EMBL-EBI webserver (http://www.ebi.ac.uk/Tools/msa/muscle/). These alignments were manually refined by comparison with previously published results from phylogenetic analyses including structural and protein sequence-analytical features (27,28); N- and C-terminally extending sequences beyond the first and last heme-binding motif (CxxCH), respectively, were trimmed and the final alignment was subjected to a Bayesian inference of phylogeny using the BEAST package (v1.7.5 of BEAUti, BEAST and TreeAnnotator; FigTree v.1.4; 29). By utilizing unique sites, tree likelihoods (ignoring ambiguities) were determined for the alignment by creating a Monte-Carlo Markov Chain (10,000,000 generations) in three independent runs. The searches were conducted assuming an equal distribution of rates across sites, sampling every 1000th generation and using the WAG empirical amino acid substitution model (30). The resulting 10,000 trees (omitting the first 350 trees as burn-in) were used to construct a phylogenetic consensus tree (Fig. S4) that was used as the basis to discuss the ammonification-relevant multiheme cytochrome c proteins in context with their evolution as defined in (27,28).

Proteomics Sample Preparation

Proteomics measurements were carried out from aliquots taken out at three time points during the incubation period: day 189 (BK1), day 207 (BK2) and day 336 (BK3) of the nitrate reducing incubation. Based on the protein estimation results from cultures, an aliquot corresponding to 300 µg total protein was used for proteomics sample preparation via the Filter-aided Sample Prep method (FASP) as previously described (18). Briefly, to 82 µl of sample in an Eppendorf tube, 30

µl of HPLC grade water, 30 µl of 10 % SDS, and 8 µl of 1 M DTT were added. The tube was then boiled at 95 °C for 10 min. The sample was cooled to room temperature, and the crude lysate was put on top of a 30 kDa molecular-weight cut-off (MWCO) filter provided with FASP Kits (Expedeon Inc., San Diego, CA, USA). The kits were operated in the standard manner specified for handling GELFrEE fractions. Briefly, the lysed sample was first washed with 200 µl of 8 M urea in 100 mM Tris-HCl (pH 8.5) at 14 000 x g for 25 min. The step was repeated twice. Following urea washes, the proteins were alkylated with IAA treatment by incubation in dark for 30 min. Then, the sample was washed three times with 100 µl of 50 mM ammonium bicarbonate solution by centrifuging at 14 000 x g for 10 min. Protein digestion was carried out first for 4 h at 37 °C using trypsin (Promega) in 1:20 protease to protein ratio. A second aliquot of trypsin was added following first 4 hours and the sample was incubated at 37 °C for an overnight digestion. Peptides were then collected in a fresh tube after washing the filter with two washes of 50 mM ammonium bicarbonate and a final addition of 0.5 M NaCl and spinning at 14 000 x g. The pH of resulting peptides solution was adjusted to < 3 by addition of formic acid.

Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

Approximately 25 µg of peptides were pressure-loaded onto an integrated, self-packed 3 cm Reverse Phase (RP) resin (Aqua, 300 Å pore size, Phenomenex, Torrance, CA, USA) and 3 cm Strong Cation Exchange (SCX) resin in a 150 µm inner diameter fused silica back column. The peptides were desalted on the column by washing from solvent A (95 % HPLC H2O, 5 % AcN, 0.1 % Formic acid) to solvent B (30 % HPLC H2O, 70 % AcN, 0.1 % Formic acid) 3 times over a period of 25 min. The desalted back column was connected to a 15 cm-long 100 µm-I.D. C-18 RP resin PicoFrit column (New Objective, Woburn, MA, USA) and placed in line with a U3000 quaternary HPLC (Dionex, San Francisco, CA, USA). The SCX-RP LC separation was carried out by eleven salt pulses with increasing concentrations of 500 mM ammonium acetate solution. Each of the first ten salt pulses was followed with 120 minute RP gradient from 100 % solvent A to 50 % solvent B, while the last salt pulse used 150 minute RP gradient from 100 % solvent A to 100 % solvent B. The LC eluent from the front column was directly nanosprayed into an LTQ-Orbitrap Elite mass spectrometer (Thermo Scientific). The mass spectrometer was operated in a data-dependent mode under the control of Xcalibur software (Thermo Scientific). The following parameters were used for the data-dependent acquisition: collision induced dissociation was carried out for top 20 parent ions in the ion trap following a full scan in the Orbitrap at 30 000 resolution, a 0.5 m/z isolation width, 35 % collision energy was used for fragmentation; and a

dynamic exclusion repeat count of 1 with duration of 30 s. The raw MS/MS data was searched using MyriMatch as previously described (18) against a predicted protein database (63,635 sequences) constructed from the metagenome assembly, along with common contaminants (44 sequences) and reverse sequences (supplementary table S8). A fixed modification of +57.0214 Da for carbamidomethylation of cysteine and a +16 Da modification for oxidation of methionine and a +43 Da modification for N-terminal carbamylation were included as dynamic modifications in the search parameters. Identified peptides were then filtered at <1 % peptide level FDR and assembled into proteins (minimum of two peptides per protein) by IDPicker 3. For more information on the search settings see supplementary table S7. For each time point, two technical replicates were performed. Identified peptides and proteins are listed in supplementary tables S9 - S14.

Table S5.1: Substrate supply rates (mmol or C-mmol/day for electron donors and acceptors) and generation times (days) during selection in continuous incubations.

Incubations	C/N ratio	Electron donor	Electron donor	Electron acceptor nitrite	nitrate	Generation time	comment
Organic carbon	1.5	Org. C-mix	23.8	14.4	1.4	0.5	
Organic carbon	2.7	Org. C-mix	42.5	14.4	1.4	0.5	1)
Organic carbon	12	Org. C-mix	43.2	3.6	0.36	0.5	
Hydrogen	-	Hydrogen	0.4 ml/min	3.6	0.36	0.5	+ CO2
Lactate	2.7	Lactate	20.9	3.6	0.36	0.5	
Organic carbon + sulfide	2.7	Org. C-mix and sulfide	10.8 + 0.7 mmol/d	3.6	0.36	0.5	
Organic carbon	2.7	Org. C-mix	4.4	1.5	0.15	1.7	
Feast and famine	2.7	Org. C-mix	4.4	1.5	0.15	1.7	2)
Nitrate + Organic carbon	**2.7**	**Org. C-mix**	**4.4**	**-**	**1.5**	**1.7**	
Hydrogen + formate	2.7	Formate + hydrogen	4.4 + 0.4 ml/min	1.5	0.15	1.7	
Organic carbon + sulfide	2.7	Org. C-mix and sulfide	4.4 +0.3 mmol/d	1.5	0.15	1.7	
Feast and famine	2.7	Org. C-mix	4.4	1.5	0.15	1.7	2)
No copper addition	2.7	Org. C-mix	4.4	1.5	0.15	1.7	3)
Low pH (6.8)	2.7	Org. C-mix	4.4	1.5	0.15	1.7	
Natural seawater	2.7	Org. C-mix	4.4	1.5	0.15	1.7	4)
400 day nitrate + organic carbon	3	Org. C-mix	33.0	-	11.0	See Fig. 2	5)
Short generation time	3	Org. C-mix	43.2	-	14.4	See Fig. 7	
Nitrite instead of nitrate	3	Org. C-mix	17.3	5.8	-	3.4	
Organic carbon limiting	2	Org. C-mix	11.5	-	5.8	3.4	

[1)] Data on the enrichment have been published in Strous et al, 2012
[2)] Medium was fed in pulses leading to the transient accumulation of nitrite. The generation time refers to the overall average.
[3)] No copper was supplied with the trace elements
[4)] The substrates were added to sterilized seawater from the sampling site.
[5)] Substrate influx given for a generation time of 1.7 days

Table S5.2: Characteristics and abundance estimates of the 11 metagenomic bins based on the metagenomic analyses and CARD-FISH.

		Bin										
		A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**
Affiliation		Denitrovibrio	Clostridiales	Firmicutes	Desulfuromusa	Clostridiales	Arcobacter	Spirochaetales	Sulfurospirillum	Bacteroidetes	Rhodobacteraceae	Alteromonadales
Bin size (Mb)		2.9	6.4	5.1	4.2	4.4	3.1	3.3	3.3	4.3	4.2	3.8
GC content (%)		42	34.3	37.3	45.7	37.5	28.1	46.2	31	40.7	63.4	57.7
N50 contig length (kb)		260.9	87.6	59.3	23.5	77.5	4.8	31.8	0.9	0.6	95.8	27.9
Contigs (#)		22	224	235	294	105	1693	182	4944	7709	76	192
coverage (x)		95.9	36.2	34.1	34.3	33.9	23.2	17.6	12.5	6	17	11.3
Transfer RNA's (#)		35	49	36	42	39	29	46	10	38	46	38
Completeness (%)		72	94	92	99	86	91	92	68	63	86	91
Redundancy (%)		0	6	2	2	1	6	1	6	11	2	2
Rel. abundance metagenomes (%)	Day 54	0	0.1	5.4	0.1	0	6.6	0	1.3	0.2	33.4	5.6
	Day 152	0	35.7	6.9	21.5	6.4	1.0	0	13.7	1.1	0	0
	Day 189	0.1	3.4	16.8	24.1	18.1	14.6	0.2	1.7	0.9	0.1	0.3
	Day 336	38.0	19.3	11.8	0.4	8.6	0.2	7.7	0.3	2.4	0	0.3
	Short Td	14.2	1.8	8.5	4.9	35.8	15.6	0.0	1.6	0	0	0.1
	Nitrite	5.1	0.7	2.7	0	2.1	0	12.1	0	5.0	25.7	16.5
Rel. abundance transcriptomes (%)	Day 189	0.1	1.6	26.3	18.5	15.4	16.9	0.2	0.3	0.3	0.5	0
	Day 199	0.0	2.4	19.8	19.2	28.9	7.7	0	0.6	1.2	0.3	0
	Day 207	0.1	2.1	8.5	39.6	17.5	9.3	0	1.4	1.3	0.4	0
	Day 336	34.2	13.2	12.3	0.3	11.6	0	7.1	0	1.1	0.3	0
	Day 368	35.2	3.3	9.3	0.3	35.5	2.5	1.6	0.1	0.7	0.4	0.2
Rel. abundance proteomes (%)	Day 189	0.2	4.2	20.0	25.9	14.5	33.8	0.1	1.2	0	0	0
	Day 207	0.1	5.7	17.6	33.4	13.1	26.6	0	3.4	0	0	0
	Day 336	40.5	25.6	16.3	1.1	9.9	0.3	6.1	0.1	0	0	0

Population		**Denitrovibrio**	**Arcobacter**	**Epsilon proteobacteria**	**Delta proteobacteria**	**Clostridiales**
Probes used (Table S4)		**N2460A**	**ARC94+ ARC1430**	**Epsy682**	**DELTA495abc**	**Lac0435 +CLO864**
Rel. abundance CARD-FISH (%)	Day 189	0	62	63	15	17
	Day 336	57	0	1	2	2

Table S5.3: Average (n=8) normalized transcriptional/translational activities of key metabolic pathways and enzyme complexes for the abundant populations selected in the continuous incubations (see Table S14 for gene annotations and activities for all bins).

Bin	A	B	C	D	E	F	G	H	I	J	K
Pathway or enzyme complex	Denitrovibrio	Lachnospira	Firmicutes	Desulfuromusa	Lachnospira	Arcobacter	Sphaerochete	Sulfurospirillum	Bacteroidetes	Rhodobacter	Alteromonas
Nitrate reductase	5±2	0±0	0±0	4±1	0±0	7±4	0±0	12±10	0±0	0±0	1±1
Denitrification	0±0	0±0	0±0	0±0	0±0	8±2	0±0	0±0	3±1*	21±17	4±0
Ammonification	14±9	5±3*	3±3*	11±11	1±1*	0±0	0±0	0±0	11±10*	0±0	0±0
Respiratory chain	12±0	5±4	5±0	15±3	7±4	20±5	4±1	14±8	13±16	27±39	7±10
ATP synthase	7±2	7±5	22±4	6±2	6±3	12±6	2±2	4±1	11±6	12±14	8±4
Hydrogen/formate oxidation	5±4	6±3	7±2	4±3	5±1	6±3	4±1	17±8	4±5	1±1	0±0
Sulfur metabolism	9±4	2±0	1±1	3±1	1±1	7±3	0±0	3±4	9±2	0±0	0±0
Acetate metabolism/fermentation	1±1	3±2	4±3	2±1	6±4	5±1	3±2	1±0	4±3	2±3	1±1
Citric acid cycle	28±3	3±3	4±2	23±11	4±3	19±4	1±1	15±9	28±5	29±23	63±31
Glycolysis/Pentose phosphate	8±7	17±14	23±12	5±4	19±13	5±4	15±9	6±4	9±1	0±0	6±0
Substrate import	10±3	51±31	30±24	27±7	51±24	10±1	71±7	28±8	9±1	8±11	10±14

Table S5.4: List of oligonucleotide probes and primers used (see www.microbial-ecology.net/probebase/).

Probe	Sequence (5'→3')	Target organisms	FA[1] (%)	Ref
EUB338 I[2]	GCTGCCTCCCGTAGGAGT	Most Bacteria	35	Amann *et al.*, 1990
EUB338 II[2]	GCAGCCACCCGTAGGTGT	Planctomycetales	35	Daims *et al.*, 1999
EUB338 III[2]	GCTGCCACCCGTAGGTGT	Verrucomicrobiales	35	Daims *et al.*, 1999
NON338	ACTCCTACGGGAGGCAGC	Control	35	Wallner *et al.*, 1993
Epsy682	CGGATTTTACCCCTACAC	Epsilonproteobacteria	35	Moussard *et al.*, 2006
ARC94	TGCGCCACTTAGCTGACA	*Arcobacter*	25	Snaidr *et al.*, 1997
ARC1430	TTAGCATCCCCGCTTCGA	*Arcobacter*	25	Snaidr *et al.*, 1997
DELTA495a (& competitor)[2]	AGTTAGCCGGTGCTTCCT (AGTTAGCCGGTGCTTCTT)	Most Deltaproteobacteria and most Gemmatimonadetes	25	Lueker et al.,2002, Loy et al., 2007
DELTA495b (& competitor)[2]	AGTTAGCCGGCGCTTCCT (AGTTAGCCGGCGCTTC(T/G)T)	Some Deltaproteobacteria	25	Lueker et al.,2002, Loy et al., 2007
DELTA495c (& competitor)[2]	AATTAGCCGGTGCTTCCT (AATTAGCCGGTGCTTCTT)	Some Deltaproteobacteria	25	Lueker et al.,2002, Loy et al., 2007
Lac0435	TCTTCCCTGCTGATAGA	Lachnospira	35	Kong *et al.*, 2010
CLO864	TTCCTCCTAATATCT ACGCA	Clostridiales	30	This study
Denitrovibrio N2460A	GAACCATTTCTTCCCTGCTG	Denitrovibrio	5	Myhr & Torsvik 2000

[1]Formamide concentration in the CARD FISH hybridization buffer
[2]Used in the mix of EUB I to III or DELTA495a –c

Table S5.5: Squared Pearson product-moment correlation coefficients between bin coverages and 16S rRNA gene sequencing coverages over sequenced samples. Correlations agreed well with taxonomic signatures of bins except for bins B, C and bin G. This was caused by low read counts (bin G) and by higher Firmicutes diversity binned into bins B and C.

	Denitrovibrio	**Clostridiales**	**Firmicutes**	**Clostridiales**	**Desulfuromusa**	**Clostridium**	**Arcobacter**	**Spirochaetales**	**Sulfurospirillum**	**Bacteroidetes**	**Rhodobacteraceae**	**Alteromonadales**
A	**0.97**	0.55	0.47	0.12	0.14	0.23	0.04	0.02	0.13	0.20	0.15	0.04
B	0.06	**0.54**	0.02	0.03	0.03	0.01	0.21	0.05	0.67	0.04	0.31	0.17
C	0.07	0.10	**0.69**	**0.61**	0.57	0.43	0.39	0.55	0.00	0.04	0.43	0.38
D	0.18	0.00	0.02	0.71	**0.88**	0.03	0.16	0.04	0.53	0.28	0.33	0.19
E	0.00	0.02	0.06	0.00	0.13	**0.84**	0.68	0.02	0.00	0.19	0.33	0.19
F	0.11	0.25	0.01	0.14	0.28	0.44	**0.95**	0.03	0.02	0.34	0.04	0.18
G	0.18	0.04	0.01	0.24	0.26	0.07	0.29	**0.00**	0.23	0.95	0.04	0.62
H	0.11	0.06	0.09	0.06	0.10	0.05	0.06	0.19	**0.98**	0.29	0.13	0.13
I	0.04	0.01	0.00	0.13	0.12	0.17	0.30	0.00	0.09	**0.83**	0.04	0.74
J	0.12	0.32	0.40	0.15	0.36	0.58	0.17	0.01	0.21	0.09	**0.99**	0.42
K	0.07	0.22	0.26	0.23	0.26	0.40	0.18	0.04	0.20	0.45	0.50	**0.97**

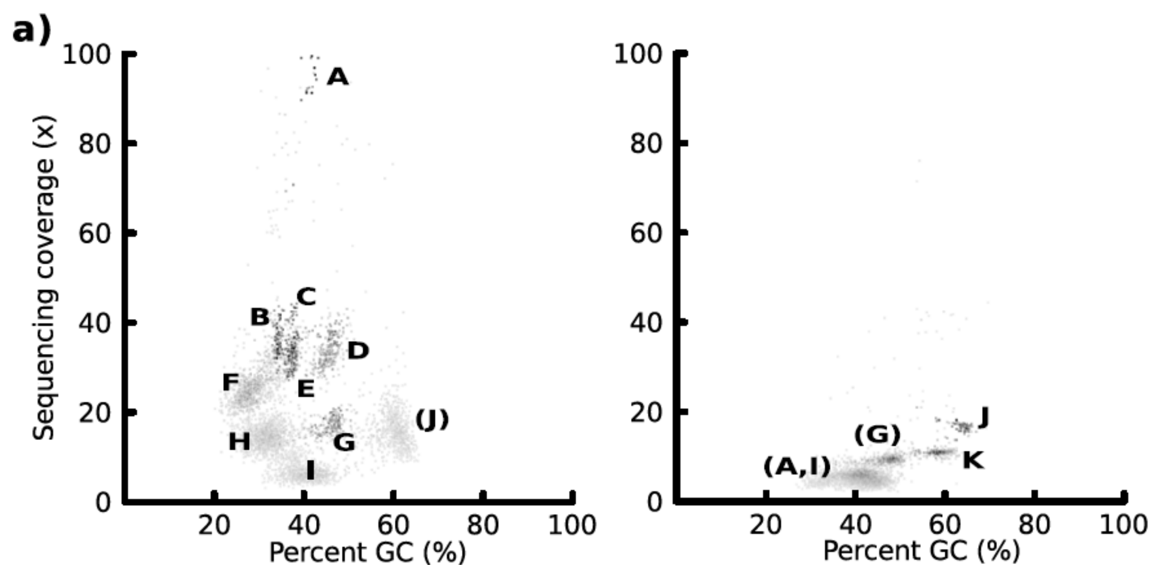
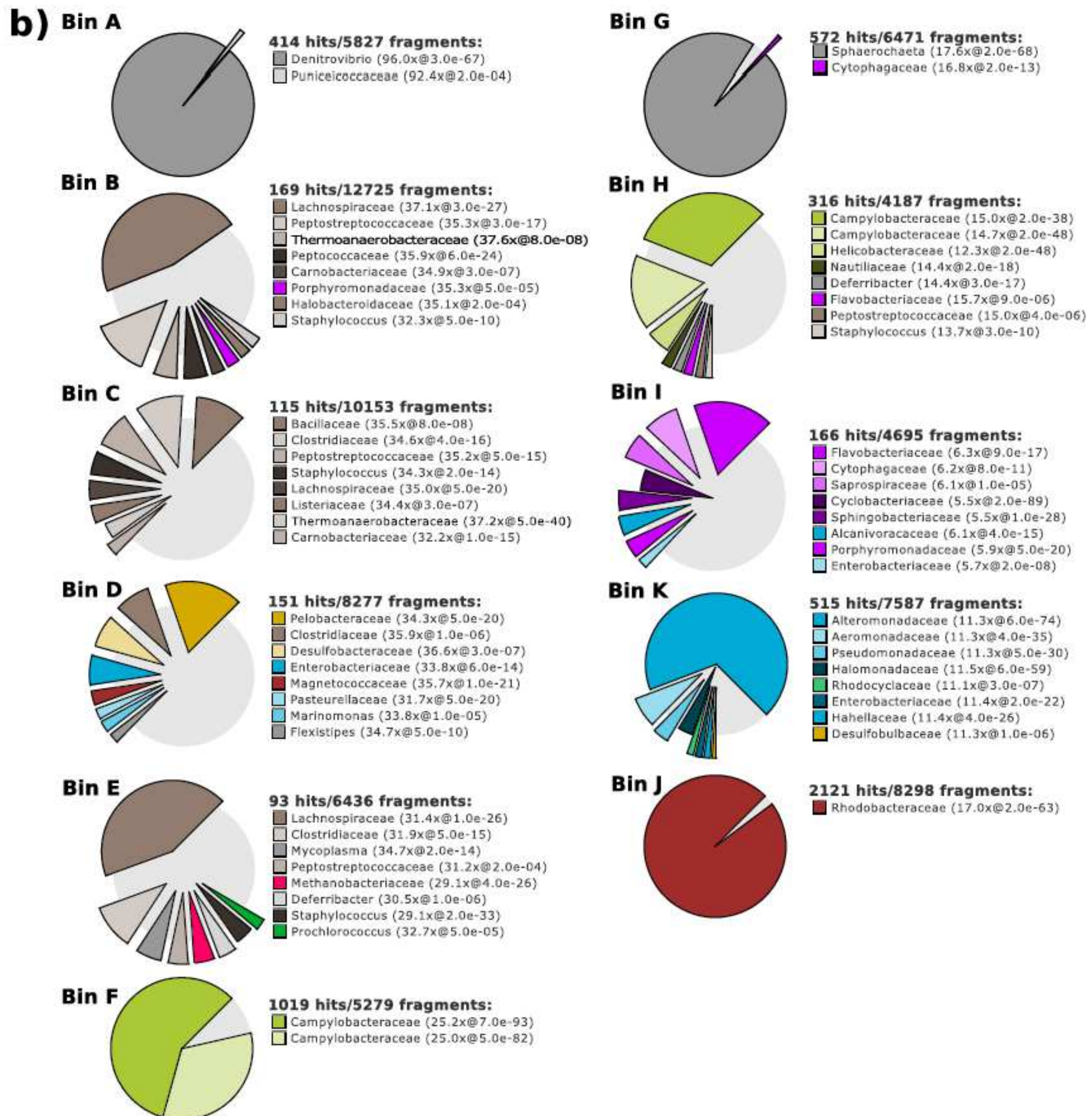


Figure S5.1: Binning of contigs to the most abundant populations in the samples of different time points by combined tetranucleotide and interpolated Markov Model binning: a)(*this page*) The distribution of the contigs on a sequencing coverage versus GC plot with each "cloud" corresponding to a different bin and population. Left: combined assembly for metagenomes of day 54,152, 189 and 336 (Figure 5.2). Right: assembly of day 52 of the incubation with nitrite (Figure S5.7b). Brackets indicate that a bin was detected in the sample but was better assembled in another sample. b) (*facing page*) The pie charts show the taxonomic distribution of blast hits of fragmented contigs to reference genomes. The distance of each slice from the center of the pie is a measure for the median e-value of the associated hits (larger e-values correspond to larger distances from the center).


b)
Bin A
414 hits/5827 fragments:
Denitrovibrio (96.0x@3.0e-67)
Puniceicoccaceae (92.4x@2.0e-04)
Bin B
169 hits/12725 fragments:
Lachnospiraceae (37.1x@3.0e-27)
Peptostreptococcaceae (35.3x@3.0e-17)
Thermoanaerobacteraceae (37.6x@8.0e-08)
Peptococcaceae (35.9x@6.0e-24)
Carnobacteriaceae (34.9x@3.0e-07)
Porphyromonadaceae (35.3x@5.0e-05)
Halobacteroidaceae (35.1x@2.0e-04)
Staphylococcus (32.3x@5.0e-10)
Bin C
115 hits/10153 fragments:
Bacillaceae (35.5x@8.0e-08)
Clostridiaceae (34.6x@4.0e-16)
Peptostreptococcaceae (35.2x@5.0e-15)
Staphylococcus (34.3x@2.0e-14)
Lachnospiraceae (35.0x@5.0e-20)
Listeriaceae (34.4x@3.0e-07)
Thermoanaerobacteraceae (37.2x@5.0e-40)
Carnobacteriaceae (32.2x@1.0e-15)
Bin D
151 hits/8277 fragments:
Pelobacteraceae (34.3x@5.0e-20)
Clostridiaceae (35.9x@1.0e-06)
Desulfobacteraceae (36.6x@3.0e-07)
Enterobacteriaceae (33.8x@6.0e-14)
Magnetococcaceae (35.7x@1.0e-21)
Pasteurellaceae (31.7x@5.0e-20)
Marinomonas (33.8x@1.0e-05)
Flexistipes (34.7x@5.0e-10)
Bin E
93 hits/6436 fragments:
Lachnospiraceae (31.4x@1.0e-26)
Clostridiaceae (31.9x@5.0e-15)
Mycoplasma (34.7x@2.0e-14)
Peptostreptococcaceae (31.2x@2.0e-04)
Methanobacteriaceae (29.1x@4.0e-26)
Deferribacter (30.5x@1.0e-06)
Staphylococcus (29.1x@2.0e-33)
Prochlorococcus (32.7x@5.0e-05)
Bin F
1019 hits/5279 fragments:
Campylobacteraceae (25.2x@7.0e-93)
Campylobacteraceae (25.0x@5.0e-82)
Bin G
572 hits/6471 fragments:
Sphaerochaeta (17.6x@2.0e-68)
Cytophagaceae (16.8x@2.0e-13)
Bin H
316 hits/4187 fragments:
Campylobacteraceae (15.0x@2.0e-38)
Campylobacteraceae (14.7x@2.0e-48)
Helicobacteraceae (12.3x@2.0e-48)
Nautiliaceae (14.4x@2.0e-18)
Deferribacter (14.4x@3.0e-17)
Flavobacteriaceae (15.7x@9.0e-06)
Peptostreptococcaceae (15.0x@4.0e-06)
Staphylococcus (13.7x@3.0e-10)
Bin I
166 hits/4695 fragments:
Flavobacteriaceae (6.3x@9.0e-17)
Cytophagaceae (6.2x@8.0e-11)
Saprospiraceae (6.1x@1.0e-05)
Cyclobacteriaceae (5.5x@2.0e-89)
Sphingobacteriaceae (5.5x@1.0e-28)
Alcanivoracaceae (6.1x@4.0e-15)
Porphyromonadaceae (5.9x@5.0e-20)
Enterobacteriaceae (5.7x@2.0e-08)
Bin K
515 hits/7587 fragments:
Alteromonadaceae (11.3x@6.0e-74)
Aeromonadaceae (11.3x@4.0e-35)
Pseudomonadaceae (11.3x@5.0e-30)
Halomonadaceae (11.5x@6.0e-59)
Rhodocyclaceae (11.1x@3.0e-07)
Enterobacteriaceae (11.4x@2.0e-22)
Hahellaceae (11.4x@4.0e-26)
Desulfobulbaceae (11.3x@1.0e-06)
Bin J
2121 hits/8298 fragments:
Rhodobacteraceae (17.0x@2.0e-63)

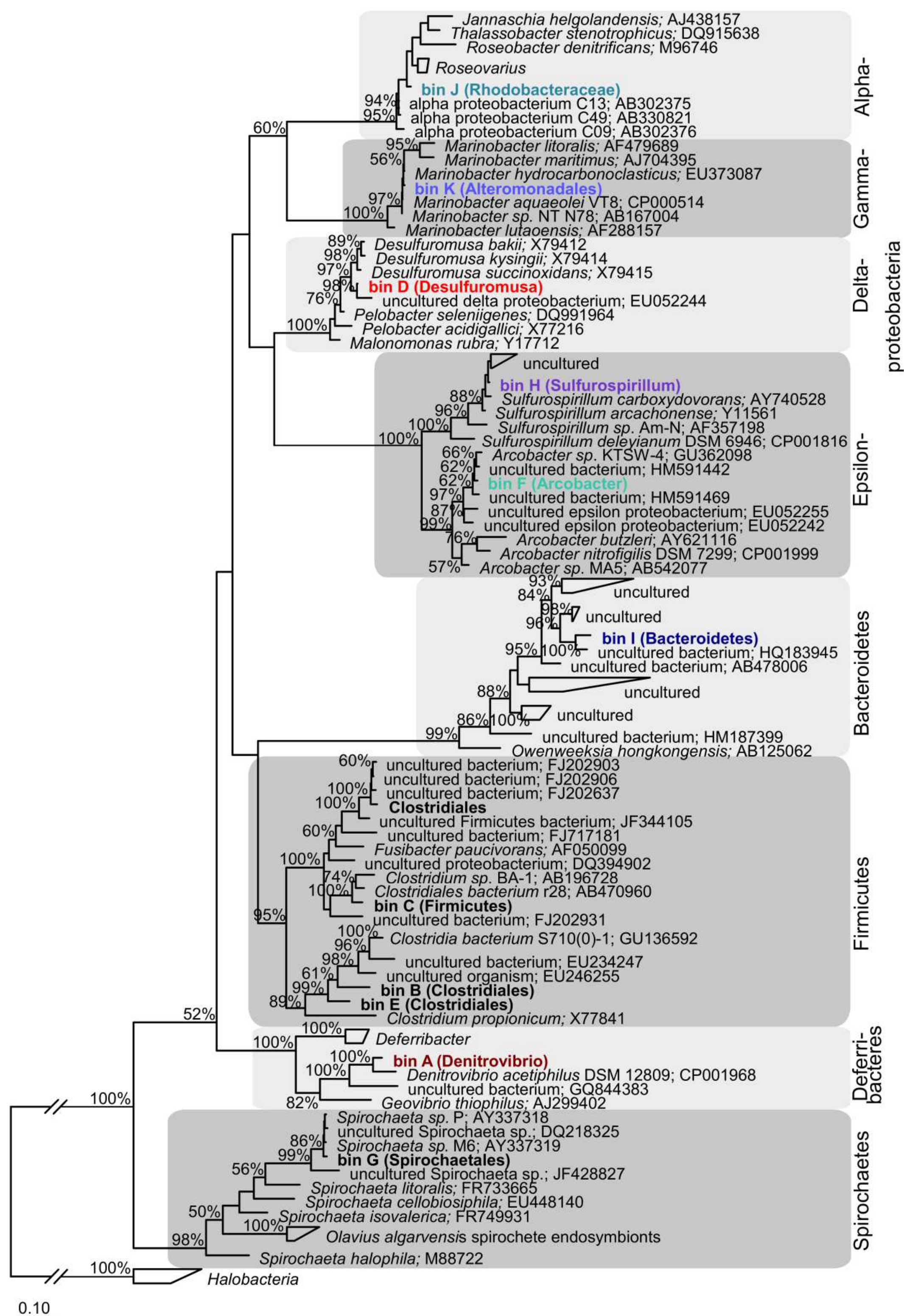


Figure S5.2 Phylogenetic tree of bacterial 16S rRNA sequences determined by maximum likelihood (RAxML). Bootstrap Values higher than 50% are given. Sequences from this study are written in bold.

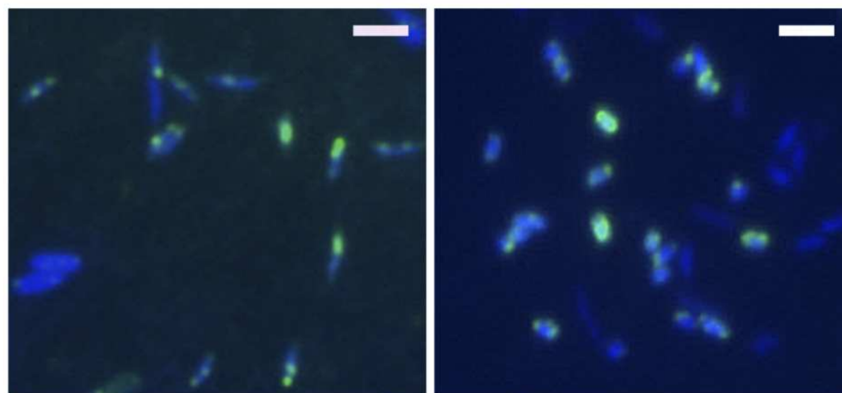


Figure S5.3 Epifluorescence microscopy image (CARD-FISH) of the dominant denitrifying (Arcobacter, probes ARC94, ARC1430, left) and arnrnonifying populations (*Denitrovibrio*, probe N246oa, right) present in the incubation of Figure 5.2 (probes see table S5.4). Green: hybridized cells; blue: DAPI-stained cells. Scale bar: 2 µm.

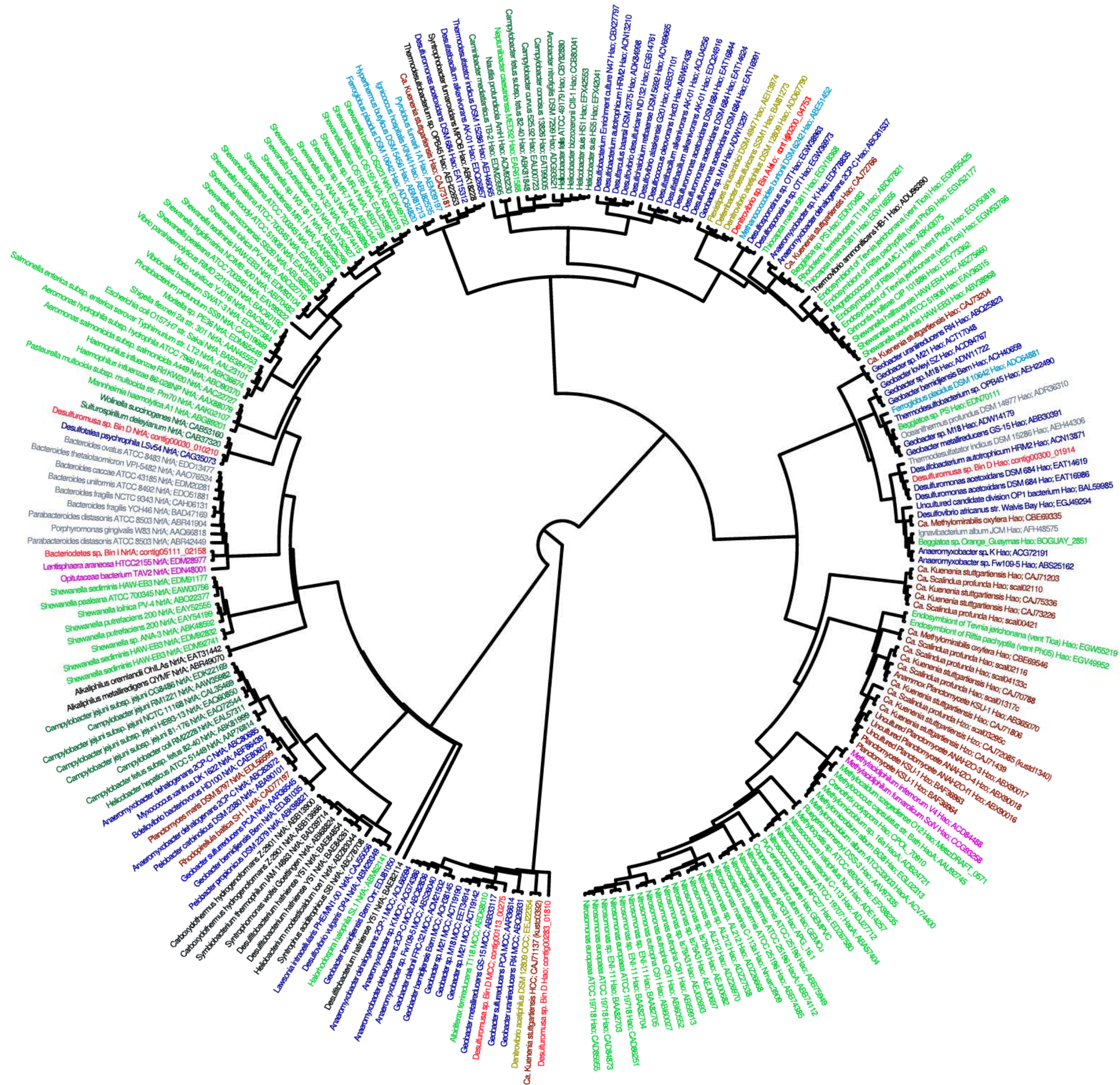


Figure S5.4. Phylogenetic consensus tree constructed after Bayesian inference of phylogeny from a MUSCLE alignment of multiheme cytochrome *c* (MCC) protein sequences. Experimentally derived MCC sequences were aligned and analyzed together with sequences of pentaheme cytochrome *c* nitrate reductase (NrfA) and octaheme cytochrome *c* (OCC) oxidoreductase proteins obtained from public databases, some of which have been experimentally identified as octaheme cytochrome *c* nitrite reductases (ONR), hydroxylamine (HAO) or hydrazine (HZO) dehydrogenases. MCC protein sequences from a different member of the superfamily were used as out-group for tree construction. Sequences from this study are colored in red, Alpha-, Beta- and Gammaproteobacteria in light green, Deltaproteobacteria in dark blue, Epsilonrnicrobia in dark green, Planctomycetes and NC10 in dark red, Verrucomicrobia in pink, Firrnicutes in black, Deferribacteres in dark yellow and Archaea in light blue, others in grey.

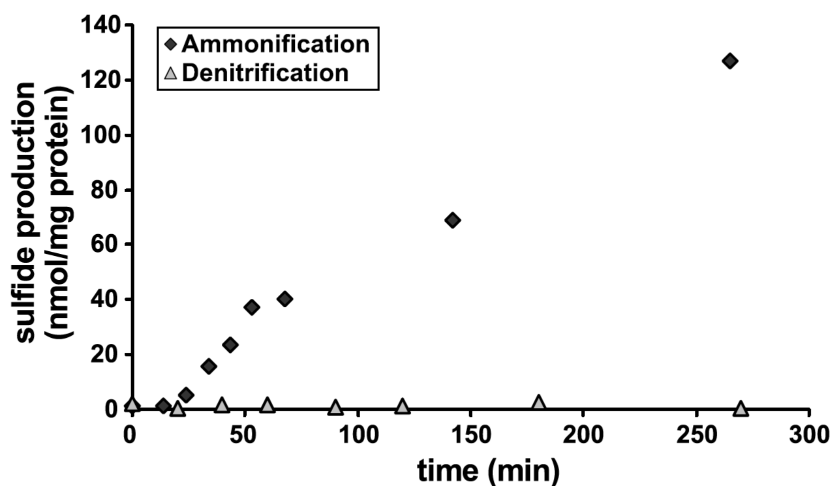


Figure S5.5 Sulfate-reduction activity of denitrifying and ammonifying cultures determined by radiotracer labeling experiments. The radio tracer experiment with denitrification as the dominating pathway, did not yield significant production of labeled sulfide (performed on day 61 of the transfer with short generation time, figure S5.7a), but the unlabeled sulfide pool was consumed after the experiment. This suggests that reduction and oxidation reactions of sulfur species were closely linked and that sulfide was produced by microbial sulfate reduction and directly oxidized again, coupled to nitrate reduction. This is supported by experimentally determined high potential rates of sulfide-dependent reduction of nitrate and nitrite (table 5.1) and the finding that sulfide accumulated in experiments without nitrate or nitrite, confirming ongoing sulfate reduction.

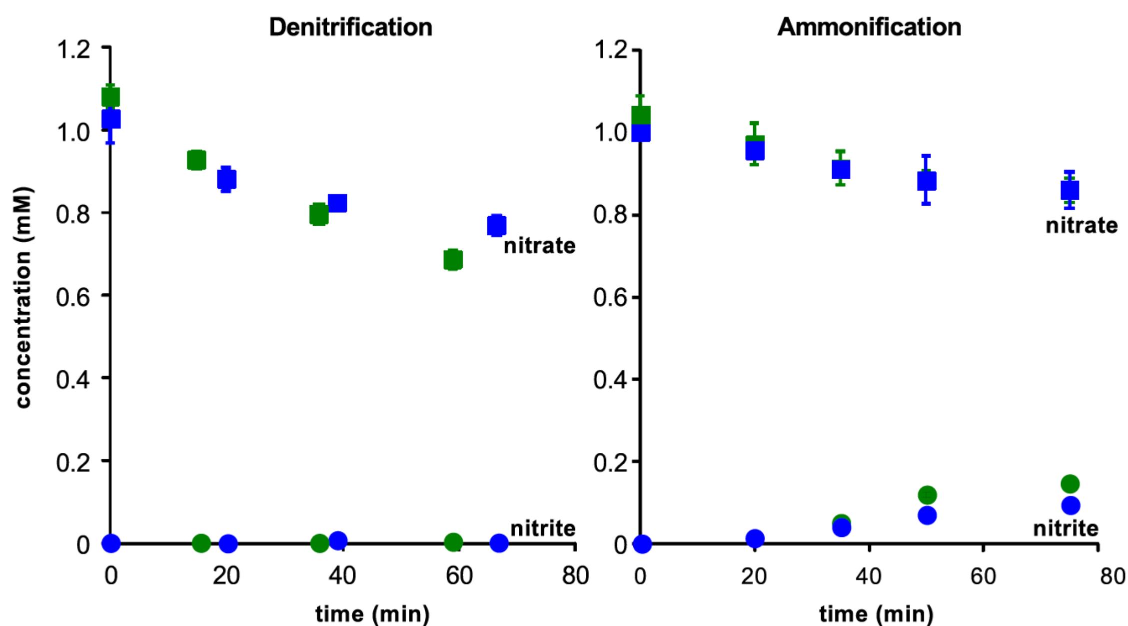


Figure S5.6: Sulfate-reduction activity of denitrifying and ammonifying cultures determined by radiotracer labeling experiments. The radio tracer experiment with denitrification as the dominating pathway, did not yield significant production of labeled sulfide (performed on day 61 of the transfer with short generation time, figure S5.7a), but the unlabeled sulfide pool was consumed after the experiment. This suggests that reduction and oxidation reactions of sulfur species were closely linked and that sulfide was produced by microbial sulfate reduction and directly oxidized again, coupled to nitrate reduction. This is supported by experimentally determined high potential rates of sulfide-dependent reduction of nitrate and nitrite (table 5.1) and the finding that sulfide accumulated in experiments without nitrate or nitrite, confirming ongoing sulfate reduction.

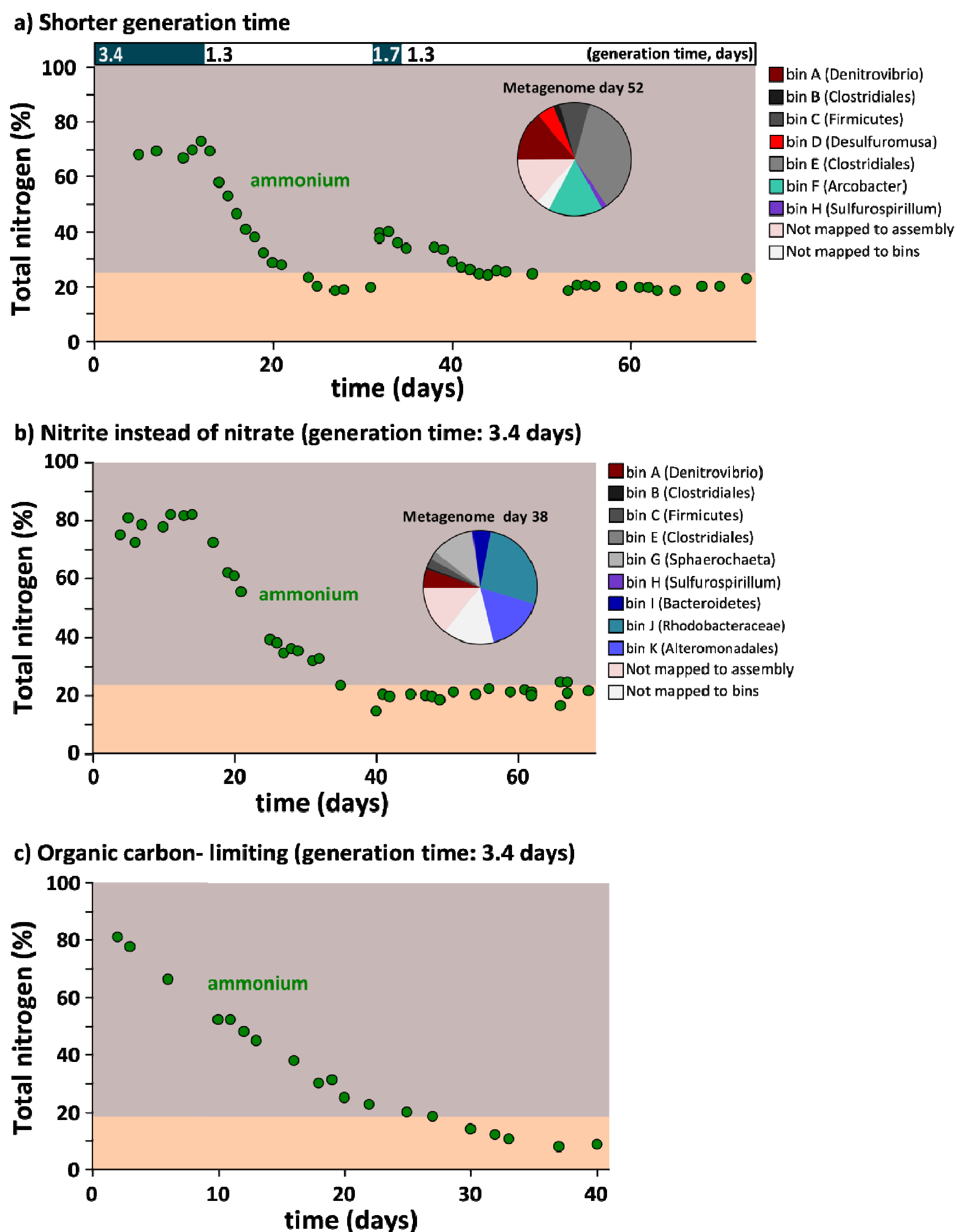


Figure S5.7: Fate of nitrate/nitrite in incubations inoculated with bacterial biomass from the incubation of figure 5.2. after 400 days. The shaded areas indicate the composition of nitrogen compounds in the inflowing medium, bronze for nitrate/nitrite and apricot for organic nitrogen from the amino acids provided as substrates. Green circles indicate the percentage of inflowing nitrogen that was converted to ammonium. If the amount of ammonium produced did not exceed the amount of inflowing organic nitrogen, it most likely originated from the degradation of organic nitrogen supplied in the medium. This was confirmed by the detection of nitrogen (N_2) as the main product. Nitrite and nitrate concentrations were <0.5 µM and <10 µM respectively. S.D. (n=3) values did not exceed symbol diameters.

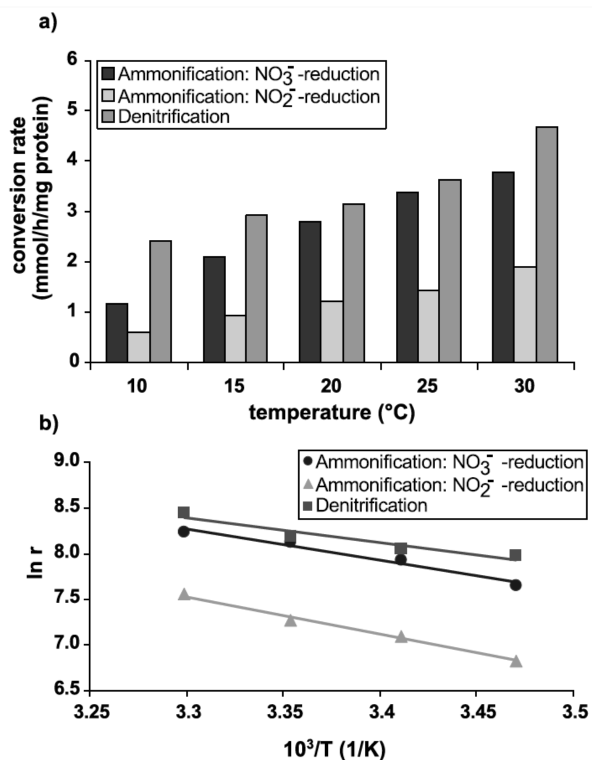


Figure S5.8: Temperature dependence of denitrification and ammonification. a) Temperature dependence of nitrate reduction rates. As nitrite accumulated for incubations when ammonification dominated, in this case, the conversion rate for nitrite as substrate was determined as well; b) Arrhenius plot. r: nitrate reduction rate, T: absolute temperature.

The additional data table listed below can be found at

http://www.sciencemag.org/content/345/6197/676/suppl/DC1

Additional Data table S6 (separate file)

Settings used by MyriMatch (2.1.111) and IDPicker (3.0.537) to search and filter MS data

Additional Data table S7 (separate file)

Summary of MyriMatch/Idpicker results from searching two technical replicates for each time point against the predicted metaproteome.

Additional Data table S8a (separate file)

List of proteins identified in replicate 1 of sample BK1 (Day 189) by MyriMatch/Idpicker platform by searching raw data against the predicted metagenome.

Additional Data table S8b (separate file)

List of proteins identified in replicate 2 of sample BK1 (Day 189) by MyriMatch/Idpicker platform by searching raw data against the predicted metagenome.

Additional Data table S9a (separate file)

List of proteins identified in replicate 1 of sample BK2 (Day 207) by MyriMatch/Idpicker platfrom by searching raw data against the predicted metagenome.

Additional Data table S9b (separate file)

List of proteins identified in replicate 2 of sample BK2 (Day 207) by MyriMatch/Idpicker platfrom by searching raw data against the predicted metagenome.

Additional Data table S10a (separate file)

List of proteins identified in replicate 1 of sample BK3 (Day 336) by MyriMatch/Idpicker platfrom by searching raw data against the predicted metagenome.

Additional Data table S10b (separate file)

List of proteins identified in replicate 2 of sample BK3 (Day 336) by MyriMatch/Idpicker platfrom by searching raw data against the predicted metagenome.

Additional Data table S11a (separate file)

List of peptides identified in replicate 1 of sample BK1 (Day 189) by MyriMatch/Idpicker platfrom by searching raw data against the predicted metagenome.

Additional Data table S11b (separate file)

List of peptides identified in replicate 2 of sample BK1 (Day 189) by MyriMatch/Idpicker platfrom by searching raw data against the predicted metagenome.

Additional Data table S12a (separate file)

List of peptides identified in replicate 1 of sample BK2 (Day 207) by MyriMatch/Idpicker platfrom by searching raw data against the predicted metagenome.

Additional Data table S12b (separate file)

List of peptides identified in replicate 2 of sample BK2 (Day 207) by MyriMatch/Idpicker platfrom by searching raw data against the predicted metagenome.

Additional Data table S13a (separate file)

List of peptides identified in replicate 1 of sample BK3 (Day 336) by MyriMatch/Idpicker platfrom by searching raw data against the predicted metagenome.

Additional Data table S13b (separate file)

List of peptides identified in replicate 1 of sample BK3 (Day 336) by MyriMatch/Idpicker platfrom by searching raw data against the predicted metagenome.

Additional Data table S14 (separate file)

Annotated provisional genomes for each bin with transcriptomic and proteomic activities for each gene.

5.4.2 References

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Chapter 6

6 Integration and perspectives

This thesis contributes to a more comprehensive understanding of the ecophysiology of two different pathways of nitrate respiration, denitrification and DNRA. To achieve this, the enrichment of microbial communities under nitrate reducing conditions was combined with metagenomic sequencing with the aim of identifying the metabolic potential of the enriched populations. In order to improve the analysis of the metagenomic datasets obtained, a new tool for the binning of metagenomic contigs was developed (chapter 3).

First, the relationship between functional and compositional stability over time within one nitrate reduction pathway was investigated (chapter 4). A heterotrophic denitrifying microbial community was enriched from a marine intertidal flat in a continuous culture. By monitoring the community composition over time it could be shown that strong community dynamics were occurring under constant conditions and during stable conversion of substrates. This indicated that for functional stability only the presence of the overall metabolic potential was important, independent of the community composition. Denitrifiers were co-enriched with fermenting populations and a stable metabolic interaction between the two microbial guilds persisted throughout the experiment unaffected by the ongoing population dynamics.

Once the ongoing competition between different populations within one nitrate reducing pathway was evaluated, the competition between different nitrate-respiration pathways could be addressed. Three environmental controls on the outcome of microbial nitrate reduction were identified (chapter 5). DNRA was the prevalent respiratory pathway when nitrate (but not nitrite) was the main electron acceptor, when the microbial generation time was long and when nitrate (but not carbon) was in limited supply. With the identification of these environmental conditions that decide which of the pathways dominates, new insights into the controls that regulate the fate of fixed nitrogen in the environment were obtained. Furthermore, sulfide, produced by microbial sulfate reduction, was identified as an important electron donor for denitrification and DNRA. Sulfide oxidation was responsible for reducing ~25% of the nitrate reduction revealing a close coupling between nitrate reduction and cycling of sulfur.

While changes in the nitrate reduction process itself and the associated microbial populations were induced based on the factors listed above, the general functional community composition and metabolic partitioning between fermenting and nitrate reducing populations stayed the same.

6.1 Functional stability is independent of community composition

Inherent to all enriched microbial communities was the syntrophy of fermenting and nitrate reducing populations. In all cases fermentation products were utilized as substrates by the nitrate-respiring populations. This functional structure was stable over time and over all different realizations of enrichments in which this was addressed (chapters 3, 4 and 5). Furthermore, this syntrophy was established over a range of different generation times and was independent of the predominating nitrate reduction pathway.

In contrast to the functional stability, the microbial community composition was unstable: The persistence of population dynamics under stable conditions was shown for two denitrifying enrichments and with different independent methods (chapters 3 and 4). Although enriched communities were highly dynamic, the diversity of the dominant populations was low: Fluctuations occurred between few different populations and generally the same populations were enriched in the different chemostat enrichments. The denitrifying populations affiliated with *Arcobacter*, *Pseudomonas*, *Rhodobacterales*, *Pseudovibrio* and *Bacteroidetes* and the DNRA-performing populations with *Denitrovibrio* and *Desulfuromusa*. The dominant fermenting populations affiliated with *Vibrio*, *Clostridiales*, *Fusobacter* and *Psychromonas*. Fluctuations also occurred between different subpopulations of *Arcobacter*, *Rhodobacterales* and *Clostridiales.* Functional redundancy among different populations within a community is thought to be a cornerstone of a stable performance, as populations able to perform the same ecological function may replace the dominant population of the same microbial guild in the case of its break down, e.g. due to disturbances or viral lysis (Briones and Raskin, 2003; Curtis and Sloan, 2004, Norberg *et al*., 2001, Allison and Martiny, 2008). Functional redundancy was not only observed on the level of populations that possessed the same metabolic capacities but also on the enzymatic level. Subpopulations of *Pseudovibrio* with the genes for different types of NO-forming nitrite reductases, *nirS* and *nirK*, were co-enriched (chapter 4). Upon the different shifts from denitrification to DNRA, not only different microbial populations built up, but they also

carried out different pathways for the reduction of nitrate to ammonium (chapter 5). A *Desulfuromusa* population possessed the nitrite reductase NrfA, while the *Denitrovibrio* population apparently reduced nitrite via an octaheme cytochrome *c* to hydroxylamine and then further to ammonium via the hybrid cluster protein hydroxylamine reductase. However, it remains to be determined whether these two pathways are indeed functionally redundant or whether the enzymes are characterized by different enzyme kinetics.

6.2 Population dynamics are driven by deterministic and neutral processes

In chapter 4, it was argued that the community composition was influenced by niche-based selection as well as neutral processes, with niche-based sorting being responsible for the selection of denitrifying and fermenting microbes and neutral processes for the specific populations of the respective guild encountered at a certain time point. The reasoning behind this was that the conditions encountered in these chemostat experiments were constant and thus changes in community composition can be regarded as neutral. The comparison of the microbial community composition between enrichment experiments confirms this hypothesis. The combination of the applied experimental conditions reproducibly selected for a specific set of populations that was best adapted to the environment created in the chemostats. There was no evident driving force that controlled which populations dominated at a certain time point.

Several previous studies also came to the conclusion that a combination of neutral and deterministic processes governs the assembly and further development of microbial communities over time (Zhou *et al*., 2013, Ofiteru *et al*., 2010, Zumstein *et al*., 2000, Langenheder and Szekely, 2011, Fernandez *et al*., 1999). Other studies reported contrasting results and concluded that community dynamics were primarily governed by deterministic processes (Vanwonterghem *et al*., 2014, McGuiness *et al*., 2006, Falk 2009). In this context, it should be taken into account that the experimental set-up may very likely influence the experimental outcome. Some studies made use of fed-batch systems (Vanwonterghem *et al*., 2014, Falk *et al*., 2009) and a reactor set-up that facilitates attachment and biofilm formation such as granules, electrodes, suspended substrate particles (Zumstein *et al*., 2000, McGuinesss, Zhou *et al*., 2012, Vanwonterghem *et al*., 2014).

In wastewater treatment plants or other large-scale applications the inflowing substrate concentration and composition is often not constant (Valentín-Vargas *et al*., 2012, Ofiteru *et al*., 2010). Thus, changes in community composition may also have been influenced by the change of external conditions. A biofilm community may highly differ from the suspended biomass of the same reactor (Briones and Raskin, 2003) and biofilms can be highly spatially structured (Battin *et al*., 2007). Attached cells may be exposed to a different set of community structure shaping processes. These factors would explain the differences in community assembly and function-structure relationships observed in comparison to the enrichments described in this thesis. The community behavior observed in chapter 4 mostly resembled the results of a study by Fernandez *et al*. (1999), which was also performed in a well controlled laboratory chemostat with highly constant conditions over time.

In a chemostat functional redundancy and the ability of one population to build up to replace another may be more important for stable functioning and survival of the whole community compared to a biofilm-supporting environment.

6.3 Environmental controls that govern nitrate-respiration

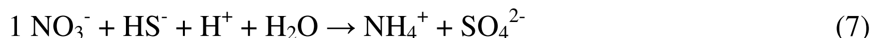
The generation time, supply of nitrite relative to nitrate and the carbon/nitrogen ratio (C/N-ratio) were identified as the selective forces that determined the outcome of the competition between denitrification and DNRA (chapter 5). Of these three controls, only the C/N-ratio has been previously proposed. Denitrifying bacteria conserve more energy per electron, which makes them more competitive when electron donors are in limited supply (chapter 1 and 2, Tiedje *et al*., 1982). The effect of the other two controls can be explained by the kinetics of the nitrite reductase active in either pathway. If the ammonium-producing nitrite reductases (NrfA and OCC) have a lower affinity for nitrite compared to the nitric oxide-forming nitrite reductases (NirS and NirK), at a high conversion rate, the DNRA-performing microbes would reduce nitrate to nitrite, which then would be lost to competing denitrifiers. A comparison of the enzyme kinetics of purified enzymes measured for different nitrite reductases from certain model organisms such as *Wolinella succinogenes, Desulfovibrio desulfuricans, Escherichia coli* or *Paraccocus pantotrophus* does not yield a clear trend (Clarke *et al*., 2005, Einsle, 2011, Kondo *et al*., 2012, Richter *et al*., 2002, Tikhonova *et al*., 2012). It is difficult to directly compare and evaluate the affinity of isolated enzymes because for example generally artificial electron donors are used. Furthermore, the actual *in vivo*

affinity may not only be determined by the enzyme itself, but the electron transfer to the enzyme may also play a role. For example, the ammonium-producing nitrite reductase NrfA requires six electrons, which are provided by three quinol molecules that sequentially dock at the quinol-binding site. In contrast, the NirS and NirK enzymes involved in denitrification only depend on the delivery of individual electrons. A slower operation rate of the ammonium-forming pentaheme and octaheme cytochrome *c* nitrite reductases compared to the NO-forming NirS and NirK enzymes would lead to a lower affinity for nitrite, and this explains both the dependence of DNRA on a supply of nitrate and on a lower generation time.

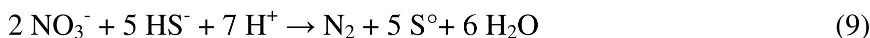
Up to 25% of the nitrate reduction was coupled to sulfide oxidation (chapter 5). The utilization of sulfide as electron donor next to organic carbon substrates for nitrate reduction adds an additional component to the bioenergetic considerations. Sulfide may be completely oxidized to sulfate or only partly to sulfur or thiosulfate. Metagenomics and metaproteomics, batch incubations with biomass from the chemostat enrichments and chemical analysis of sulfur compounds indicated that sulfur cycling most likely preceded via sulfur or sulfate (reactions 5-12).

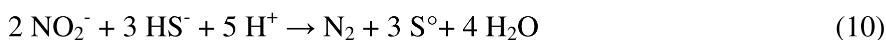
$$8\ NO_3^- + 5\ HS^- + 3\ H^+ \rightarrow 4\ N_2 + 5\ SO_4^{2-} + 4\ H_2O \qquad (5)$$

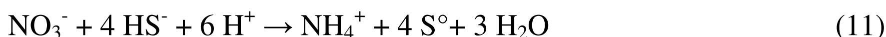
$$8\ NO_2^- + 3\ HS^- + 5\ H^+ \rightarrow 4\ N_2 + 3\ SO_4^{2-} + 4\ H_2O \qquad (6)$$

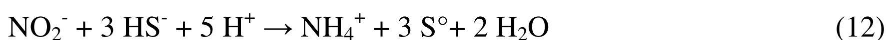

$$1\ NO_3^- + HS^- + H^+ + H_2O \rightarrow NH_4^+ + SO_4^{2-} \qquad (7)$$

$$4\ NO_2^- + 3\ HS^- + 5\ H^+ + 4\ H_2O \rightarrow 4\ NH_4^+ + 3\ SO_4^{2-} \qquad (8)$$


$$2\ NO_3^- + 5\ HS^- + 7\ H^+ \rightarrow N_2 + 5\ S^\circ + 6\ H_2O \qquad (9)$$


$$2\ NO_2^- + 3\ HS^- + 5\ H^+ \rightarrow N_2 + 3\ S^\circ + 4\ H_2O \qquad (10)$$


$$NO_3^- + 4\ HS^- + 6\ H^+ \rightarrow NH_4^+ + 4\ S^\circ + 3\ H_2O \qquad (11)$$


$$NO_2^- + 3\ HS^- + 5\ H^+ \rightarrow NH_4^+ + 3\ S^\circ + 2\ H_2O \qquad (12)$$

Remarkably, with sulfide as electron donor DNRA is not necessarily the bioenergetically more favorable pathway when the electron acceptor is limiting (Table 6.1). This is only the case if nitrate is the electron donor and sulfide is oxidized to sulfur. When sulfide is oxidized to sulfur and nitrite is the electron acceptor denitrification and DNRA have the same energy yield per electron acceptor. When sulfide is oxidized to sulfate and the electron acceptor is limiting denitrification is even slightly more favorable than DNRA. This

gives an additional explanation for the dominance of denitrification provided that sulfide is available.

Furthermore, the proportion, in which organic carbon compounds, hydrogen and sulfide are utilized as electron donor, may additionally influence the outcome of the competition between DNRA and denitrification.

Table 6.1: Theoretical energy yield of denitrification and DNRA and the electrons transferred with nitrate and nitrite as electron acceptor and sulfide as electron donor. ($G^{0'}$ values were taken from Thauer *et al.*, 1977.)

Reaction	Electron acceptor	Sulfide oxidized to	$\Delta G^{0'}$ (kJ/mol) per e^--donor	$\Delta G^{0'}$ (kJ/mol) per e^--acceptor	Electrons transferred per e^--acceptor
Denitrification	NO_3^-	S°	-197	-491	5
	NO_3^-	SO_4^{2-}	-745	-465	5
	NO_2^-	S°	-237	-356	3
	NO_2^-	SO_4^{2-}	-908	-340	3
DNRA	NO_3^-	S°	-181	-728	8
	NO_3^-	SO_4^{2-}	-448	-448	8
	NO_2^-	S°	-118	-355	6
	NO_2^-	SO_4^{2-}	-431	-323	6

6.4 An alternative pathway for DNRA

In the nitrate-reducing chemostat enrichment of chapter 5, a *Denitrovibrio* population was enriched when DNRA was the predominating nitrate reduction process. In the metagenomic bin of the *Denitrovibrio* population no gene encoding for NrfA, the pentaheme cytochrome *c* nitrite reductase typically associated with respiratory ammonification could be detected. Instead, an octaheme cytochrome *c* (OCC) protein-encoding gene was highly transcribed and translated together. The capacity to reduce nitrite has been shown for certain OCC proteins (Tikhonova *et al.*, 2006, Atkinson *et al.*, 2007), but only recently the first experimental evidence has been shown that an organism (*Nautila profundicola*) was reducing nitrate to ammonium with such an OCC protein as the responsible enzyme for the reduction of nitrite

(Hanson *et al*., 2013). The OCC proteins belong to the same large multiheme cytochrome *c* protein superfamily as NrfA (Klotz *et al*., 2008**,** Kern *et al*., 2011).

The sequence encoding the OCC from the metagenomic bin of the *Denitrovibrio* population clustered with sequences of the OCCs with proven nitrite reducing capabilities. It further shared structural characteristics such as the absence of a CxxCK motif at the active site, axial His residues aligning well with those of OCCs, a tyrosine forming an intermolecular cross-linking to the catalytic heme. Together with the OCC protein a hybrid cluster protein was highly expressed and most likely had a hydroxylamine reducing function. In the absence of other genes with a known function in nitrite reduction to ammonium in this bin or any other bin important on that time point, the detected OCC protein-encoding gene and the putative hydroxylamine reductase encoding gene are the only candidate genes encoding enzymes that together are capable of catalyzing nitrite reduction to ammonium.

It has been proposed that ammonium generation via the OCC protein in *Nautila profunidcola* is a novel nitrate assimilation pathway. In this thesis, I provide evidence that OCCs are involved in respiratory nitrate reduction and constitutes an alternative to the conventional pathway via NrfA. It remains to be determined how wide spread this pathway actually is in the environment. Hydroxylamine is highly toxic for the cell. Therefore, in nitrifying microbes the oxidation of hydroxylamine is tightly coupled to its production (Stein *et al*., 2012). Rate measurements of hydroxylamine oxidation indicate that this is also the case for hydroxylamine reduction in *Nautila profunidcola* (Hanson *et al*., 2013). This can explain why hydroxylamine has not been detected as intermediate in DNRA. So far, DNRA has mainly been studied for a few model organisms. Little is known about the identity of DNRA-performing microorganisms in natural habitats and the pathways that they perform. It would be interesting to investigate whether a niche differentiation between the DNRA pathways exists e.g. whether the two nitrite reductases have differences in affinity. Besides rate measurements, the importance of DNRA relative to denitrification is often evaluated by the abundance of relevant functional genes (chapter 2). Because usually only *nrfA* is used as a marker gene for DNRA, the importance of this pathway may have been substantially underestimated.

6.5 Can the identified controls explain the distribution of nitrate respiration pathways occurring *in situ*?

The different studies included in this thesis did not aim at reproducing the conditions and communities found *in situ*, but tested hypotheses that should apply to (marine) microbial metabolism and communities in general. However, this implies that these hypotheses and the identified controls should also, if not above all, hold true for the habitat the inoculum was taken from. Denitrification is the main nitrate reduction pathway at the sampling site, the tidal flat Janssand, while DNRA, although present, only constitutes up to 10% of the total nitrate reduction (Gao *et al*., 2012, Behrendt *et al*., 2013).

The dissolved organic carbon concentrations in the pore water of the sampling site accounts for several hundred micromoles per liter (Beck *et al*., 2008, Beck *et al*., 2009, Seidel *et al*., 2014), while nitrate concentrations of up to 60 µM have been measured (Gao *et al*., 2012). Thus, the C/N ratio is well in the range that would enable DNRA. However, this assessment relies on the assumption that most of the dissolved organic carbon is bioavailable.

Nitrate and nitrite concentrations are often determined together as total NO_x^-. This was also the case for the sampling site (Billerbeck *et al*., 2006, Gao *et al*., 2010, Gao., 2012). Although it can be assumed that most NO_x^- was present in the form of nitrate, no conclusion can be drawn with regard to the influence of nitrite on the nitrate reduction pathway. This example depicts the importance of the conceptual approach of this study: the role of the availability of nitrite as important driving force has so far not been recognized and consequently corresponding data were not recorded.

The generation time was identified as important control of the nitrate respiration pathway. In the environment this translates into the retention time of the system, which is difficult to determine. The tidal flat, where our sampling site was located, experiences a high advective flow (50-100 $l/m^2/d$) leading to high turnover rates of nutrients (Precht and Huettel, 2003, Billerbeck *et al*., 2006). However, the main part of the microbial community is probably attached to sediment particles, and consequently generation times would not be determined by the rates of advective pore water flow through the sediment. A further potential control of the bacterial generation time is grazing, but again it is difficult to estimate the resulting generation time *in situ*. The average generation time of a microbial community can be estimated based on the codon usage bias in its metagenome (Viera and Rocha, 2010). The estimate for the generation time of the microbial community on Janssand is with 0.4

days remarkably lower than the generation time at the transition point from DNRA to denitrification (chapter 5). This is consistent with the prevalence of denitrification on the tidal flat, even though it has to be considered that the estimated generation time holds true for the average of the whole *in situ* community and deviations may occur. All in all, the combined selecting forces identified in chapter 5 are able to explain the dominance of denitrification observed at the sampling site within the constraints of data availability for *in situ* nitrite concentrations. Nevertheless, the general validity of the identified selecting forces remains to be verified. To test for this, continuous culture enrichments could be repeated with inocula from a variety of different ecosystems including limnic and terrestrial habitats. Furthermore, future *in situ* studies should differentiate between nitrate and nitrite concentrations and try to address the retention time of the habitat.

6.6 Behavior of microbial communities close to the tipping point between denitrification and DNRA

Dynamic systems can abruptly shift from one stable state into another (Scheffer *et al*., 2001), although the underlying environmental changes causing this loss of resilience may seem gradual and minor. The observation of several distinct shifts from a state of denitrification to a state of DNRA in the experiments described in chapter 5 indicates a potential for such alternative stable equilibria in nitrate-reducing communities. Because the continuous culture enrichments were performed at several different generation times and C/N-ratios (chapter 5), we have a good indication for the range in which the respective tipping points lie. This gives us important clues on how to force a system from one state into the other. Although threshold values are likely habitat specific, general patterns could occur, which could be the focus of future experiments. From a systems ecology perspective, it would also be interesting to observe the behavior of the microbial communities in proximity to the tipping points more closely in order to identify indicators that may predict when a shift from denitrification to DNRA or vice versa is about to occur.

So far, only gradually changing indicators for the loss of resilience, as for example the slowing down of the recovery rate from perturbations and consequent rises in autocorrelation, have been identified (Veraart *et al*., 2012, van Nes and Scheffer, 2007). The

next challenge is to find indicators that provide a measure of how close a system is to a tipping point, as well as the type of transition that will occur (Kefi *et al.*, 2012). The competition between different nitrate reducing pathways in a chemostat could deliver valuable insights as the underlying driving mechanisms are known and under experimental control. For example, one could observe differences between shifts from e.g. denitrification to DNRA upon a change in generation time for a range of different C/N-ratios.

6.7 Potential application in sustainable management of nitrogen fertilizer

The anthropogenic input of fixed nitrogen to the environment is immense (chapter 1) and it is expected to increase in the future (Canfield, 2010). Regionally, severe impacts on natural ecosystems can already be observed (Howarth and Marino, 2006, Smith *et al.*, 1999).

Agricultural run-off is by far the biggest source of anthropogenic nitrogen in the environment (Fowler *et al.*, 2013). Therefore, there is a large interest in strategies that reduce the nitrogen leakage from agricultural areas. The sustainable management of fertilizers is not only of ecological importance but also would constitute an economical benefit for the agricultural sector (Seitzinger, 2006). Several attempts are already being made to improve the nitrogen use efficiency of crops (the ratio between the crop yield attributed to fertilization and the amount of nitrogen fertilizer applied) (Cherry *et al.*, 2008). Those strategies try to reduce the amount of nitrogen fertilizer used through e.g. crop rotation including legumes, a better prediction of the field- and crop-specific nitrogen requirements and optimization of the time of fertilization or the chemical inhibition of nitrifying bacteria (Robertson and Vitousek, 2009, Canfield, 2010). They mainly target the uptake of nitrogen by plants and mostly do not address the importance of microbial communities as the integral component of the nitrogen cycle.

The controls determined in chapter 5 may be helpful for the development of a more sustainable management of fertilizer usage that is cost efficient and reduces the export of fixed nitrogen to neighboring areas. A desirable application could be the stimulation of denitrification in run-off areas that experience high nitrogen loads and of DNRA on the agricultural fields. Nevertheless, though easy to manipulate in a continuous culture set-up, it is challenging to address these controls within an ecosystem. However, approaches that aim to recapture nitrogen lost from fields in agricultural watersheds could be a promising starting point. Water that is drained from agricultural fields often contains high loads of fixed

nitrogen. It can be captured in (artificial reconstructed) wetland reservoirs (Tan *et al*., 2007). Here, the retention time could be manipulated in order to stimulate DNRA. That way the fixed nitrogen would be preserved and the captured water can be used for irrigation of the crop, and hence the contained nitrogen would be recycled. Also the application of bioreactors is conceivable (Blowes *et al.,* 2004). That way, surface water ecosystems would not be impacted by elevated ammonium concentrations.

In contrast to agriculture, wastewater treatment strategies have rapidly adopted new advances in knowledge about the microbiology of the nitrogen cycle. Several examples demonstrate the successful application of different microbial nitrogen transformation pathways for the effective improvement of nitrogen removal such as the combination of partial nitrification and denitrification or anammox (Van Kempen *et al*., 2011, Van der Star *et al.*, 2007, Kumar and Lin 2010, Winkler *et al*., 2012). The factors identified in chapter 5 are relatively easy to control in a wastewater treatment plants. In the coupling of nitrification and anammox and denitrification, for example, retention times are kept short to prevent the enrichment of nitrite oxidizing microbes (Van der Star *et al.*, 2007, Van Kempen *et al*., 2011). In certain cases, for example when wastewater treatment plants are located close to agricultural areas, they could manipulate nitrate respiration towards DNRA and thus keep fixed nitrogen available for further re-use.

In any case, the consequences of different management practices need to be well thought of and experimentally addressed to fully understand the complex interaction and potential feedback loops among different transformations within the nitrogen cycle.

6.8 Application of the experimental approach on the competition between other important biogeochemical processes

The combination of multiple parallel enrichments in chemostats with rate measurements, meta-genomics and, where required, -transcriptomics and -proteomics has proved to be a successful approach to study interactions and dynamics in microbial communities and the ecophysiology of microbes in conjunction with their natural interaction partners. This way, it was possible to extract the environmental factors that shape such interactions.

It would be interesting to apply this strategy to similar questions. For example, the competition for the electron donors acetate and hydrogen by sulfate-reducing prokaryotes and methanogens and for the latter also by homoacetogens in deep sediments has been intensively studied for years. Generally, sulfate-reducers are expected to prevail as long as sulfate is available, and methanogens should win over homoacetogens due to differences in substrate affinities (Lovley & Goodwin, 1988, Muyzer and Stams, 2008). However, also in this competition between different microbial guilds the outcome seems to be more complex than that: Homoacetogens may successfully co-exist with methanogens and sulfate-reducers (Conrad *et al*., 1986, Emde & Schink, 1987, Hoehler *et al*., 1999). Close syntrophies between sulfate-reducers and methanogens (Schink, 1997) and the capacity of some sulfate-reducing prokaryotes to perform homoacetogenesis themselves obscure the picture (Klemps *et al*., 1985, Ramamoorthy *et al*., 2006, Kraft *et al*., 2012). Multiple parallel continuous culture incubation of microbial communities at e.g. different sulfate to hydrogen ratios and generation times may show if this competition is influenced by similar controls as nitrate respiration. Therefore, I suggest addressing the complex interaction and competition between microbial sulfate-reduction, methanogenesis and homoacetogenesis in the same way as the competition between nitrate respiration pathways was addressed in this thesis.

6.9 Where do other NO_x^- -reducing pathways enter in the competition?

Discoveries in the past 15 years added a lot more complexity to the nitrogen cycle, which has become rather a network than a cycle with complex interactions and feedback loops among the different pathways (reviewed in chapter 1 and 2). Alone two further microbial pathways reduce nitrate or nitrite to dinitrogen: Anammox and NO-dismutation. The identification of three controls that determine the outcome of the competition between DNRA and denitrification in this thesis leads to the questions if such controls also can be determined for other NOx-reducing pathways and to what extend the identified controls also influence anammox and NO-dismutation. Where do they enter in the competitions? The C/N-ratio and the affinity for nitrite, for example, have been proposed to influence the predominance of anammox versus denitrification in oxygen minimum zones (Kalvelage *et al*., 2013, Babbin *et al.*, 2014, Kartal *et al.,* 2013).

6.10 References

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ERKLÄRUNG

Hiermit erkläre ich, dass ich die Arbeit mit dem Titel:

Competition in nitrate-reducing microbial communities

selbstständig verfasst und geschrieben habe und außer den angegebenen Quellen keine weiteren Hilfsmittel verwendet habe.

Ebenfalls erkläre ich hiermit eidesstattlich, dass es sich bei den von mir abgegebenen Arbeiten um 3 identische Exemplare handelt.

....

(Beate Kraft)