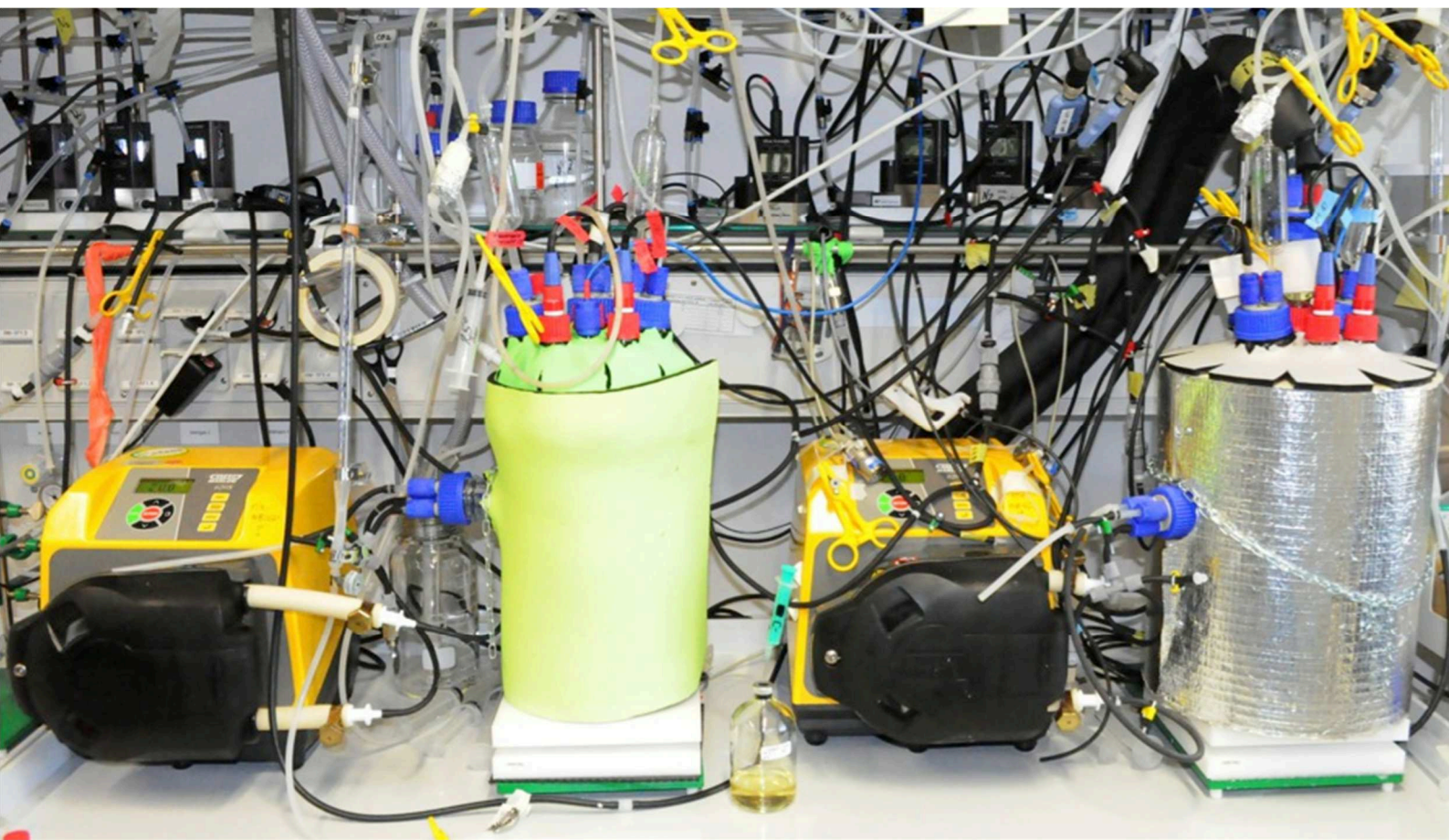# Continuous Cultivation of Janssand Microbial Communities

Response to Varying Oxygen Concentrations and Temperatures

Anna Hanke

1. Gutachter: **Prof. Dr. Friedrich Widdel**

2. Gutachter: **Prof. Dr. Marc Strous**

Datum des Promotionskolloquiums: 19. September 2014

"It is fundamentally the confusion between effectiveness and efficiency that stands between doing the right things and doing things right. There is surely nothing quite so useless as doing with great efficiency what should not be done at all."

(Peter Ferdinand Drucker, 1909 - 2005)

Continuous Cultivation of
Janssand Microbial Communities

Response to Varying
Oxygen Concentrations and Temperatures

Dissertation zur Erlangung des

Doktorgrades der Naturwissenschaften

- Dr. rer. nat. -

dem Fachbereich Biologie

der Universität Bremen

vorgelegt von

Anna Hanke

aus Bremen

Bremen, 30. Juli 2014

Diese Arbeit wurde zwischen November 2009 und Juli 2014 im Rahmen des Graduiertenprogramms "The International Max Planck Research School of Marine Microbiology" (mar*mic*) in der Arbeitsgruppe "Mikrobielle Fitness" am Max-Planck-Institut für Marine Mikrobiologie in Bremen angefertigt.

1. Gutachter: **Prof. Dr. Friedrich Widdel**

2. Gutachter: **Prof. Dr. Marc Strous**

3. Prüfer: **Prof. Dr. Wilhelm Hagen**

4. Prüfer: **Dr. Gaute Lavik**

Datum des Promotionskolloquiums: 19. September 2014

Abstract

The Wadden Sea is the largest contiguous system of tidal sand and mud flats worldwide. It forms a variety of (micro-)habitats, which create ecological niches with an outstanding richness of thousands of species. The Wadden Sea is an area of intense biogeochemical cycling and mineralization processes such as the nitrogen cycle, which comprises the transformation of nitrogen containing molecules. Denitrification, the stepwise reduction of nitrate (NO_3^-) to dinitrogen (N_2), is such a transformation process usually employed in the reduction of nitrate concentrations of wastewater and a source of nitrous oxide (N_2O), a long-living, potent greenhouse gas.

This thesis addresses the effect of temperature and dynamic oxic/anoxic conditions on marine denitrifying bacteria. The experimental approach consisted of the long-term continuous cultivation of microbial communities sampled from tidal Wadden Sea sediments. Continuous cultivation enabled the natural selection of simplified microbial communities under defined, stable and environmentally relevant conditions. Compared to more complex communities, these simple communities were traceable with genomics, transcriptomics and proteomics approaches in order to identify the involved organisms and determine their metabolic traits. Furthermore, fluorescence *in situ* hybridization (FISH-) approaches, determination of metabolites in culture liquid and off-gas, and stoichiometric modelling completed the picture.

In part one, the development of the experimental setup for continuous cultivation is described. This chapter shows that with the presented setup it was possible to select a microbial community comprised of representatives of populations that were important *in situ*. One of the selected populations represented a clade of potential sulfur oxidizing *Gammaproteobacteria* that were highly abundant *in situ*. Another population was a member of the uncultured BD1-5/SN-2 division that was shown to translate the stop codon UGA as the amino acid glycine. Its enrichment also enabled microscopy images of a bacterium belonging to this enigmatic clade.

In part two, the developed experimental setup was used to study the outcome of natural selection under tidal (oxic/anoxic) conditions. It was shown that the

resulting community facilitated the parallel occurrence of thermodynamically unsorted redox processes: fermentation, sulfate reduction, denitrification and aerobic respiration. Oxygen sensitive enzymes were protected by a combination of cellular aggregation and active oxygen consumption by cells performing anaerobic metabolism.

In part three, the effect of temperature on natural selection of denitrifying communities was investigated. The results were consistent with those of chapter 2, and showed co-selection of fermentative and denitrifying bacteria. However, whereas the fermentative/denitrifying consortia selected at 25 °C denitrified effectively, this was not the case at 10 °C, leading to a reduced denitrification activity at this temperature. Temperature was shown to have a strong selective effect on the denitrifying populations. Although the observations could not be explained conclusively, we speculate that at 10 °C, cells were selected that performed denitrification and fermentation in parallel, whereas at 25 °C division of labor was more pronounced. Thus, at 10 °C fermentation may have outcompeted denitrification at the single cell level while at 25 °C fermentative and denitrifying microbes were able to co-exist.

Overall, the work of this thesis pioneers a new approach to the microbial ecology of denitrification in the context of other redox processes. The research provided new insight about how denitrification interacts with other redox processes and opened the way for direct, hypothesis-based assessment of these interactions in nature.

Zusammenfassung

Das Wattenmeer vor Deutschland, Dänemark und den Niederlanden ist die größte zusammenhängende, tidenabhängige Wattfläche der Erde. Es ist Grundlage für eine Vielzahl verschiedener (Mikro-)Habitate, die ökologische Nischen für Tausende von Arten darstellen. Das Wattenmeer ist durch vielfältige biochemische Kreisläufe und Mineralisierungsprozesse gekennzeichnet. Einer dieser Kreisläufe ist der Stickstoffkreislauf, der chemische Umwandlungen stickstoffhaltiger Moleküle umfasst. Denitrifikation, die schrittweise Reduktion von Nitrat (NO_3^-) zu molekularem Stickstoff (N_2), ist Teil des Stickstoffkreislaufes. Denitrifikation wird häufig zur Reduktion hoher Nitratkonzentrationen im Rahmen der Abwasseraufbereitung eingesetzt und ist eine Quelle des langlebigen und hochwirksamen Treibhausgases Distickstoffoxid (N_2O).

Die vorliegende Arbeit untersucht den Einfluss von Temperatur und dynamisch oxisch/anoxischen Bedingungen auf marine denitrifizierende Bakterien. Die Untersuchungen basierten auf der kontinuierlichen Kultivierung mikrobieller Gemeinschaften aus Wattsediment, die die natürliche Selektion simpler mikrobieller Gemeinden unter definierten, stabilen und umweltrelevanten Bedingungen ermöglichte. Diese simplen Gemeinschaften konnten mittels Metagenom-, Metatranskriptom- und Metaproteom-Analysen auf die Zusammensetzung und die metabolischen Eigenschaften ihrer Mitglieder untersucht werden. Vervollständigt wurde das Bild durch Fluoreszenz-*in-situ*-Hybridisierung (FISH), Bestimmung von Stoffwechselintermediaten und –produkten in Kulturflüssigkeit und -gas, sowie durch stöchiometrische Modellierung der metabolischen Vorgänge in der Kultur.

Der erste Teil der Arbeit beschreibt den für die kontinuierliche Kultivierung von Mikroorganismen verwendeten Versuchsaufbau. Es demonstriert die Nutzbarkeit des Setups zur Anreicherung von mikrobiellen Gemeinschaften, die aus Repräsentanten *in situ* relevanter Populationen bestehen. Eine der angereicherten Populationen gehörte zu einem Stamm potentiell Schwefel-oxidierender Gammaproteobakterien, die auch *in situ* einen großen Teil der mikrobiellen Gemeinschaft ausmachten. Eine andere angereicherte Population bestand aus Mitgliedern des bisher nicht kultivierten BD1-5/SN-2-Stammes. Es konnte nachgewiesen werden,

dass die angereicherten BD1-5/SN-2-Bakterien das Stop-Codon UGA zur Codierung der Aminosäure Glycin verwendeten. Die Anreicherung der BD1-5/SN2-Bakterien ermöglichte mikroskopische Fluoreszenzaufnahmen dieser bisher wenig bekannten Gruppe von Mikroorganismen.

Im zweiten Teil wurde der entwickelte Versuchsansatz dazu genutzt das Ergebnis natürlicher Selektion unter wechselnd oxisch-anoxischen Bedingungen zu untersuchen, die für tidenabhängige marine Sedimente charakteristisch sind. Es zeigte sich, dass die angereicherte mikrobielle Gemeinschaft thermodynamisch ungeordnete Redoxprozesse, nämlich Fermentation, Sulfatreduktion, Denitrifikation und aerobe Atmung, parallel vollzog. Bei der gleichzeitigen Ausführung aerober und anaerober Stoffwechselprozesse konnten sauerstoffempfindliche Enzyme anaerober Zellen mithilfe einer Kombination von exzessivem Sauerstoffverbrauch und der Bildung von Zellaggregaten geschützt werden.

In Teil drei wurde der Einfluss von Temperatur auf die natürliche Selektion denitrifizierender mikrobieller Kulturen untersucht. Die Ergebnisse stimmten im Wesentlichen mit den Ergebnissen aus Kapitel 2 überein, da sie die simultane Anreicherung fermentierender und denitrifizierender Bakterien innerhalb derselben Kultur beschreiben. Allerdings wies die bei 10 °C angereicherte denitrifizierende/fermentierende Gemeinschaft eine deutlich geringere denitrifizierende Aktivität auf als die bei 25 °C angereicherte. Temperatur hatte offensichtlich einen starken selektiven Einfluss auf die angereicherten denitrifizierenden Populationen. Auch wenn die gewonnenen Ergebnisse nicht abschließend interpretiert werden können, ist anzunehmen, dass bei 10 °C eine Artengemeinschaft angereichert wurde, deren einzelne Mitglieder parallel fermentieren und denitrifizieren konnten, während die bei 25 °C angereicherte Kultur aus Arten bestand, die hinsichtlich ihres Stoffwechsels Arbeitsteilung betrieben. Bei 10 °C konnte sich der fermentierende Stoffwechselweg auf dem Level einzelner Zellen gegen die Denitrifikation durchsetzen, während bei 25 °C denitrifizierende und fermentierende Organismen nebeneinander bestehen konnten.

Zusammengefasst präsentiert diese Arbeit eine neue Herangehensweise zur Untersuchung der mikrobiellen Ökologie der Denitrifikation im Kontext verschiedener Redoxprozesse. Die vorliegende Forschung liefert neue Einblicke in die Wechsel-

wirkung der Denitrifikation mit anderen Redoxprozessen und bereitet den Weg für weitere direkte, Hypothesen-basierte Untersuchungen dieser Wechselwirkungen in natürlichen Habitaten.

Content

List of Figures

List of Tables

List of Abbreviations

ADP	adenosine monophosphate
approx.	approximate(ly)
ARISA	automated ribosomal intergenic spacer analysis
ATP	adenosine triphosphate
BLAST	basic local alignment search tool
BSA	bovine serum albumin
Cand.	candidatus
CARD-FISH	catalyzed reporter deposition-FISH
CH_4	methane
CLASI-FISH	combinatorial labeling and spectral imaging-FISH
CO	carbon monooxide
CO_2	carbon dioxide
COD	chemical oxygen demand
CODH	carbon monooxide dehydrogenase
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
DNRA	dissimilatory nitrate reduction to ammonia
DOC	dissolved organic carbon
DOPE-FISH	double labeling of oligonucleotide probes-FISH
DTT	dithiothreitol
EMIRGE	expectation maximization iterative reconstruction of genes from the environment
esp.	especially
et al.	*et alii*
FASP	filter aided sample preparation
Fe_2^+	bivalent iron oxide (also Fe(II))
Fe_3^+	trivalent iron oxide (also Fe(III))
FISH	fluorescence *in situ* hybridization
GC	gas chromatography
GC content	% guanine, cytosine content
GELFrEE	a system for the fractionation of biological samples
GHG	greenhouse gas
H_2O	water

H_2S	hydrogen sulfide
HMM	Hidden Markov Model
HPLC	high performance liquid chromatography
HPO_4^{2-}	phosphate ion
IMM	Interpolated Markov Model
IRMS	isotope ratio mass spectrometry
K_m	Michaelis Constant
LC	liquid chromatography
LC-MS/MS	liquid chromatography tandem mass spectrometry
MAR-FISH	microautoradiography-FISH
MilliQ	demineralized water in Millipore quality
Mn_4^+	tetravalent manganese oxide (also Mn(IV))
MS	mass spectrometry
MWCO	molecular weight cut off
Na	sodium
NAD(H)	nicotinamide adenine dinucleotide (with hydrogen)
NH_3	ammonia
NH_4^+	ammonium ion
N_2	dinitrogen gas
N_2O	nitrous oxide
NO	nitric oxide
NO_2^-	nitrite
NO_3^-	nitrate
NO_x	nitrogen oxides such as nitrate, nitrite, nitric oxide
O_2	oxygen
OD	optical density
OEM	original equipment manufacturer
OM	organic matter
ORF	open reading frame
OTU	operational taxonomic unit
PCR	polymerase chain reaction
PGM^{TM}	personal genome sequencer^{TM}
pH	*pondus hydrogenii*; the negative $\log_{10}$ of the proton concentration
PHA	polyhydroxyalkanoate
P_i	inorganic phosphate

PSU	practical salinity unit
rDNA	ribosomal deoxyribonucleic acid
RNA	nibonucleic acid
RP	reverse phase
rpm	rounds per minute
rRNA	ribosomal ribonucleic acid
RT	room temperature
SCX	strong cation exchange
SDS	sodium dodecyl sulfate
SILVA	a high quality ribosomal RNA database
SO_4^{2-}	sulfate ion
sp. nov.	*species nova*
SRB	sulfate reducing bacteria
SRR	sulfate reduction rate
SSU	small subunit
TGGE	thermal gradient gel electrophoresis
(**T**-)**RFLP**	(terminal) restriction fragment length polymorphism
Tris-**HCL**	Tris-(hydroxymethyl)-aminomethane
V	Volt
VCl_3	Vanadium(III)chloride
vs.	*versus*
W	Watt
WGA	whole genome amplification
x g	times [acceleration of gravity]

Introduction

The Wadden Sea

Heut bin ich über Rungholt gefahren,

Die Stadt ging unter vor fünfhundert Jahren.

Noch schlagen die Wellen da wild und empört

Wie damals, als sie die Marschen zerstört.

Die Maschine des Dampfes schütterte, stöhnte,

Aus den Wassern rief es unheimlich und höhnte:

Trutz, Blanke Hans.

(…)

Mitten im Ozean schläft bis zur Stunde

Ein Ungeheuer, tief auf dem Grunde.

Sein Haupt ruht dicht vor Englands Strand,

Die Schwanzflosse spielt bei Brasiliens Sand.

Es zieht, sechs Stunden, den Atem nach innen

Und treibt ihn, sechs Stunden, wieder von hinnen.

Trutz, Blanke Hans.

Doch einmal in jedem Jahrhundert entlassen

Die Kiemen gewaltige Wassermassen.

Dann holt das Untier tiefer Atem ein

Und peitscht die Wellen und schläft wieder ein.

Viele tausend Menschen im Nordland ertrinken,

Viele reiche Länder und Städte versinken.

Trutz, Blanke Hans.

(…)

Ein einziger Schrei - die Stadt ist versunken,

Und Hunderttausende sind ertrunken.

Wo gestern noch Lärm und lustiger Tisch,

Schwamm andern Tags der stumme Fisch.

Heut bin ich über Rungholt gefahren,

Die Stadt ging unter vor fünfhundert Jahren.

Trutz, Blanke Hans?

The preceding poem "Trutz, blanke Hans", written by Detlev von Liliencron in approximately 1882, gives an impression of the immense forces of nature, which create and determine the area of and life and death in the European Wadden Sea. The poem describes the drowning of a place called Rungholt in the night from January 16th to 17th in 1362 during a storm tide, the so-called "De Grote Mandränke" (literally: great drowning of men). Rungholt was a congregation located on a small island offshore from the North Friesian Coast, south-east of the North Friesian Island Pellworm. As per legend, it is supposed to ascend every 7 years for one night to be redeemed by a Sunday's child entering the place where it once drowned (Duerr 2005). This is reflected in its nickname "The Atlantis of the North".

Due in part to this tragedy and the resulting curiosity emerging in people to find out more about Rungholt's doomed fate, the Wadden Sea is currently among the best-studied coastal areas in the world (Dittmann 1999). Indeed, from a biological point of view the Wadden Sea is highly interesting because of its unique position and the influence of variety of environmental factors (e.g. tides, wind, river discharges), which create a unique habitat for micro- and macroorganisms. The area has accordingly been in the focus of natural scientists for more than 130 years (Bietz 2004). First documented studies stem from the zoologist Karl August Möbius who investigated the disappearance of the oyster *Ostrea edulis* in 1877 and later derived the terminus technicus "Lebensgemeinde"/"biocenosis", an ecological paradigm (Möbius 1877). Today, Arthur Hagmeier (1886-1957), Erich Wohlenberg (1903-1993), and Otto Linke (1909-2002) are considered pioneers in German Wadden Sea research. Their elaborate ecological studies were the first investigations that used an interdisciplinary approach to extensively focus on the living conditions in the Wadden Sea (Bietz 2004).

The Wadden Sea, located in the south-eastern part of the North Sea, stretches from the Den Helder peninsula (The Netherlands) along the Dutch coast, the German Bight, and the Danish Coast to the Skallingen peninsula (Denmark). This stretch covers a distance of about 500 km and is one of the best-studied coastal areas on the planet (CWSS 2008). The German tidal flat area is located between the north-western German coastline and the Eastern Friesian Islands serving as a barrier from the open ocean, separated by tidal inlets.

28

The Wadden Sea is the largest contiguous system of tidal sand and mud flats worldwide with a height difference from its highest to lowest points deepest within 50 m above and below sea level. The area comprises of about 14,700 square kilometers and its highly dynamic natural processes remain vastly undisturbed. The latter create a constantly changing landscape of flats, barrier islands, channels, saltmarshes, and other coastal and sedimentary features. These transitions are characterized by semi-diurnal flood and ebb tides and high fluctuations in salinity and temperature. The result is the formation of many different (micro-)habitats creating ecological niches for a variety of species adapted to these extreme environmental conditions. This multitude of (transitional) zones between land, sea, and freshwater habitats serve as the basis for an outstanding richness of an estimated 10,000 species (from unicellular organisms to animals). This accounts for a primary and secondary productivity ranking among the highest biomass productivity worldwide (CWSS 2008).

The entire Wadden Sea consists of a sand-mud tidal system. Supported by estuaries, marshes and a wide intertidal zone, the Wadden Sea serves as a gigantic coastal filter system in which fresh and marine waters are mixed back and forth with the tides. This transport of huge amounts of nutrients, biomass and sediment creates the basis of the Wadden Sea's trophic system. The imported organic material is re-mineralized by microorganisms serving as an additional nutrient source leading to the area's exceptionally high productivity (CWSS 2008).

From a broader perspective, rivers only marginally influence the entire Wadden Sea, not enough to categorize it as an estuary. It is neither an open oceanic coast but rather hydrologically intermediate, with a characteristic salinity between 20 psu and 30 psu. Wave exposure is mitigated by barrier islands as well as meso- to macrotidal conditions with a mean tidal range of 1.4 to 4.0 m and an extremely gentle slope from land to sea (CWSS 2008).

However, on a small scale, local parts of the Wadden Sea are highly affected by input from the rivers Elbe and Rhine as well as some smaller ones. These estuaries, small in number and size, compared to the entire marine Wadden Sea, are defined as transition zones between marine and riverine environments and are highly influenced by the tides. Taken together, all rivers discharging into the

Wadden Sea come from a catchment area of 230,000 square kilometers and transport a discharge volume of 60 cubic kilometers every year. These inputs are of importance for the Wadden Sea ecosystem as they cause very high local fluctuations in salinity. Also, they supply nutrients, mainly nitrogen species, to the coastal waters. Resulting from their variable characteristics they form a distinctive habitat for a variety of obligate brackish-water species. However, the estuaries have been influenced by human activities like fertilization and are only partly protected as nature reserves (CWSS 2008).

The Wadden Sea region covers a latitude of 53 to 55 ° N which in combination with the influence of the Gulf Stream creates a climate comparable to that of the Gulf of Maine, Vancouver Island, for example. Average water temperatures range from 4 °C in winter to 15 °C in summer. Extreme values measured in the tidal area during the past 60 years range from -2.3 °C to +23 °C, respectively. In summer, water temperature rarely exceeds 20 °C while the sediments with their residual waters can reach 32 °C on sunny days during low tide which constitutes an adaptation challenge for inhabiting organisms (CWSS 2008).

The entire area is under constant protection and monitoring since approximately 66 % of the Wadden Sea ecosystem (including the Dutch Wadden Sea Conservation Area and the German Wadden Sea National Parks of Lower Saxony and Schleswig-Holstein – all together nearly 10,000 square kilometers) have been declared a World Heritage Area in June 2009. This highlights the importance of a clear understanding of all physical, chemical and biological processes interacting to form and sustain this elemental ecosystem. Two key documents guide the overall management operations that also include the Danish part of the Wadden Sea: the "*Wadden Sea Plan*" as a legally binding document adopted in 1997 and an *"Integrated Coastal Zone Management (ICZM) Strategy"* which addresses recommendations from the European Parliament on coastal zone conservation and management (CWSS 2008). In June 2014 the Danish part of the Wadden Sea and a small German extension were additionally declared a World Heritage Area. Now the Wadden Sea UNESCO World Heritage finally covers an area of roughly 11,500 square kilometers (CWSS 2012).

Climate, Fertilization, and the Nitrogen Cycle (Publication I)

Anna Hanke[1], and Marc Strous, Ph.D.[1,2]

[1]Max Planck Institute of Marine Microbiology Bremen and CeBiTec Bielefeld, Germany.
[2]Max Planck Institute of Marine Microbiology, Celsiusstr. 1, 28359 Bremen, Germany.

Status: published in *Journal of Cosmology*

Own contribution: AH collected and reviewed the available literature on the topic and wrote the manuscript together with MS.

Abstract

The concentration of greenhouse gases in the atmosphere and the global climate is discussed in relation to the activities of microorganisms and the nitrogen cycle. The human impact on the carbon cycle (currently around 5 % of the natural background) is still relatively small compared to our contribution to nitrogen cycling where the anthropogenic input may be larger than the natural background. To understand the effects of this input on the climate, it is essential to unravel the different microbial interconversions of nitrogen compounds in nature. Here we review the current microbiological understanding of the complicated nitrogen network, its reactions, microorganisms and genes. Although this network features many recently discovered processes, knowledge on the more conventional processes such as denitrification and dissimilatory nitrate reduction to ammonia is lagging behind.

Introduction

The relationship between the concentration of greenhouse gases in the atmosphere and the global climate is a current topic of scientific research and cause for alarm (Cairns 2010; Moriarty and Honnery 2010). Since the start of the industrial era around 1750, the concentrations of these gases have increased dramatically and the role of the anthropogenic carbon dioxide emissions has been widely discussed. However, the human impact on the carbon cycle is still relatively small compared to our contribution to nitrogen cycling (Fig. I-1). To feed the growing human population we have become completely dependent on the use of synthetic fertilizers; nowadays approximately one out of three nitrogen atoms that enter the biosphere originate from the fertilizer industry. Combined with ammonia deposition caused by fossil fuel burning, the anthropogenic input may even be higher than the natural background (Fig. I-1). The consequences for the climate are poorly understood but we argue that fertilization most likely reinforces global warming by increasing the atmospheric concentrations of methane and nitrous oxide, two powerful greenhouse gases. To understand this possible reinforcement, and make predictions for the future, it is essential to experimentally unravel the complex interactions of the biogeochemical nitrogen network. What follows is a step-by-step review of these interactions, and finally, the open questions and consequences for the climate are discussed.
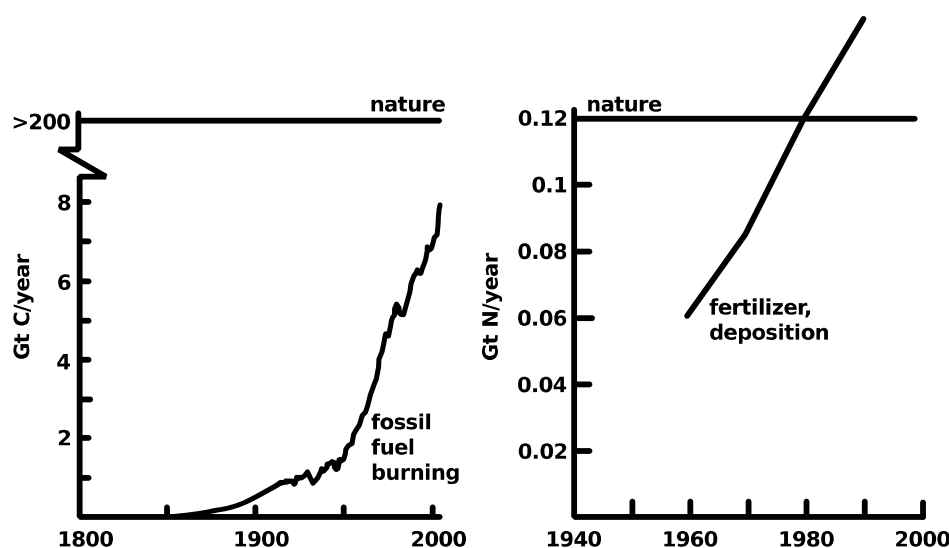
Figure I-1 I Estimated trends for anthropogenic inputs to the global carbon and nitrogen cycles compared to the natural background (natural carbon and nitrogen fixation; Galloway *et al.* 2008; Duce *et al.* 2008; Vitousek *et al.* 1998; Lequere *et al.* 2009; Gruber *et al.* 2008).

The Nitrogen Cycle

The nitrogen cycle consists of transport processes and chemical reactions; the latter are mainly catalyzed by bacteria. Except for nitrogen fixation, bacteria perform these reactions to gain energy for chemotrophic growth. The phrase "nitrogen cycle" is generally used, but together the reactions actually form a more complicated "nitrogen network" (Fig. I-2). To gain insight into this network it is essential to combine two complementary experimental approaches: The first approach, ^{15}N labeling, can provide information about the rates of the individual processes.
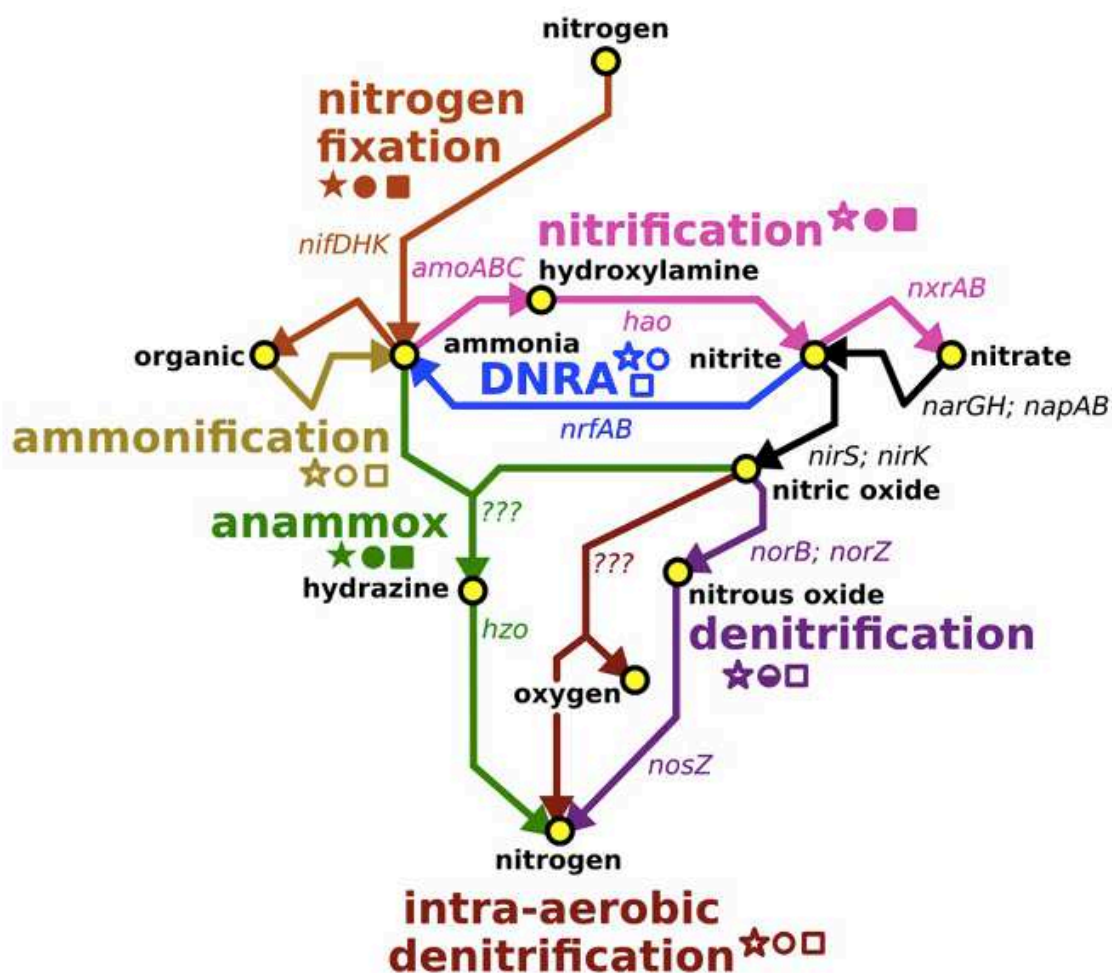
Figure I-2 I Current understanding of the biogeochemical nitrogen network. Nitrogen compounds (yellow circles), processes (bold) and known structural genes (italics) are indicated. DNRA: Dissimilatory nitrate reduction to ammonia. Stars indicate the current status of ^{15}N tracer technology (open: not available; filled: mature; half-filled: possible but rarely used). Circles indicate the current status of molecular ecology (open: primers/probes not available or gene targets unknown; filled: mature; half-filled: the used primers/probes do not match the known biodiversity). Squares indicate knowledge about environmentally significant microbial players (open: unknown; filled: known).

Although other approaches have been used for this purpose in the past (for example use of inhibitors such as acetylene) ^{15}N labeling is the only one that is still useful in the context of the full complexity of Figure I-2. The second complementary and independent approach, molecular ecology, provides information about the presence and activity of the associated bacteria and genes. Because many processes are performed by unrelated bacteria, we are generally dependent on the detection and quantification of functional genes (Fig. I-3). Technical progress along these lines, for example the application of novel tracer technology and continued mining for functional gene markers, is extremely important.
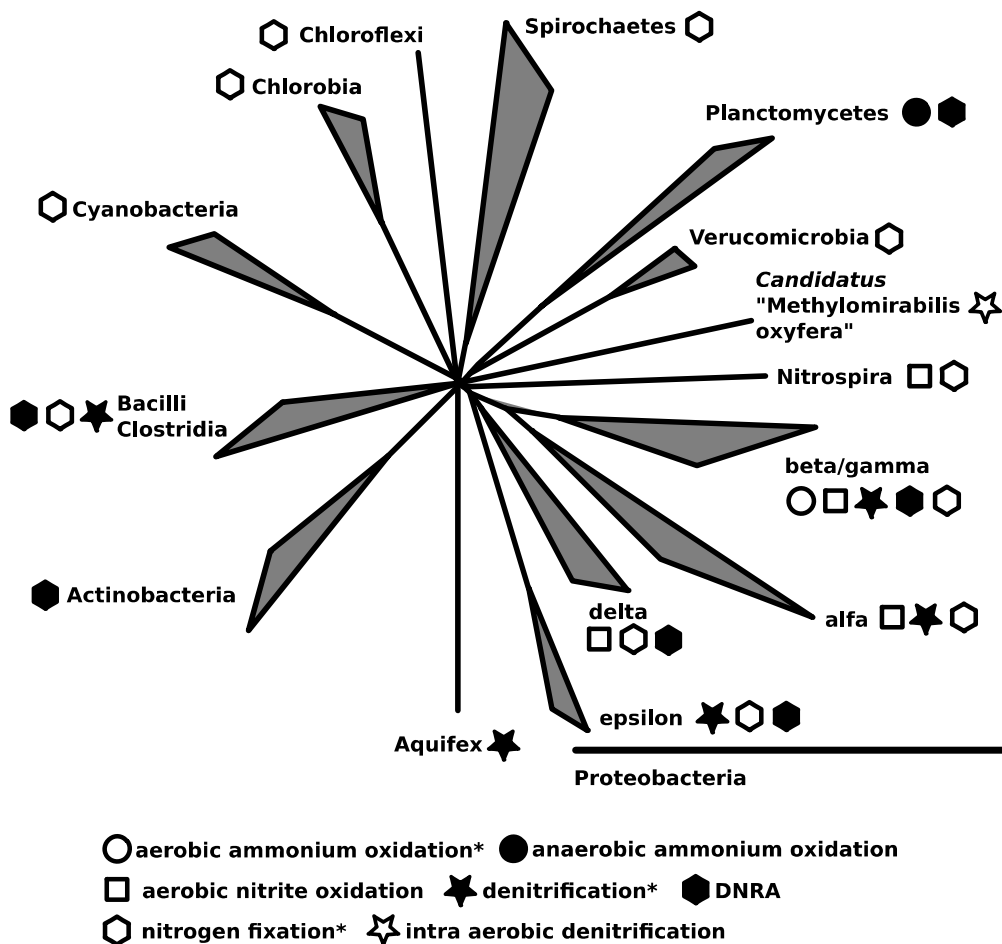
Figure I-3 I Evolutionary tree of bacterial *phyla* relevant to the nitrogen cycle. The known participation of members of these *phyla* in the different nitrogen conversions is indicated. Some conversions are performed by many different *phyla* (e.g. nitrogen fixation, denitrification), which makes the development of molecular ecological approaches more difficult. * indicates that the process is also performed by *archaea*. It is expected that many more microbial players representing different *phyla* and participating in different processes remain to be discovered.

Nitrogen Fixation and Primary Production

All life depends on atomic nitrogen because it is an essential component of amino acids, nucleic acids, porphyrins, amino sugars, etc. Dinitrogen gas in the atmosphere is the largest reservoir of nitrogen available to life on Earth, and this may even be a consequence of life itself (Capone *et al.* 2006). Dinitrogen is accessed by microorganisms in a reaction known as nitrogen fixation. In this reaction dinitrogen is reduced to ammonia (NH_3) by the enzyme nitrogenase. Although the reaction is exergonic, the activation energy is very high and it requires 16 molecules of ATP per N_2 fixed. It is especially difficult to fix nitrogen in the presence of oxygen (O_2) because the nitrogenase is destroyed by oxygen. Therefore, during most of the geological history of the Earth the difficulty of accessing atmospheric nitrogen has been one of the factors that constrained primary production.

Only a small proportion of known bacterial species is able to fix nitrogen and possesses the structural genes for nitrogenase (*nifDHK*). Environmentally important nitrogen fixing organisms are plant symbionts such as *Rhizobium* and free-living organisms such as the cyanobacterium *Trichodesmium*. Nitrogenase is phylogenetically widespread, e.g. many evolutionary unrelated species have acquired the genes for nitrogenase, although there is little evidence for recent lateral gene transfer (Zehr *et al.* 2003). For this reason, the gene *nifH* is used as a functional marker to identify nitrogen-fixing bacteria in nature, independent of organismal phylogeny as defined by the 16S ribosomal gene. For nitrogen fixation, tracer studies with ^{15}N labeled N_2 and molecular ecology (targeting *nifH*) are established methods. Despite the availability of these methods, oceanic nitrogen budgets indicate that we may so far have overlooked key nitrogen fixers in the oceans (Codispoti *et al.* 2001). Technically, nitrogen fixation is carried out by the Haber Bosch process - essentially the same chemical reaction as nitrogen fixation. The widespread use of fertilizers in agriculture leads to high (hundreds of micromolars) concentrations of nitrate (NO_3^-) in many freshwater and coastal surface waters (Mulholland *et al.* 2008). Here, nitrate has replaced dinitrogen as the main source of nitrogen sustaining growth of bacteria and plants. This is leading to eutrophication, loss of biodiversity and higher rates of primary production (fixation of atmospheric carbon dioxide).

It is unlikely that fertilization actually drives a net removal of carbon dioxide from the atmosphere, because surface waters contain no long-term sink for carbon dioxide. The extra biomass that is produced is rapidly consumed and recycled - to carbon dioxide. Long-term studies addressing this issue have even reported additional release of carbon dioxide by enhanced mineralization of refractile organic matter in the presence of nitrate (Mack *et al.* 2004). The only long term biological sink for atmospheric carbon dioxide is the biological pump – which requires sinking of biomass into the deep ocean and storage of the carbon in deep marine sediments.

Therefore, ammonia deposition to the open ocean is more important as a possible negative feedback on global warming than fertilization. However, even in the open ocean the beneficial climate effects caused by increased primary production is likely to be neutralized by increases in nitrous oxide production (Duce *et al.* 2008).

In surface waters it is more likely that fertilization reinforces global warming by stimulating biological methane production (and nitrous oxide production, see below). Increased primary production leads to more build-up of biomass in shallow sediments where most of the biomass is degraded anaerobically, leading to the production of methane. Because shallow sediments are already a major source of methane emissions to the atmosphere (e.g. wetlands, rice fields) it is likely that a part of the extra carbon dioxide removed from the atmosphere by fertilization is returned as methane. As a greenhouse gas, methane is much stronger than carbon dioxide.

The nitrogen incorporated into biomass by primary producers enters the biological food chain. At each trophic level of the food chain most of the biomass is used as an energy source; sugars, proteins and lipids are mainly oxidized to carbon dioxide and only a small part is used for growth. Therefore, most of the nitrogen in the biomass is set free as ammonia. This ammonia release is known as ammonification. Presumably, many organisms are involved in this process. It is rarely investigated experimentally. Rates of ammonification are generally inferred from Redfield stoichiometry.

Nitrification

Ammonia can be oxidized to nitrate with oxygen. This aerobic process is known as nitrification and consists of two steps performed by two different groups of chemolithoautotrophs: the ammonium oxidizers oxidize ammonia to nitrite and the nitrite oxidizers oxidize nitrite to nitrate. Environmentally important ammonium oxidizers are affiliated with beta- (e.g. *Nitrosomonas europaea*) and *Gammaproteobacteria* (e.g. *Nitrosococcus oceani*) and *Crenarchaea* (e.g. *Nitrosopumilus maritimus*, Könnecke *et al.* 2005). Presently, five different unrelated groups of nitrite oxidizers (Fig. I-3) are known, all affiliated to different bacterial *phyla*.

Biochemically, ammonia is activated by the enzyme complex ammonia monooxygenase. The genes encoding this complex are *amoABC* and they serve as functional genetic markers to assess the diversity and abundance of ammonia oxidizers. Hydroxylamine is oxidized to nitrite by the octaheme enzyme hydroxylamine oxidoreductase, encoded by the gene *hao* (Klotz *et al.* 2008). However, this gene is not present in the genome of the crenarchaeal ammonia oxidizers (Walker *et al.* 2010). Nitrite oxidation is catalyzed by the enzyme complex nitrite:nitrate oxidoreductase, a member of the molybdopterin oxidoreductase superfamily. The genes are known as *nxrAB* but they are homologous to the genes used by denitrifiers to perform the reverse reaction (e.g. *narGH* see below).

Fertilizer is generally applied in the form of ammonium. Ammonium is positively charged and binds to (negatively charged) clay. After the nitrifiers convert ammonium to nitrate (negatively charged) it desorbs from the clay and is readily transported to surface or groundwater. For this reason, nitrification inhibitors are frequently added to the fertilizer mixture but these are only partially effective (Welte, 1994).

Nitrification is also a major source of nitrous oxide (N_2O) emissions. Although nitrous oxide is not an intermediate of nitrification, it is still produced by ammonia oxidizers, either as a by-product of the hydroxylamine oxidoreductase or by reduction of the produced nitrite ("nitrifier-denitrification") by denitrification

enzymes expressed by ammonia oxidizers at low oxygen levels (Meyer *et al*. 2008). Both the application of ^{15}N tracers and molecular gene markers are established for nitrification (e.g. Lam *et al*. 2009).

Denitrification

The first process that recycles nitrate back to dinitrogen gas is known as denitrification or "nitrate respiration". The latter term results from the fact that nitrate instead of oxygen serves as the final electron acceptor. During denitrification, nitrate (NO_3^-) is reduced *via* nitrite (NO_2^-), nitric oxide (NO), and nitrous oxide (N_2O) to dinitrogen (N_2). Denitrification generally occurs in the absence of oxygen but may sometimes proceed even when oxygen is present (Gao *et al*. 2009).

Denitrification is carried out by many different unrelated species. The "model denitrifiers" are affiliated with *Proteobacteria* (e.g. *Pseudomonas*) but environmentally important denitrifiers have yet to be found. The first step, the reduction of nitrate to nitrite, is catalyzed by the enzyme complex nitrate reductase encoded by *narGH* or *napAB*. The next step, the reduction of nitrite to nitric oxide, is catalyzed by the enzyme nitrite reductase. Nitrite reductase occurs in two forms, a multicopper oxidase type enzyme encoded by *nirK* or *aniA* and a heme cd enzyme encoded by *nirS*. The reduction of two nitric oxide molecules to nitrous oxide is catalyzed by nitric oxide reductase encoded by *norB* or *norZ*. This enzyme is part of the heme/copper family of oxygen reductases. Nitrous oxide is reduced to dinitrogen by the copper enzyme nitrous oxide reductase, encoded by *nosZ*. *NirK, nirS* and *nosZ* are most often used as functional gene markers (Jones *et al*. 2008). However whole genome sequencing is providing evidence that with the primers currently in use (e.g. Baker *et al*. 1997) a substantial portion of these functional genes may be overlooked in the environment.

Recently, it was shown that denitrification might also proceed *via* a different pathway. In this pathway, performed by the bacterium Candidatus "*Methylomirabilis oxyfera*", nitrate is first reduced to nitric oxide as described above. Subsequently, two molecules of nitric oxide (NO) are dismutated into dinitrogen (N_2) and oxygen (O_2) (Ettwig *et al*. 2010). Oxygen can then be respired

aerobically or used in a monooxygenation reaction to activate hydrocarbons such as methane. It is unknown which enzymes and genes are responsible for this dismutation reaction. It is also unknown how important this reaction is in nature. With all present tracer methods it cannot be distinguished from "normal" denitrification. The interesting implication of the discovery of this pathway is that methane emissions resulting from fertilization may be restrained this way.

Together with nitrification, denitrification is an important source of nitrous oxide emissions to the atmosphere. Apparently, under some conditions denitrification is incomplete and nitrous oxide is not further reduced to dinitrogen. The chemical or biological conditions that affect nitrous oxide production are actively researched but no clear causal relationships have become apparent so far.

Anammox

The second process that recycles nitrate back to dinitrogen gas is known as anaerobic ammonium oxidation (anammox), a relatively recent discovery (Mulder *et al*. 1995). In this process, ammonia and nitrite are combined into dinitrogen. As far as we know, anammox is performed by one monophyletic group of bacteria associated with the *phylum Planctomycetes* (Strous *et al*. 1999).

The pathway presumably proceeds *via* nitric oxide and hydrazine (N_2H_4) and is inhibited by oxygen. Functional gene markers are currently being established (Strous *et al*. 2006). For example, hydrazine is presumably oxidized by a homologue of hydroxylamine oxidoreductase encoded by *hzo* (Schmid *et al*. 2008). A general property of anammox bacteria is that they can also reduce nitrate to ammonia (dissimilatory nitrate reduction, see below). For this reason it can be difficult in tracer studies to discriminate their overall activity from denitrification (Kartal *et al*. 2007).

However, because anammox is performed by only a single group of bacteria and these bacteria have a unique lipid biomarker in the form of ladderanes (Sinninghe Damste *et al*. 2002), anammox bacteria can be detected in the environment with relative ease targeting both the 16S gene and the ladderanes. Presently, it is estimated that in the marine environment approximately 50 % of the dinitrogen is

produced by the anammox bacteria of the genus *Scalindua* (e.g. Lam *et al.* 2009; but see Ward *et al.* 2010).

Dissimilatory Nitrate Reduction to Ammonia

Where denitrification and anammox close the nitrogen cycle by recycling nitrate to dinitrogen, dissimilatory nitrate reduction closes the cycle by recycling nitrate to ammonia. In contrast to denitrification and anammox, this process does not remove the nitrogen from the habitat – it remains available to primary producers. Like denitrification and anammox, it is a form of anaerobic respiration, where nitrate is used as electron acceptor instead of oxygen. Many different unrelated bacteria are capable of this process, but *Proteobacteria* have been most extensively studied; the best-known dissimilatory nitrate reducers are *Escherichia coli* and giant sulfur bacteria such as *Thioploca* (Otte *et al*. 1999).

The first step of the pathway is shared with denitrification, the reduction of nitrate to nitrite by a molybdopterin enzyme complex. Next the six-electron reduction of nitrite to ammonia is performed by pentaheme nitrite reductase encoded by *nrfAB*. Recently, it was found that octaheme enzymes evolutionary related to hydroxylamine oxidoreductase (see nitrification above) are reversible and can also reduce nitrite to ammonia (Atkinson *et al*. 2007; Klotz *et al*. 2008). Thus, a functional gene marker for this process is still work in progress. Dissimilatory nitrate reduction rates can be measured in natural ecosystems by tracer studies, but this is rarely performed and the same is true for the use of functional gene markers (e.g. Lam *et al*. 2009; Dong *et al*. 2009). Therefore, it is unknown how important this process is compared to denitrification. It is also unknown how much this process contributes to nitrous oxide emissions.

Conclusion and Discussion

Humanity mainly impacts the nitrogen cycle by agricultural fertilization and fossil fuel burning (resulting in ammonia deposition). Together, these anthropogenic inputs are estimated to be more important than natural nitrogen fixation (Fig. I-1). From field and budget studies the current effects of these emissions can be estimated: It is possible that oceanic ammonia deposition has minor negative feedback on the atmospheric carbon dioxide concentration. However, the positive feedback in the form of increased emissions of nitrous oxide and methane, as well as increased mobilization of stored terrestrial carbon is more important. It can be calculated that the total anthropogenic nitrogen inputs currently contribute 5-10 % of the current enhanced greenhouse effect, with methane and nitrous oxide contributing approximately equally. It is difficult to estimate the contribution of enhanced mobilization of refractile organic carbon in soils.

It is impossible to draft scenarios for future trends because our current understanding of the microbial nitrogen network is far from complete: For many processes environmentally significant microorganisms and genes are simply unknown (Figs. I-2 and I-3). It is also unknown how the environmental conditions affect the interplay between the different processes and the outcome in the form of changes in primary productivity, and emission of methane and nitrous oxide. These unknowns can only be addressed by disentangling the different branches of the nitrogen network and by identifying the missing microbial players. To do just that, the combination and continued development of ^{15}N labeling approaches and molecular ecology is essential.

References

Atkinson, S. J., Mowat, C. G., Reid, G. A., Chapman, S. K. (2007). An octaheme ctype cytochrome from *Shewanella oneidensis* can reduce nitrite and hydroxylamine. *FEBS Letters*, 581 (20), 3805-3808.

Baker, S. C., Saunders, N. F., Willis, A. C., Ferguson, S. J., Hajdu, J., Fülöp, V. (1997). Cytochrome CD1 structure: unusual haem environments in a nitrite reductase and analysis of factors contributing to beta-propeller folds. *Journal of Molecular Biology*, 269, 440-455.

Capone, D. G., Popa, R., Flood, B., Nealson, K. H. (2006) Follow the nitrogen. *Science*, 312 (5774), 708-709.

Cairns, J. (2010). Threats to the biosphere: Eight interactive global crises. *Journal of Cosmology*, 8, In press.

Codispoti, L. A., Brandes, J. A., Christensen, J. P., Devol, A. H., Naqvi, S. W. A., Paerl, H. W., Yoshinari, T. (2001). The oceanic fixed nitrogen and nitrous oxide budgets: Moving targets as we enter the anthropocene? *Scientia Marina*, 65, 85-105.

Dong, L. F., Smith, C. J., Papaspyrou, S., Stott, A., Osborn, A. M., Nedwell, D. B. (2009). Changes in benthic denitrification, nitrate ammonification, and anammox process rates and nitrate and nitrite reductase gene abundances along an estuarine nutrient gradient (the Colne estuary, United Kingdom). *Applied and Environmental Microbiology*, 75 (10), 3171-3179.

Duce, R. A. and colleagues (2008). Impacts of atmospheric anthropogenic nitrogen on the open ocean. *Science*, 320 (5878), 893-897.

Ettwig, K. F., and 22 colleagues (2010). Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. *Nature*, 464, 543-548.

Fuhrman, J. A., Capone, D. G. (1991). Possible biogeochemical consequences of ocean fertilization. *Limnology and Oceanography*, 36, 1951-1959.

Gao, H. and colleages (2009). Aerobic denitrification in permeable Wadden Sea sediments, *The ISME Journal*, 4 (3), 417-426.

Galloway, J. N., and 10 colleagues. (2008). Transformation of the nitrogen cycle: recent trends, questions, and potential solutions. *Science*, 320 (5878), 889-892.

Gruber, N., Galloway, J. N. (2008). An Earth-system perspective of the global nitrogen cycle. *Nature*, 451 (7176), 293-296.

Jones, C. M., Stres, B., Rosenquist, M., Hallin, S. (2008). Phylogenetic analysis of nitrite, nitric oxide, and nitrous oxide respiratory enzymes reveal a complex evolutionary history for denitrification. *Molecular Biology and Evolution*, 25, 1955-1966.

Kartal, B., Kuypers, M. M. M., Lavik, G., Schalk, J., Op den Camp, H.J. M., Jetten, M. S. M. Strous, M. (2007). Anammox bacteria disguised as denitrifiers: nitrate reduction to dinitrogen gas *via* nitrite and ammonium. *Environmental Microbiology*, 9 (3), 635-642.

Klotz, M. G., Schmid, M. C., Strous, M., den Camp, H. J. M., Jetten, M. S. M., Hooper, A. B. (2008). Evolution of an octahaem cytochrome c protein family that is key to aerobic and anaerobic ammonia oxidation by bacteria. *Environmental Microbiology*, 10, 3150-3163.

Könneke, M., Bernhard, A. E., de la Torre, J. R., Walker, C. B., Waterbury, J. B., Stahl, D. A. (2005). Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature*, 437, 543-546.

Lam, P., Lavik, G., Jensen, M. M., van de Vossenberg, J., Schmid, M., Woebken, D., Gutierrez, D., Amann, R., Jetten, M. S. M., Kuypers, M. M. M. (2009). Revising the nitrogen cycle in the peruvian oxygen minimum zone. *Proceedings of the National Academy of Sciences*, 106, 4752-4757.

Le Quere, C., Raupach, M. R., Canadell, J. G., Marland, G. (2009) Trends in the sources and sinks of carbon dioxide. *Nature Geoscience*, 2 (12), 831-836.

Mack, M. C., Schuur, E. A. G., Bret-Harte, M. S., Shaver, G. R. Chapin, F. S. (2004). Ecosystem carbon storage in arctic tundra reduced by long-term nutrient fertilization. *Nature*, 431 (7007), 440-443.

Meyer, R. L., Allen, D. E., Schmidt, S. (2008). Nitrification and denitrification as sources of sediment nitrous oxide production: A microsensor approach. *Marine Chemistry*, 110, 68-76.

Moriarty, P., Honnery, D. (2010). Why technical fixes won't mitigate climate change. *Journal of Cosmology*, 8, in press.

Mulder, A., van de Graaf, A. A., Robertson, L. A. Kuenen, J. G. (1995). Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor. *FEMS Microbiology Ecology*, 16, 177-183.

Mulholland, P. J. and 30 colleagues (2008). Stream denitrification across biomes and its response to anthropogenic nitrate loading. *Nature*, 452 (7184), 202-205.

Otte, S., and 9 colleagues (1999). Nitrogen, carbon, and sulfur metabolism in natural *Thioploca* samples. *Applied and Environmental Microbiology* , 65 (7), 3148-3157.

Schmid, M. C., and 8 colleagues (2008). Environmental detection of octahaem cytochrome c hydroxylamine/hydrazine oxidoreductase genes of aerobic and anaerobic ammonium-oxidizing bacteria. *Environmental Microbiology*, 10 (11), 3140- 3149.

Sinninghe Damsté, J. S., and 7 colleagues (2002). Linearly concatenated cyclobutane lipids form a dense bacterial membrane. *Nature*, 419 (6908), 708-712.

Strous, M., Kuenen, J. G., Jetten, M. S. M. (1999). Key physiology of anaerobic ammonium oxidation. *Applied and Environmental Microbiology*, 65, 3248-3250.

Strous, M., and 36 colleagues (2006). Deciphering the evolution and metabolism of an anammox bacterium from a community genome. *Nature*, 440, 790-794.

Vitousek, P. M., and 7 colleagues (1998). Human Alteration of the Global Nitrogen Cycle: Sources and Consequences. *Ecological Applications*, 7 (3), 737- 750.

Walker, C. B., and 23 colleagues (2010). *Nitrosopumilus maritimus* genome reveals unique mechanisms for nitrification and autotrophy in globally distributed marine *crenarchaea*. *Proceedings of the National Academy of Sciences of the USA*, 107, 8818-8823.

Ward, B. B., Devol, A. H., Rich, J. J., Chang, B. X., Bulow, S. E., Naik, H., Pratihary, A., Jayakumar, A. (2009). Denitrification as the dominant nitrogen loss process in the Arabian Sea. *Nature* , 461(7260), 78-81.

Welte, E. (1994). Agricultural chemistry - evaluation of an agricultural science in presence and future. *Landbauforschung Voelkenrode*, 44(3), 243-255.

Zehr, J. P., Jenkins, B. D., Short, S. M. Steward, G. F. (2003) Nitrogenase gene diversity and microbial community structure: a cross-system comparison. *Environmental Microbiology*, 5(7), 539-554.

Sampling site "Janssand"

Localization and characterization

Sediment samples, which served as the basis for all conducted continuous culture experiments described in this thesis, were taken from the intertidal flat "Janssand" located in the German Wadden Sea National Park of Lower Saxony. Janssand, about 11 square kilometers in size, is located in the backbarrier area of the Island Spiekeroog (Fig. 4) where the semi-diurnal tidal range is 2.6 m (Flemming & Davis 1994).
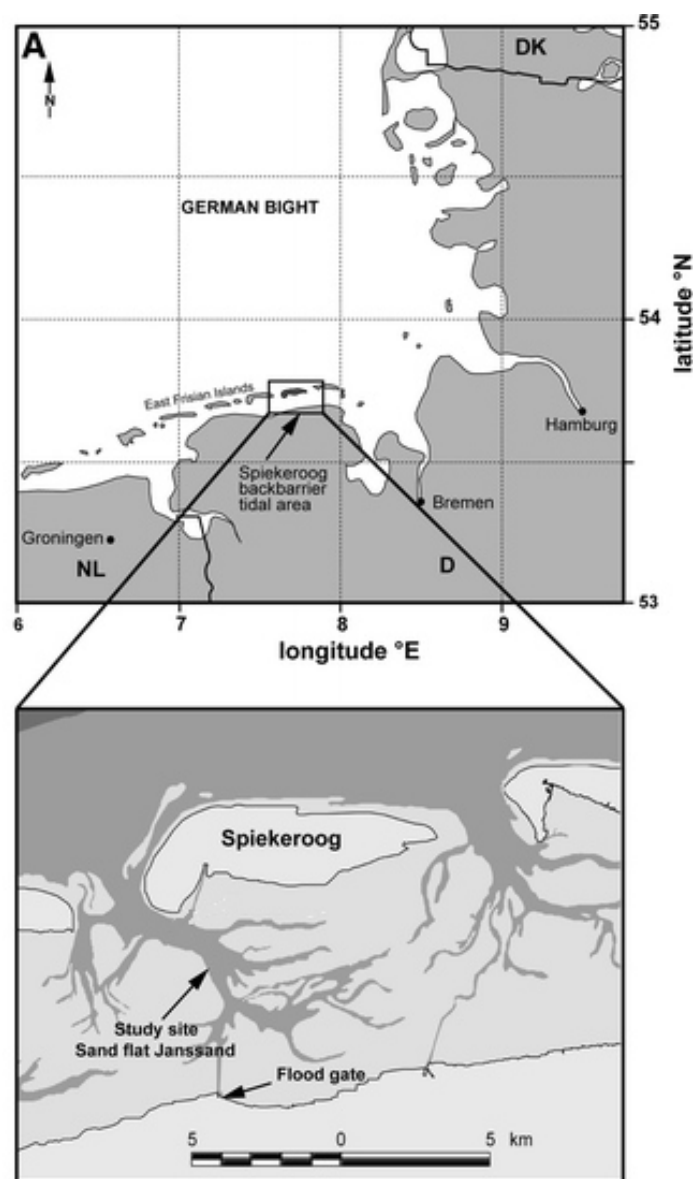
Figure 4 I Geographical location of "Janssand", an intertidal flat in the backbarrier area of the Eastern Friesian Island Spiekeroog (German Wadden Sea). (Modified from Beck *et al.* 2008).

Sampling occurred at low tide on the upper sand flat approximately 50 m from the water line. During high tide, the flat is covered by 1.5–2 m water for approximately 6 hours before it becomes exposed to the atmosphere for about the same period of time, depending on tidal range and wind direction. The flat surface is almost horizontal, except for the margin, and during low tide the difference in altitude from the water line to the sampling site is about 1.5 m (Beck *et al.* 2008, Huettel *et al.* 1996).

The Spiekeroog barrier complex has been studied intensively over the past several decades in the areas of sedimentology (Flemming and Davis 1994), nutrient cycling (Billerbeck *et al.* 2006, Gao *et al.* 2010, Gao *et al.* 2012, Beck & Brumsack 2012, Behrendt *et al.* 2013), barrier island evolution (Lüders 1953, Flemming & Davis 1994), and human interventions by either land reclamation (Brockamp & Zuther 2004) or indirect fertilizer deposition (Colijn *et al.* 2002, Fock 2003, Daehnke *et al.* 2010).

Sandy sediments cover about 70 % of all continental shelves and most beaches worldwide (Boudreau *et al.* 2001) and the intertidal regions of the Wadden Sea also mostly consist of sandy sediments (Flemming and Ziegler 1995). Sandy sediments are often considered "biochemical deserts" since they are characterized by relatively coarse sand grains, small bacterial numbers and low organic matter content (Llobet-Brossa *et al.* 1998, Rusch *et al.* 2003). In fine-grained muddy sediments, transport mechanisms are restricted to molecular diffusion, bioirrigation, and bioturbation (Aller 1982, Janssen *et al.* 2005). In sandy sediments, however, the permeability improves with increasing grain size. In sediments with permeabilities of k >10-12 m^{-2}, horizontal pressure gradients at the sediment-water interface cause advective pore water flow (Huettel & Gust 1992, Huettel *et al.* 1996). This is the flow of water through the sediment's interstices. For example, oxygenated water intruding the ripple troughs forces the upwelling of anoxic deep pore water to the sediment surface (Precht *et al.* 2004). In sandy sediments the efficient exchange between pore water and the water body is not only due to bioturbation (Wethey *et al.* 2008), bioirrigation (Reise 2002), and diffusion (as it is the case for muddy sediments) but mostly because of pore water advection, waves (Precht & Huettel 2003, Precht *et al.* 2004), and tidal pumping (Beck *et al.* 2008a). In this way, advective transport in sandy sediments can

exceed diffusion-controlled transport of muddy sediments by several orders of magnitude (Huettel & Webster 2001). In particular, advective pore water flow causes the influx of oxygen (Ziebis *et al*. 1996, Precht *et al*. 2004, Forster *et al*. 1996), nutrients (Huettel *et al*. 1998), and organic material (Rusch & Huettel 2000) from the surrounding water into the sediments. Similar to a very large filter system, the suspended material as well as algae and bacteria are trapped in the sediment pores (Rusch *et al*. 2003, Jansen *et al*. 2009). The permanent delivery of oxygen and other reactants by pore water flow facilitates the decomposition of the suspended material by the trapped microorganisms and leads to mineralization rates similar to that seen in nutrient-rich muddy sediments (De Beer *et al*. 2005). Although counterintuitive, in the surface layer of the permeable sediment, which delivered the samples for inoculation of the continuous cultures investigated in the course of this thesis, oxygen penetration is high at high tide (up to several centimeters (Jansen *et al*. 2009)), resulting from pore water flow, but restricted to a few millimeters at low tide (De Beer *et al*. 2005, Jansen *et al*. 2009). At low tide, intrusion of air into the sediment is prevented by its fine grain size, which enables capillary forces to keep the sediment pores filled with water and thereby prevents ventilation (Atherton *et al*. 2001, Billerbeck *et al*. 2006). Furthermore, efficient bacterial respiration rates in combination with limitations of diffusion-controlled transport in the water-saturated sediment during low tide result in anoxia even in the upper sediment layers.

Despite the low organic matter content of sandy sediments the high aerobic and anaerobic degradation and mineralization rates (Forster *et al*. 1996, D'Andrea *et al*. 2002, De Beer *et al*. 2005) resulting from pore water flow promote a fast recycling of organic matter and metabolic products within hours and/or days (Beck *et al*. 2008, Jansen *et al*. 2009).

Consequently, for sediment dwelling microorganisms the living conditions in this type of sediments are not only shaped by physical influences like hydrodynamic forces but also by biogeochemical processes resulting from the movement of pore water and soluble and particulate substances. These organisms have to develop strategies to cope with shear stress (for example *via* attachment to sand grains (Rusch *et al*. 2003)), gradients in salinity and temperature, and frequent changes in the availability of nutrients, oxygen and other electron acceptors.

Nutrient conversion pathways, rates, and bacterial key players

Janssand, like most tidal flats, is an area of high primary production and organic matter mineralization. Generally, pore water bodies inside the flats are richer in nutrients than the surrounding seawater bodies (Beck *et al.* 2009). Microbial activity in the sediment remineralizes organic matter (OM) produced in the flat area as well as material imported from the North Sea. Inside the sediment, the organic matter is degraded by a cascade of redox processes. These involve aerobic respiration, followed by the subsequent reduction of the electron acceptors nitrate/nitrite, manganese (Mn) and iron (Fe) oxides, sulfate, and finally by methanogenesis. This cascade follows a gradual decrease in redox potential of the mentioned oxidants (Aller 1982, Jørgensen 2006). Exceptions from this theory will be discussed later. Mineralization products such as nutrients (phosphate (HPO_4^{2-}), ammonia (NH_4^+), and trace gases like methane (CH_4), and nitrous oxide (N_2O)) are released from the sediments into the overlying water column by pore water transport. This forms the basis for a continuously high primary production in the Wadden Sea and theoretically contributes to global warming by GHG production (Carpenter *et al.* 2012).

The type of prevalent remineralization by oxidation of carbon substrates in the sediments generally depends on the availability of the above mentioned electron acceptors and the corresponding bacteria capable of using these substrates. However, the availability of these nutrients in areas like the backbarrier region of Spiekeroog varies depending on multiple factors including tides, seasons, rainfall events, advective pore water transport, phytoplankton dynamics, bacterial respiration activities, bioirrigation, and human activities (like the use of flood gates and fertilizers in the hinterland). Additionally, the organismal communities utilizing a given substrate are localized in different zones and niches of the sediment bulk not necessarily next to a community specialized on the same substrate (Wilms *et al.* 2006a). Due to this temporal and spatial variability in tidal flat sediments, the use of the mentioned electron acceptors does not follow the strict theoretical pattern according to the substrates' energy yields, as may be the case for less permeable muddy sediments. In diffusion-controlled muddy sediments, a horizontally layered geochemical stratification according to the redox tower can sometimes (e.g. in the absence of disturbance by macrobenthos) be observed

(Aller 1982). In highly dynamic habitats like permeable sands, however, the geochemical stratification and hence the classical redox cascade is disturbed. Here, advective pore water flows create a complex three-dimensional geochemical zonation (Huettel *et al.* 1998, Beck & Brumsack 2012).

It is therefore impossible to strictly spatialize certain conversions or to quote nutrient conversion rates for a site like Janssand. However, since Janssand has been the focus of research for microbiologists and biogeochemists for several decades, the volume of data on nutrient cycling in the area is, nevertheless, quite remarkable.

Oxygen respiration

Oxygen respiration dominates organic matter degradation in the uppermost layer of Janssand, up to a depth of 3-5 cm (Jansen *et al.* 2009) depending on the sediment's permeability and the resulting oxygen penetration depth. However, oxic micro-niches could be detected down to a depth of 20 cm along the burrows of the zoobenthos in coastal sediments (Reise 2002). As microbial respiration activity is generally higher in summer, and as oxygen dissolves to a lower extent in warm water, oxygen penetration and according aerobic OM degradation is restricted to higher levels in summer than in winter (Jansen *et al.* 2009). Highest oxygen consumption rates were determined in tidal flat margin sediments flushed with seawater each tidal cycle and were in the range of up to 200 mmol m^{-2} d^{-1} (Billerbeck *et al.* 2006).

Photosynthesis

Photosynthesis is present in the sediment surface as well. Benthic photosynthesis decreases with increasing water depth due to diminishing light penetration in the turbid Wadden Sea (Colijn & Cadée 2003). Oxygen production rates of up to 17 mmol m^{-2} d^{-1} were determined by Jansen *et al.* (2009). Investigation of photosynthesis is complicated and depends on many factors, therefore different rates are determined under different conditions. Interestingly, photosynthesis does not substantially increase the oxygen penetration depth in the sediment (Jansen *et al.* 2009).

Denitrification & aerobic denitrification

Fixed nitrogen (N) in form of nitrate (NO_3^-) and nitrite (NO_2^-) is primarily removed from coastal ecosystems *via* microbial mediated denitrification, which takes place on the seafloor (Seitzinger 1988). The importance of denitrification and aerobic denitrification (the (aerobic) reduction of nitrate to dinitrogen (N_2), for the sake of OM remineralization) is less well understood than the oxygen dependent degradation process in Janssand sediments. Denitrification has a minor contribution to OM degradation in sandy sediments (Vance-Harris & Ingall 2005). However, research data have shown (Gao *et al.* 2010) denitrification rates on Janssand being among the highest potential denitrification rates in marine environments with ≥ 0.19 mmol N m^{-2} h^{-1}. Obviously, the denitrifying bacteria in this sediment are well adapted to tidally induced changing redox oscillations (aerobic *vs.* anaerobic conditions) although for a long time denitrification was thought to be inhibited by oxygen (Sacks & Barker 1949) (Hernandez & Rowe 1988). The organisms seem to have developed strategies to continue denitrification in the presence of up to 90 µM oxygen, being able to denitrify with a rate of >50 µmol m^{-2} h^{-1} (Gao *et al.* 2010). According to Llobet-Brossa *et al.* (1998), nitrate respiring members of the genus *Arcobacter* were shown to be present in upper sediments layers of Dangast (Jade Bay, German Wadden Sea) to a depth of 3.5 cm with total numbers of $>10^7$ cells m^{-3}. This is in accordance to a nitrate penetration depth of 3.6 mm in the same sediments (Llobet-Brossa *et al.* 2002).

Anaerobic ammonia oxidation

Anaerobic ammonia oxidation (anammox), another dissimilatory pathway that removes fixed nitrogen from the environment and releases it in form of dinitrogen, has only recently been proven in marine environments. In 2002 Thamdrup & Dalsgaard published the first direct evidence for the occurrence of anammox in a natural environment, namely in sediments of the Skagerrak, Denmark. Anammox rates of up to 11 nmol cm^{-3} h^{-1} (26 % contribution to total N_2 production) were determined in sediments of Randers Fjord, Denmark (Risgaard-Petersen *et al.* 2004). These authors detected a bacterium affiliated with the anammox organism candidatus *Scalindua sorokinii* (99 % overall 16S *rRNA* gene sequence similarity). This organism belongs to the *phylum Planctomycetes* whose autotrophic members

are responsible for the anammox process (Strous *et al.* 1999). In studies by Thamdrup & Dalsgaard (2002) and Engström *et al.* (2005) anammox activity in Skagerrak sediments was responsible for up to 67 % and 79 % of total N_2 production, respectively. Thus, anammox has a higher impact on the removal of fixed nitrogen than denitrification in these sediments. However, on a larger scale the relative contribution of anammox to fixed nitrogen removal is highly variable. The failure of detection of anammox activity in sediments of the Norsminde Fjord (Denmark) by Risgaard-Petersen *et al.* (2004) indicates that this process is not ubiquitous.

Dissimilatory nitrate reduction to ammonia

A third pathway that removes fixed nitrogen is called dissimilatory nitrate reduction to ammonia (DNRA). DNRA also has a minor influence on nitrate consumption in marine sediments compared to denitrification (Behrendt *et al.* 2013). DNRA converts nitrate to ammonia. The latter is recycled either directly within the sediment or in the water column into which it diffuses. DNRA and denitrification compete for their substrate nitrate and the outcome of this competition, either ammonia (and nitrate after recycling; *via* DNRA), or dinitrogen (*via* denitrification), determines whether the sediment acts as a source or a sink of fixed nitrogen. This in turn determines the trophic status of the whole system. A DNRA-based ammonia production rate of 0.13 ± 0.02 nmol N m^{-2} h^{-1} could be determined in incubations of sediment cores from Janssand (Behrendt *et al.* 2013). Rates determined in slurry incubations from the same site were substantially higher (Behrendt *et al.* 2013). Throughout the study, highest rates were obtained on sites with very high concentrations of ammonia and total sulfide. DNRA activity was shown to be highest within and below the layer of NO_3^- reduction.

Manganese & iron oxide reduction

Manganese and iron oxide reduction are to a minor degree contributing to carbon degradation in permeable sediments, however they gain importance in Mn or Fe-rich sediment layers (Thamdrup *et al.* 1994). According to molecular biological approaches conducted by Mußmann *et al.* (2005), an Fe(III)-reducing strain that affiliated with the *Pelobacter-Desulphuromonas* group was detected in up to 6 %

of total cell numbers in an intertidal flat in the Jade Bay. This group of bacteria might play a substantial role in dissimilatory Fe(III) reduction.

Sulfate reduction

Deep in the flat, sulfate reduction, as one of the main terminal pathways of anaerobic OM degradation in Wadden Sea sediments, occurs and leads to depletion of sulfate. This facilitates methanogenesis in this deep zone of Janssand (Jansen *et al*. 2009). Sulfate reduction has been studied intensively in the Wadden Sea tidal flat areas and accordingly, sulfate reducing bacteria (SRB) constitute a highly active and abundant population in anoxic sediments (Llobet-Brossa *et al*. 2002). Key players of this process in Wadden Sea sediments include members of the *Desulfovibrio* and *Desulfosarcina-Desulfococcus-Desulfofrigus* groups. These were often detected in marine sediments *via* fluorescence *in situ* (FISH) approaches and appear to be a numerically abundant and active group of SRB in this habitat (Llobet-Brossa *et al*. 2002). Perhaps, another important player detected in these experiments may be a large filamentous bacterium affiliated with the genus *Desulfonema,* which in terms of numbers contributed little to the SRB detected but due to its size significantly in terms of the total bacterial biomass in the sediments (Llobet-Brossa *et al*. 1998). Sulfate reduction rates (SSR) of up to 9.5 mmol $m^{-2} d^{-1}$ were determined (Billerbeck *et al*. 2006) in the upper flat sediment of Janssand. In this central region of the flat, sulfide as the product of sulfate reduction is released by pore water flow at the seeps where it is oxidized by chemical or microbial processes (Jansen *et al*. 2009).

Methanogenesis

Methane (CH_4), the end product of methanogenesis, is released mostly during low tide from deeper sediment layers, in which methanogenesis occurs, and is partly oxidized by microbes and partly set free to the atmosphere (Jansen *et al*. 2009). Maximum methane concentrations in the water column next to Janssand range from 140 to 570 nM (in autumn 2005; Grunwald *et al*. 2010). However, CH_4 detectable in this area might not be exclusively produced by methanogens but might also be imported by freshwater contributed to the back barrier area *via* opening of the flood-gate in Neuharlingersiel (Grunwald *et al*. 2010).

Methanogenesis is assumed to occur mainly in depths of >5 m on Janssand (Grunwald *et al.* 2010). In layers above 5 m, methanogens directly compete with sulfate reducers for electron donors. Although methanogens are assumed to escape this competition by utilization of methylated substrates, which are not used by sulfate reducers (Madigan 2006), hotspots for methanogenesis are found in sulfate depleted sediment depths (Wilms *et al.* 2006a, Grunwald *et al.* 2010). Elevated CH_4 concentrations measured above 5 m depth, co-existing with sulfate concentrations, are assumed to result from the upward diffusion of this gas (Grunwald *et al.* 2010). Methanogens identified in Janssand sediments belong to the typically hydrogenotrophic *Methanomicrobiales* (Wilms *et al.* 2006a). According to the measurable release of CH_4 from the sediments, methane oxidation in the upper layers can obviously not cope with its production rate to eliminate methane in a dynamic environment like tidal sediments (Jansen *et al.* 2009). This means that intertidal flats are considerable sources of this powerful greenhouse gas. A model calculated by Grunwald *et al.* (2010) reveals an estimate for the CH_4 budget of the Spiekeroog backbarrier tidal flat area of 3.3-3.6×10^6 mol a^{-1}.

Bacterial key players

The availability and quality of organic carbon seems to have the most substantial effect on bacterial community compositions of Janssand. The availability of electron acceptors, however, seems to have a minor influence, at least in subsurface sediments (Wilms *et al.* 2006a). At this site, fermenters and syntrophic bacteria dominate the bacterial community (Wilms *et al.* 2006a). Since these microorganisms are independent of sulfate they are uniformly distributed in the upper 2 m of the sediment, including the sulfate minimum zone. The bacteria detected were affiliated to *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Spirochaetes*, and *Chloroflexi*. *Gammaproteobacteria* were almost exclusively found in the upper layers while *Firmicutes*, *Bacteroidetes*, and *Chloroflexi* were mainly detected at and below 220 cm (Wilms *et al.* 2006a). In a second publication the authors mention a striking population shift at 200 cm depth of the Janssand area sediments (Wilms *et al.* 2006b). The layer above 200 cm was dominated by *Gamma-* and *Deltaproteobacteria* known to mostly consume

easily degradable organic substances, which generally occur in the upper, younger layers of these marine sediments. The layer below 200 cm, however, was shown to be dominated by members of subdivision two of *Chloroflexi*, a widespread phylogenetic group that has often been detected in deep biosphere subsurface sediments. Bacteria affiliated to *Chloroflexi* subdivision two are assumed to degrade kerogen for carbon retrieval and in deeper sediment layers they are thought to compete with methanogens for hydrogen (Wilms *et al.* 2006b). The microbial community in the oxic Janssand sediments is dominated by populations of *Gammaproteobacteria* among which potential sulfur oxidizing *Gammaproteobacteria* make up for an important part (approx. 39.6 %) (Lenk *et al.* 2011). Llobet-Brossa *et al.* (1998) found bacteria belonging to the *Cytophaga-Flavobacterium* cluster to be the most abundant phylogenetic group in German Wadden Sea sediments (samples taken at Jade Bay, Germany). This group accounted for 15-25 % of all detected cells. The highest abundance was observed in the uppermost layer of the sediment. These organisms are all organotrophs, aerobic, microaerophilic, and facultatively anaerobic, respecttively, gram negative, and mainly specialized in the degradation of complex biomacromolecules (Reichenbach 2006). Furthermore, Llobet-Brossa *et al.* (1998) found low numbers of *Alpha*-, *Beta*- and *Gammaproteobacteria*, *Planctomycetes*, and gram-positive bacteria with high GC-content. *Planctomycetes* were also detected on Janssand and shown to be highly abundant at all depths of Janssand sediments with a maximum abundance at 3-4 cm (Jansen *et al.* 2009).

Thermodynamics

Microbially mediated geochemical cycling sustains life on Earth as we know it. For example, 2 billion years ago, evolution led to the development of the biogeochemical process that formed the prerequisite for human existence: oxygenic photosynthesis. This process released oxygen to the atmosphere, slowly generating the atmospheric conditions we face today.

Geochemical processes on Earth follow the basic thermodynamic laws and strive for a state of maximum entropy and most stable mineral phases. The redox reactions carried out in course of the geochemical processes like for example

photosynthesis, denitrification or methanogenesis may proceed by purely chemical reactions. However, they are enzymatically catalyzed in microorganisms and therefore often accelerated up to 10^{20}-fold relative to the non-biological reaction rate (Jørgensen 2006). Microorganisms inhabited Earth long before humans appeared. With their versatile enzymatic machinery, microorganisms in combination with mass transfer processes control the rates of element cycles and so determine the (spatial and temporal) accumulation of elements like oxygen. The diversity of microbes' catalytic capabilities are still not fully explored and our knowledge of these capabilities still expands with the discovery of new organisms and metabolisms.

Previously unknown geochemical processes can be derived from the discovery and investigation of "new" organisms such as a *Planctomycete* responsible for the anammox process (Strous *et al.* 1999). The other way around, the observation of certain chemical characteristics in a natural habitat can lead to the discovery of a previously unknown geochemical process. This in turn can trigger the search for and finally the discovery of so far unknown microbial species such as anoxygenic phototrophic iron oxidizers (Widdel *et al.* 1993) and anaerobic methane oxidizing sulfate reducers (Boetius *et al.* 2000).

Another way towards the discovery of new unknown geochemical processes is by modelling them thermodynamically. Thermodynamic models can reveal theoretically (thermodynamically) possible reactions, which have not been found in nature (Broda 1977, Van de Leemput *et al.* 2011, de Jonge *et al.* 2002). Modelling of so far unknown metabolic scenarios allows the directed search for such processes. Furthermore, unknown metabolic pathways can be derived from information of metabolic potential gained in sequencing approaches as described for the detection of nitrite driven anaerobic methane oxidation by Ettwig *et al.* (2010).

Apart from human dependency on microbial activity in geochemical cycling, microbes perform these processes because they provide them with chemical energy for cell functions such as growth, cell division, movement, and synthesis of cellular components. Microbes conserve energy by coupling the energy derived from catalyzing thermodynamically favorable redox processes to the formation of a

proton motive force, which finally drives the formation of high-energy phosphate bonds in compounds such as ATP. Another possibility is the formation of such bonds by phosphorylation of carbon compounds.

Thermodynamically favorable redox reactions are exergonic reactions characterized by a negative change in free energy (ΔG^0). Larger negative ΔG^0 values indicate that the more energy is dissipated from the corresponding reaction. The free energy change ΔG^0 depends on the number of electrons transferred and on the redox potentials (E_0'; standard conditions) of the involved redox pairs (e.g. $2H^+/H_2$: -0,42 V). For standard conditions ΔG^0 can be calculated according to the following equation:

$$\Delta G^0 = -n \ F \ \Delta E_0` \ [kJ \ mol^{-1}] \qquad (1)$$

where n is the number of electrons transferred, F is Faraday's constant (96,485 Coulomb mol^{-1}), and ΔE_0 is the difference between the standard redox potentials of both half reactions.

Under *in situ* conditions the given physical and chemical parameters can furthermore influence the free energy change of a reaction. A redox reaction favorable under standard conditions might be not favorable *in situ*. Thermodynamic modeling approaches therefore must account for the prevailing conditions in a certain habitat.

The redox potential E_0', expressed in Volts (V) or Millivolts (mV), is the tendency of a substrate to either donate or accept electrons. Good electron donors ("reductants", e.g. organic substances like glucose) have rather low E_0' and good electron acceptors ("oxidants", e.g. oxygen) in turn have high redox potentials. The larger the difference in E_0' between an electron donor and an electron acceptor, the more energy is released. Accordingly, the oxidation of organic compounds with oxygen is a very favorable reaction. Generally, respiration processes (either aerobic with O_2 as oxidant or anaerobic with electron acceptors like NO_3^- or NO_2^-) deliver more energy in form of ATP than fermenting processes like acetate or lactate fermentation. Products of respiration processes are, next to the oxidized substrate (CO_2), the reduced electron acceptors, for example Fe^{2+}, H_2S, N_2, and H_2O.

In contrast to respiration processes, fermentation pathways, which are very important in marine sediments, do not make use of external electron acceptors. Instead, during fermentation the substrate serves as electron donor and acceptor simultaneously. This leads to the disproportionation of the substrate, producing reduced and oxidized compounds at the same time. Energy conservation in form of ATP does not occur *via* chemiosmosis, as it is mostly the case for respiration, but through substrate level phosphorylation. During the latter, intermediates are subsequently coupled to a phosphate molecule, which in turn is used to transform ADP into ATP. The yield of ATP produced from fermentation is rather low but still high enough to enable activities like growth, maintenance, and motility. A prerequisite for this process is, however, that the cells prevent the accumulation of the reduced electron carrier NADH as this would lead to cessation of glycolysis and to the shutdown of the whole process. This is realized principally by transferring the electrons from NADH to an intermediate such as pyruvate in the oxidative branch of the fermentation process. Fermentation is very important in marine sediments as it provides the organisms with small organic molecules such as volatile fatty acids (e.g. acetate, formate, butyrate, propionate), lactate, some alcohols, hydrogen and CO_2. These molecules are in turn the main substrates for sulfate reduction in anoxic sediments and partly also for methanogenesis.

In marine sediments, like in all other habitats, redox reactions of various types do not randomly occur instantaneously at the same spot. In theory, habitats like sediments and soils (as long as they are physically mostly undisturbed) are more or less zoned or even stratified as already mentioned. This results from the poor solubility of oxygen and limited downward rates of the transport of electron acceptors.

Firstly, different microbes catalyzing different geochemical reactions have unique needs with respect to the environmental conditions. For example, some need the presence of oxygen to survive (obligate aerobes), some cannot survive in the presence of oxygen (obligate anaerobes), whilst some need low oxygen concentrations (microaerophils) or are even able to live in presence as well as absence of oxygen (facultative anaerobes). According to these restrictions microbes catalyzing certain chemical reactions inhabit regions offering conditions that match their requirements. However, some organisms even have developed

strategies to overcome the restrictions, which eventually expands their niche (for example by mechanisms to detoxify inhibiting substances).

Further, metabolic activity is constrained by the presence or absence of certain electron donors and acceptors. For example, in marine sediments the availability of oxygen is generally limited to the upper few millimeters to centimeters (in the absence of advective pore water flow and bioirrigation, as already mentioned). Accordingly, aerobic respiration can only take place in this upper layer of sediments while lower layers provide electron acceptors for microorganisms catalyzing different reactions. Consequently, most of the organic mineralization takes place within the anoxic layers of marine sediments with the help of the metabolically highly diverse prokaryotic organisms.

The typical (however not obligate, as discussed later) theoretical geochemical zonations in marine sediments with respect to carbon degradation and the consecutive reduction of oxidants as well as their reoxidation are depicted in Figure 5.
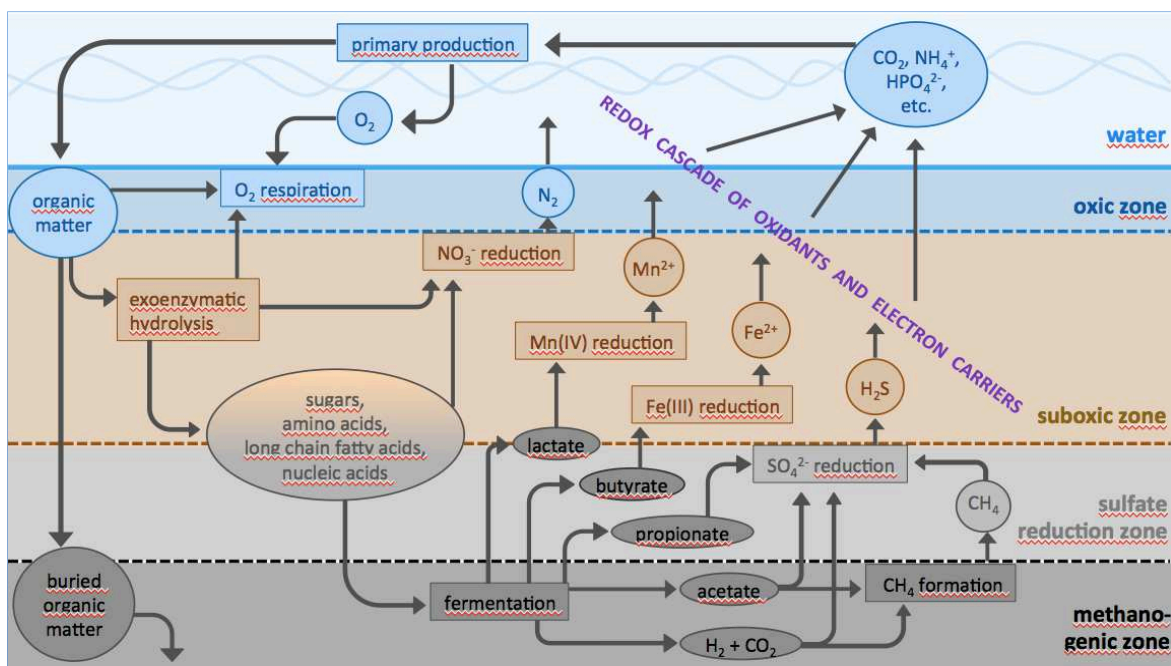
Figure 5 l Theoretical geochemical zonation of the successive reduction of oxidants during carbon degradation in marine sediments (reproduced from Jørgensen 2006).

From Figure 5 it can be concluded that (macromolecular) organic matter is primarily degraded with oxygen in the uppermost layer of the sediment. With increasing depth the availability of electron acceptors changes: as nitrate is the

thermodynamically next favorable electron acceptor its depletion takes place in the suboxic sediment layer, below the oxic zone. The organic substrates for nitrate reduction are assumed to be the same as those used in aerobic respiration. After depletion of nitrate, along with increasing depth, the redox cascade continues with the electron acceptors Mn(IV), Fe(III), SO_4^{2-}, and CO_2. This sequence is in accordance with the gradual decrease in redox potential of these substances and with the amount of free energy delivered by their respiration. In terms of electron donors, sulfate reducers and methanogens, are mostly unable to respire sugars or amino acids and are therefore dependent on the products delivered by fermentative degradation of these substrates. The tight coupling between different degradation processes in marine sediments and the subsequent use of metabolic products by different species leads to an efficient network of highly active metabolic processes in these environments (Jørgensen 2006, Madigan 2006).

Until now, the organisms responsible for catalyzing all so far known geochemical reactions have only been partially identified. New microbes are continuously being identified either with help of culturing approaches or by molecular or sequencing studies (as mentioned previously).

However, as mentioned before it is not always true that metabolic processes abide by the redox cascade. Unsorted redox processes do co-occur in nature although this comprises the overall productivity. The following examples are known:

Warburg Effect

One of the observations of the co-occurrence of unsorted redox processes is known as the Warburg Effect, leading back to the work of Otto Warburg in the first half of the 20th century (Warburg *et al.* 1924, Warburg *et al.* 1927, Warburg *et al.* 1956). The Warburg Effect was observed in some animal derived cells of proliferative and tumorous tissue (reviewed by Vander Heiden *et al.* (2009) and Ferreira (2010)). Recently, this effect has also been linked to activated inflammatory immune cells (reviewed by Palsson-McDermott & O'Neill (2013)). The Warburg Effect is sometimes called "aerobic glycolysis", indicating the counter-intuitive suppression of mitochondrial oxidative phosphorylation in the presence of oxygen. Instead of conducting bioenergetically effective oxidative

phosphorylation, as normal differentiated cells do, cells characterized by the Warburg Effect conduct fermentative glucose degradation to lactate in the presence of oxygen which leads to the conservation of substantially less energy. Oxidative phosphorylation creates only small amounts of lactate and in accordance has a high energy yield (36 mol ATP/mol glucose). Aerobic glycolysis, however, delivers not more than about 4 mol ATP per mol glucose (reviewed by Vander Heiden *et al.* (2009)). The reason why tumorous and proliferating cells perform this inefficient metabolic pathway under oxygen rich conditions and with functional mitochondria has remained under discussion. Initially, Otto Warburg hypothesized an irreversible injury to the cells' respiratory mechanism of being responsible for the cancer cells to adopt a fermentative metabolism to compensate for improper respiration capability (Warburg *et al.* 1956). This theory, however, was challenged as cancer research continued (Weinhouse 1956, Weinhouse 1972). The Warburg Effect appears to be caused by the enhanced expression of glycolysis enzymes and glucose transporters and a simultaneous down-regulation of mitochondrial metabolism (Diaz-Ruiz *et al.* 2009). At present, there are many hypotheses about the function of this mechanism for the cells. For example, it offers the cells an advantage to proliferate under hypoxia, which is a common condition in the inner cores of solid tumors. The ability to switch between both mechanisms furthermore enables the cells to adapt their metabolism to prevailing conditions, either inside a tumor or on the outside near blood vessels which supply the tumor with oxygenated blood (Sutherland 1988, Gatenby & Gillies 2004). Moreover, the possible escape from apoptosis by down-regulation of oxidative phosphorylation has been discussed as a potential background for the observations made (Tomiyama *et al.* 2006, Schlisio 2009, Gogvadze *et al.* 2010). These hypotheses are of importance with respect to the development of anti cancer and anti inflammation therapeutic strategies (Vander Heiden *et al.* 2009, Palsson-McDermott & O'Neill 2013).

Crabtree Effect

The Crabtree Effect in budding yeast e.g. 'baker's yeast' (*Saccharomyces cerevisiae*) is known as the repression of respiration under fully aerobic conditions in the presence of rapidly fermentable sugars as a carbon source (De Deken

1966). The effect is characterized by a glucose-induced repression (rather than an inhibition) of respiration enzymes in the presence of oxygen (Beck & von Meyenburg 1968). In particular, enzymes are regulated on two levels: first on the abundance level (high or low gene transcription *via* gene induction/repression, mRNA stability, translation, and protein stability) and second on the activity level (post-transcriptionally by allosteric and covalent activation or inhibition) (Rolland *et al.* 2002). The according shift from oxygen respiration towards (inefficient) fermentative metabolism leads to a loss in biomass yield. Glucose-sensitive yeasts overcome this problem by increasing their glycolytic capacity *via* induction of high amounts of glycolytic genes as well as genes encoding for glucose transporters. The accordingly achieved high glucose turnover rate, that compensates the low ATP yield derived from fermentation, enables the cells effectively to compete for survival. Their competitive advantage is increased by the release of ethanol as fermentation product, which inhibits growth of competing microorganisms. Furthermore, yeast cells can subsequently use ethanol aerobically as a non-fermentable carbon source (Rolland *et al.* 2002). *S. cerevisiae* was shown to be able to adapt its metabolism to the environmental conditions leading to a fermentative metabolism at high glucose concentrations and a respiratory metabolism under carbon limitation (reviewed by Thevelein (1994)). The Crabtree Effect in baker's yeast is economically undesired when producing yeast as a baking agent. While growing aerobically, *S. cerevisiae* gains much energy from respiring relatively small amounts of glucose, resulting in a high biomass yield per mol glucose fed. However, if *S. cerevisiae* switches to fermentative metabolism, the glucose turnover rate increases, ethanol is produced and the biomass yield decreases which is economically undesirable (Daramola & Zampraka 2008).

The term Crabtree Effect is not only used to describe a metabolism feature in yeast but has also been observed in cancer cells. In cancer research, the Crabtree Effect specifies a transient suppression of respiration and oxidative phosphorylation by glucose in tumor cells in spite of the presence of functional mitochondria and oxygen. The outcome is a reduced energy (ATP) yield. As for yeast, cancer cells presenting the Crabtree Effect have the ability to switch between fermentation and respiration (Rossignol *et al.* 2004). This feature might enable cancer cells *in vivo* to adapt their metabolism to the heterogeneous

conditions in tumorous tissues (Diaz-Ruiz *et al.* 2011). The differences of the Crabtree Effect in cancer cells *vs.* yeast are marginal as reviewed by Diaz-Ruiz *et al.* (2011). The Crabtree Effect is distinguishable from the above-mentioned Warburg Effect by its persistency: metabolic traits related to the Crabtree Effect are short-term and reversible. Warburg Effect related observations are long-term changes resulting from a metabolic reprogramming (Diaz-Ruiz *et al.* 2009, Diaz-Ruiz *et al.* 2011). As in the Warburg Effect, the mechanisms behind the Crabtree Effect in cancer cells have yet to be unraveled. The most accepted theory is that glycolysis enzymes and mitochondria compete for free cytoplasmic ADP (Weinhouse 1972, Gatt & Racker 1959) where glycolysis enzymes, overactive in cancer cells characterized by the Crabtree Effect, outcompete mitochondria. Accordingly, ADP as substrate for oxidative phosphorylation is lowered in concentration and respiration is decreased. However, also this hypothesis seems unlikely to occur *in vivo* due to a ca. 100-fold lower K_m (Michaelis constant) of the mitochondrial ADP converting enzymes in comparison to the glycolysis enzymes (Veech *et al.* 1979), which would lead to an outcompetition of glycolysis by respiration (Diaz-Ruiz *et al.* 2011). Another hypothesis suspects the cytoplasmic [ATP]/[ADP][P_i], especially the intracellular [P_i], to trigger the Crabtree Effect (Sussmann *et al.* 1980).

Cryptic sulfur cycle

Cryptic biogeochemical cycles like the cryptic sulfur cycle complicate the understanding of the complex network of biogeochemical reactions in natural habitats. They consist of reactions interacting in a way that prevents the identification of their products. The cryptic sulfur cycle (Canfield *et al.* 2010) comprises the simultaneous activity of sulfate reduction and sulfide oxidation. Active sulfate reduction occurring in the environment or a microbial culture can normally be determined by standard geochemical protocols, i.e. by the detection of sulfide accumulation or reduction of sulfate concentrations. Sulfide oxidation, on the other hand, leads to the production of sulfate, which can be determined by measuring either the accumulation of sulfate or the loss of sulfide. The tight coupling of both reactions, however, results in the consumption of each of the substances as soon as they are produced. Therefore, neither detection of sulfate

nor sulfide can be linked to a microbial activity. With respect to the calculation of carbon and nutrient budgets, the presence of cryptic biogeochemical cycles can consequently cause underestimations of the outcomes. While mass balances fail to detect these processes, RNA-based molecular and sequencing approaches are convenient ways to discover them. The identification of the genetic activity of the underlying community (transcriptomics) can reveal valuable information about active metabolic pathways that cannot be detected by geochemical protocols alone (Canfield *et al.* 2010, Kiel Reese *et al.* 2013, Kiel Reese *et al.* 2014).

Aerobic denitrification

„Since the classical experiments of Gayon & Dupetit (1886), it has been known that oxygen inhibits the reduction of nitrate and the formation of nitrogen by denitrifying bacteria" (Sacks & Barker 1949). Furthermore, according to the preferential order of electron acceptor use (Froelich *et al.* 1979), denitrification as an obligate anaerobic process was not believed to occur in the presence of oxygen. Indeed, for a long time, many experiments demonstrated that denitrification was a strictly anaerobic process as denitrification by some well-studied bacteria was arrested after exposure to oxygen (Dendooven & Anderson 1994, Zumft 1997, Codispoti *et al.* 2001, Hulth *et al.* 2005). Oxygen could be shown to inhibit both the formation of certain denitrification enzyme complexes as well as their function once they are present in the organism (Sacks & Barker 1949). The mechanism of inhibition or repression of denitrification enzymes by oxygen seems to differ among the involved reductases as well as among different denitrifying organisms (Otte *et al.* 1996). Some studies demonstrated the reversibility of this inhibition and discussed the possible advantage of bacteria being able to switch from nitrate respiration to aerobic respiration and back under transiently changing oxygen conditions, implying the significance of aerobic denitrification (Hernandez & Rowe 1988, Takaya *et al.* 2003). At that time, several publications had already presented data on bacterial reduction of nitrate under aerated conditions (Seiser & Walz 1925, Meiklejohn 1940). However, active debate persisted on the validity of these results. Krul & Veeningen (1977) and Meiberg *et al.* (1980) for the first time published hints for aerobic denitrification, which were mostly accepted by the scientific community

(Robertson & Kuenen 1984). Contradictory results of early studies in terms of oxygen influence on denitrification were supposed to result from methodological issues with respect to maintaining "aerobic conditions" (Sacks & Barker 1949). Another reason was the divergent behavior of different bacteria leading to different experimental outcomes (Sacks & Barker 1949). Nevertheless, Robertson & Kuenen (1984) and Robertson *et al.* (1988) published generally accepted data on aerobic denitrification in pure bacterial cultures which lead to the acceptance of aerobic denitrification as being a variant represented by several denitrifiers rather than an extremely rare exception (Zumft 1997). Since studies have shown that microbes can use branches of their electron transport chain to use the electrons simultaneously for the reduction of NO_3^- and O_2, aerobic respiration has been defined as the co-respiration or co-metabolism of O_2 and NO_3^- as long as oxygen does not suppress N oxide utilization (Zumft 1997). Bazylinski & Blakemore (1983) even describe an obligate aerobically denitrifying bacterium that denitrifies under microaerophilic but not anaerobic conditions and consumes oxygen while actively denitrifying. However, denitrification is almost exclusively a facultative anaerobic or microaerophilic trait, which illustrates the close interaction of the denitrification machinery and the aerobic electron transport chain (Zumft 1997). In 2010, Gao *et al.* demonstrated a significant contribution of aerobic denitrification to overall denitrification potential in the environment (marine sediments). The trait of the *in situ* studied bacteria was hypothesized to result from adaptation to recurrent tidally induced redox oscillations in these sediments.

Aerobic denitrification leads to an increase in N_2O production as shown by several studies, e.g. Körner & Zumft (1989), Otte *et al.* (1996), Chen *et al.* (submitted). This results from the sensitivity of nitrous oxide reductase towards oxygen. The inhibition of the nitrous oxide reductase can be either reversible or irreversible, depending on the enzyme variant and/or the organism (Otte *et al.* 1996). This feature has implications for the global climate as N_2O is a potent greenhouse gas.

The question of how organisms overcome the obvious sensitivity of their denitrification enzymes towards oxygen (Sacks & Barker 1949, Ferguson 1994) remains unanswered. One possibility is the creation of anaerobic micro-sites as discussed by Parkin & Tiedje (1984), Rao *et al.* (2007), Chen *et al.* (submitted). In these micro-niches intense microbial activity associated with the availability of

fresh organic matter is supposed to lead to a high oxygen respiration rate, which finally lowers the oxygen concentration faster than oxygen is able to diffuse into the center of the aggregate. This creates anaerobic conditions in environments, which are actually supposed to be aerobic. Moreover, a close coupling of denitrification and nitrification in such micro-sites has two advantages: first, nitrification produces nitrate which feeds denitrification and second nitrification as an oxygen respiring process lowers the oxygen concentration inside the micro-site to create conditions adequate for high denitrification activity. This cooperation works best in environments with limited oxygen concentrations, high enough to support nitrification but low enough to create sufficiently reducing conditions allowing denitrification to occur (Rao *et al.* 2007). Nitrification-denitrification has also been investigated and described by Risgaard-Petersen *et al.* (1994) and Eriksson & Weisner (1999).

In 1997, Zumft stated that both denitrification processes, either anaerobically or aerobically, are identical with respect to their enzymatic machinery. It is not a matter of the denitrification enzymes being sensitive to oxygen but rather the regulation at the enzyme and genetic level, which is influenced by oxygen presence or absence. Accordingly, the regulation of denitrification under oxygen is carried out on the transcriptional level. This could be proven by e.g. Körner & Zumft (1989) and Baumann *et al.* (1996). Consequently, anaerobically expressed gene products can also remain active under oxygen presence and can therefore result in aerobic denitrification.

According to Ferguson (1994), different variants of denitrification enzymes provide the organisms with the capability to conduct all steps of denitrification, including N_2O reduction, with each at least one enzyme variant not being inhibited by oxygen. Nevertheless, not all denitrifiers possess all oxygen tolerant variants. Therefore denitrification can still be considered as repressed by oxygen in certain cases.

Methods currently applied in microbial community research

To determine bacterial activity and diversity in natural microbial communities, microbiology traditionally has depended on the isolation of target microbes in pure

66

culture. Several reasons account for poor success with such isolation, including non-cultivability of estimated 99 % of all organisms existing in the environment (Amann *et al.* 1995), substrate-accelerated death after addition of a growth-limiting substrate to a starved community, induction of lysogenic phages by plating onto agar medium, or finally the lack of crossfeeding, synergism or symbiosis among the microbes (reviewed in Barer & Harwood (1999) and Overmann (2006)). Moreover, pure cultures do not deliver any insight into metabolic interactions between different species in a community. To overcome some of these restrictions, different molecular approaches have been developed which determine the biodiversity in given samples and may partly unravel the metabolism of key community members without their isolation.

16S rRNA gene based techniques

Since their advent in the late twentieth century, molecular biological techniques such as fluorescence *in situ* hybridization (FISH) and 16S rRNA gene clone libraries have greatly increased our knowledge of microbial diversity in most kinds of ecosystems as they provide tools for the analysis of entire communities including members unable to grow in the laboratory.

Llobet-Brossa *et al.* (1998) for the first time investigated the microbial composition of Wadden Sea sediment using FISH. This technique makes use of fluorescently labeled, group-specific, rRNA targeted oligonucleotides. Today, the rRNA, which is highly abundant in all cells, is widely accepted as a phylogenetic marker due to its high evolutionary conservation throughout all domains of life combined with the presence of more variable regions. The latter "diagnostic" or "signature" regions are specific for particular genera or even species and can either help identify microbes and allow one to draw conclusions on the phylogenetic relationships among different organisms. After the specific binding of a fluorescence labeled oligonucleotide probe to the rRNA of a chemically fixed target organism it can be visualized *via* fluorescence microscopy and quantified by counting the fluorescence signals, e.g. manually or *via* flow cytometry (Thiele *et al.* 2011). FISH has had an impressive technical evolution during the past years leading to applications such as CARD-FISH, MAR-FISH, DOPE-FISH, and CLASI-FISH, as reviewed by Wagner & Haider (2012) and references therein.

16S rRNA gene clone libraries make use of purified 16S rRNA gene amplification products, which are cloned into plasmid vectors and finally transformed into competent *E. coli* cells. Cells are subsequently plated onto media, raised to single clones, and eventually the plasmid containing the 16S rRNA insert is extracted for further investigation *via* sequencing approaches (Thiele *et al.* 2011). Using 16S rDNA libraries, previously uncultured organisms inhabiting marine sediments have been detected (Gray & Herwig 1996, Teske *et al.* 1996).

Pattern techniques

Additional molecular approaches for the investigation of microbial diversity are pattern techniques such as thermal gradient or denaturing gradient gel electrophoresis (TGGE/DGGE; Teske *et al.* 1996) and (terminal) restriction fragment length polymorphism ((T)-RFLP; Moyer *et al.* 1994). TGGE and DGGE provide information about variations in the GC content of rRNA genes or target genes of different organisms. After PCR-amplification the products are separated in denaturing gradient gels. Here, the motility of the fragments in the gel depends proportionally on their different melting points, which are in turn determined by the GC contents.

RFLP approaches use fluorescence labeled primers for PCR amplification of the genes of interest and a subsequent restriction digest. Fluorescence based detection of the fragments is realized with help of gel or capillary electrophoresis in which the mobility of the fragments is determined by their lengths. Fragment lengths can be deduced from the migration time and the detection of the fluorescence signal. Different 16S rDNA fragment lengths indicate different taxa and achieved banding patterns are used as fingerprints of the investigated communities (Liu *et al.* 1997, Abdo *et al.* 2006, Thiele *et al.* 2011).

These pattern techniques provide only a limited resolution to assess the overall microbial diversity and are restricted to specific genes. For this reason they only yield parts of the information of the DNA from a community (Thiele *et al.* 2011, Torsvik *et al.* 1996). This limitation can be overcome by techniques, which make use of the total DNA from complex communities such as the determination of the GC content of total DNA by thermal denaturation (Mandel & Marmur 1968).

Another possible method is the analysis of the reassociation kinetics of single stranded (denatured) DNA under appropriate conditions (Britten & Kohne 1968, Torsvik *et al.* 1996).

Although all these techniques partly permit both the culture independent monitoring of individual phylogenetic groups as well as a quantification of their abundance in the natural habitat they fail to explain metabolic interactions and dependencies among community members. This, however, is essential to understand the relationship between environmental factors, such as environmental stress and perturbations resulting from pollution and global change, on ecosystem functions.

Metagenomic genome reconstruction

Metagenomic genome reconstruction can discover members of so far uncultivated lineages as well as their metabolic characteristics (Wrighton *et al.* 2012, Castelle *et al.* 2013). Furthermore, it can prove inter-population genetic exchange demonstrating the importance of this for the adaptation to specific ecological niches (Lo *et al.* 2007) and also help resolve function-identity-relationships (Walsh *et al.* 2009). Metagenomic genome reconstruction consists of DNA extraction of a mixed community sample followed by (metagenome) sequencing and assembly of recovered sequences (reads) into larger fragments (contigs). Without assembly, using methods such as EMIRGE (Miller *et al.* 2011, Miller *et al.* 2013), full-length 16S rRNA sequences can be reconstructed directly from sequencing reads, quantified with respect to their relative abundance in the community, and finally compared to rRNA databases such as SILVA (Pruesse *et al.* 2007), which allows for a phylogenetic analysis of the community members. Additionally, contigs that apparently originate from the same source population can be clustered into "bins", for example by using the "Metawatt binner" (Strous *et al.* 2012). Contigs can be binned based on compositional features, sequencing coverage and/or on similarity to genomes of reference organisms. In Metawatt, contigs are binned based on compositional features and classified taxonomically by first comparing the (previously fragmented) contigs to a database, e.g. by using BLAST (Altschul *et al.* 1990, Camacho *et al.* 2009, Strous *et al.* 2012). To further investigate the ecological function of populations associated with bins, the provisional genomes of

each bin can be functionally annotated with help of programs like Prokka (Seemann 2014). Metagenome reconstruction alone offers information about the genetic capacity of a community. However, in combination with strain-resolved transcriptomics and proteomics it enables to distinguish the behavior of even closely related, co-existing organisms that can have quite distinct and important roles in natural ecosystems (Lo *et al.* 2007). However, metagenome reconstruction depends on a low complexity of the sample (i.e, a low species richness and a low heterogeneity in the abundance of community members) with a low frequency of genomic rearrangements and gene insertions or deletions (Tyson *et al.* 2004).

Single cell genomics

Single cell techniques range from flow cytometry *via* PCR to sequencing approaches. They help to understand complex biological phenomena at single-cell resolution. Single-cell genomics requires a highly demanding sample preparation including separation of cells, a cell membrane breakup, contaminate-free million-fold whole genome amplification (WGA) of the cells' DNA, library preparation, and finally high-throughput DNA sequencing. Nevertheless, single-cell genomics is unique among current genomic approaches with respect to the access to individual cell-genomes without need for cultivation and data-assembly from different cells or strains. Grouping of sequence reads of single cells is free of uncertainty and can resolve the finest strain structures, and phase variation at the whole-genome level (Blainey 2013, Rinke *et al.* 2013).

Stable-isotope probing

During the past years, the use of stable isotopes combined with mass spectrometry became a method of choice for analyzing the interactions between microbes and their environment (Abraham 2014). Incorporation of stable-isotope labeled substrates by organisms can be traced with help of mass spectrometry. In the mass spectrometer, the sample is ionized, accelerated in an electrical field and eventually detected by its mass. Mass spectrometry yields information about the amount of a stable isotope within a sample or even a single cell (Abraham 2014).

These techniques alone are not sufficient to accurately model the response of natural microbial networks or engineer the metabolic function and interactions of microbial communities in their natural contexts. Therefore, it is difficult to understand the ecological niche of the isolated microorganism without the ability to study it in the context of its natural habitat. This, however, is generally complicated by the dynamics and the complexity of natural environments and their inhabiting communities.

Continuous cultivation approaches

Engineering of a simplified natural ecosystem in the laboratory with help of continuous culture approaches, e.g. a chemostat, a retentostat or a sequencing batch reactor, can enable the enrichment and observation of uncultivated bacteria in the context of simplified microbial communities (Kartal & Strous 2008). By natural selection under the applied conditions in a continuous culture, a simplified microbial community assembles spontaneously from the more complex inoculum. Together with metagenomic genome reconstruction, proteomics and metabolomics this approach enables the characterization of the overall community metabolic function and microbial interactions. This way, the contribution of each member of the community can be clarified. With help of such an engineered culturing system, manipulating the applied conditions can identify key environmental factors that define the ecological niche of selected populations. A niche is for example defined by the types of energy and carbon metabolism performed, the temperature and pH range for growth, the dependencies on and interactions with other micoorganisms, the growth rate, and finally the affinity for the substrate (Kartal & Strous 2008). For enrichment or isolation of a desired organism the factors determining its niche should, at least partly, be known and applied to the culture. Such manipulations are not possible *in situ*, however, they are feasible *via* continuous culture systems. Furthermore, continuous cultivation even enables, if desired, the specific enrichment of K-strategists ("oligotrophic" organisms; successful competitors for limited resources; Andrews & Harris 1986) *via* supply of substrates at defined, low, near *in situ* concentrations. Additionally, in a continuous culture the applied conditions are stable (or dynamic, if desired) and reproducible.

The continuous culture system presented here was created to simulate the environmental conditions of tidal flat sediments in a simplified way. Apart from the dynamics, such systems are known as chemostats. The chemostat was first described by Novick & Szilard (1950) as a "device for keeping a bacterial population growing at a reduced rate over an indefinite period of time".

The principle of a chemostat is to continuously grow a well-mixed, suspended bacterial culture in a growth vessel under continuous substrate-supply and simultaneous removal of culture liquid to achieve a constant culture volume. In such a steady state, the dilution rate, which is the medium supply rate divided by the vessel volume, equals the growth rate of the cultivated bacteria. In a chemostat, the bacterial productivity is restricted by the supply of the limiting growth factor (e.g. a nutrient or a trace element). In steady state, the biomass concentration is constant (Novick & Szilard 1950).

The aim of the described chemostat was to enrich environmentally important bacteria from a natural sample under nearly *in situ* conditions. This enables us to investigate the ecology of microorganisms under controlled conditions for an indefinite amount of time. By combining metagenomic sequencing, transcriptomics, proteomics and metabolomics analyses of the microbial community growing in the chemostat, we can get insight into a metabolic network and extrapolate results to the natural environment.

Methods

To answer the questions addressed in this work, continuous culture incubations of bacterial communities from Wadden Sea sediment were conducted. All together, 6 bacterial communities were enriched in chemostats over periods between 3 and 12 months. Comparisons of bacterial diversity in untreated sediment *vs.* chemostat inoculum were addressed by ARISA.

Metabolic activity in the chemostats was studied by measuring the compositions of headspace gas and culture liquid. Gas composition was analyzed with a GAM400 mass spectrometer and *via* gas chromatography. After the medium was amended with stable-isotope labeled nutrients ($Na^{15}NO_3$), the N-isotopic composition of the headspace gas was determined with a GC/IRMS. Concentrations of most nutrients, protein content, and COD of the culture liquid were measured with help of various spectrophotometric assays. Nitrate concentration was determined with an NO_x analyzer and dissolved O_2 concentration with an optical oxygen sensor. The optical density of the culture liquid was measured photometrically. Fatty acids were determined by HPLC. Culture samples were periodically inspected with a microscope to verify the absence of grazing protists.

To investigate bacterial metabolism under conditions other than applied in the chemostats, batch incubations with biomass samples were conducted. In some cases, these incubations included feeding of stable isotope-labeled substrates.

The community composition in the chemostats was determined by both FISH/CARD-FISH and metagenome sequencing (454 GS-FLX(+), PGM™, Illumina© MiSeq®) with subsequent phylogenetic analyses of detected 16S rDNA genes. In one case, phylogenetic characterization was performed by applying 16S rDNA tag sequencing. Additionally, by analyzing functional genes from the metagenome, insight into the metabolic capability of the community members was gained. To assess the cultures' metabolic activity under specific conditions, transcriptome analyses were performed with a PGM™. Finally, proteome analyses with help of an LC/MS enabled identification of expressed genes.

Additionally, metabolic activity in the cultures was modeled stoichiometrically.

Aims

The first aim of the presented study was the development of a cost effective, reliable and handy method for long-term cultivation of microorganisms under near *in situ* conditions with full control over all technical parameters. The cultivation setup consisted of an incubation vessel itself, the technical and computational basis for the application and control to create defined and reproducible conditions, and a set of methods proven useful to investigate the outcome of the experiments.

This aim was addressed in publication II.

The second aim was to investigate the effect of periodic oxygen availability on a bacterial community enriched under denitrifying conditions. The latter conditions are defined by (1) (mostly) anoxia, (2) supply of nitrite as the main electron acceptor, and (3) supply of carbon compounds in the form of a representative mixture of monomers set free during the decay of biomass, the main electron donor *in situ*. Periodic oxygen input to the system simulated the delivery of oxygenated water to the sediments *in situ* during high tide. The major task addressed here was to discover strategies denitrifiers use to cope with the periodic presence of oxygen as an oxidant that competes with their primary electron acceptor nitrate/nitrite.

This aim was addressed in publication/manuscript II and III.

The third aim was to study the influence of different temperatures on a bacterial community enriched under denitrifying conditions (see above). As even temperature rises of a few degrees Celsius were shown to substantially affect denitrification *in situ* (Veraart *et al.* 2011) and as one intermediate of denitrification, N_2O, is an important greenhouse gas, the long-term observation of cultures grown under two different temperatures was addressed here. The influence of different temperatures on community composition and -metabolism was investigated.

This aim was addressed in publication (manuscript) IV.

Overview of enclosed manuscripts

I) Climate, Fertilization, and the Nitrogen Cycle*

Anna Hanke & Marc Strous

Status: published 2010 in *Journal of Cosmology* (8):1838-1845

II) Recoding of the stop codon UGA to glycine by a BD1-5/SN-2 bacterium and niche partitioning between *Alpha*- and *Gammaproteobacteria* in a tidal sediment microbial community naturally selected in a laboratory chemostat

Anna Hanke, Emmo Haman, Ritin Sharma, Jeanine S. Geelhoed, Theresa Hargesheimer, Beate Kraft, Volker Meyer, Sabine Lenk, Harald Osmers, Rong Wu, Kofi Makinwa, Robert L. Hettich, Jillian F. Banfield, Halina E. Tegetmeyer, Marc Strous

Status: published 2014 in *Frontiers in Microbiology* (5), article 231

III) Competition between thermodynamically unsorted redox processes limits productivity of microbial ecosystems

Jianwei Chen, Anna Hanke, Halina E. Tegetmeyer, Ines Kattelmann, Ritin Sharma, Emmo Hamann, Theresa Hargesheimer, Beate Kraft, Sabine Lenk, Jeanine S. Geelhoed, Robert L. Hettich, Marc Strous

Status: submitted to *Nature*

IV) Temperature as a factor for natural selection on marine denitrifying bacterial communities in continuous cultures

Anna Hanke, Jasmine Berg, Theresa Hargesheimer, Halina E. Tegetmeyer, Christine E. Sharp, Marc Strous

Status: in preparation

* This review is part of the introduction

Publication II

Title:

Recoding of the stop codon UGA to glycine by a BD1-5/SN-2 bacterium and niche partitioning between *Alpha-* and *Gammaproteobacteria* in a tidal sediment microbial community naturally selected in a laboratory chemostat

Authors:

Anna Hanke, Emmo Haman, Ritin Sharma, Jeanine S. Geelhoed, Theresa Hargesheimer, Beate Kraft, Volker Meyer, Sabine Lenk, Harald Osmers, Rong Wu, Kofi Makinwa, Robert L. Hettich, Jillian F. Banfield, Halina E. Tegetmeyer, Marc Strous

Status:

Published in *Frontiers in Microbiology*

Own contribution:

AH planned and conducted the cultivation experiments with help of EH, determined, analyzed and visualized the nutrient data, and wrote the manuscript with help of MS and some co-authors.

Recoding of the stop codon UGA to glycine by a BD1-5/SN-2 bacterium and niche partitioning between *Alpha-* and *Gammaproteobacteria* in a tidal sediment microbial community naturally selected in a laboratory chemostat

Anna Hanke[1], Emmo Hamann[1], Ritin Sharma[4,5], Jeanine S. Geelhoed[1], Theresa Hargesheimer[1], Beate Kraft[1], Volker Meyer[1], Sabine Lenk[1], Harald Osmers[1], Rong Wu[2], Kofi Makinwa[2], Robert L. Hettich[4,5], Jillian F. Banfield[6], Halina E. Tegetmeyer[1,3], Marc Strous[1,3,7*]

[1]Max Planck Institute for Marine Microbiology, Microbial Fitness Group, Bremen, Germany

[2]Delft University of Technology, Delft, The Netherlands

[3]Center for Biotechnology, University of Bielefeld, Bielefeld, Germany

[4]UT-ORNL Graduate School of Genome Science and Technology, University of Tennessee, Knoxville, USA

[5]Chemical Science Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee, USA

[6]Department of Earth and Planetary Science, Department of Environmental Science, Policy, and Management, University of California, Berkeley CA, USA.

[7]Department of Geoscience, University of Calgary, Calgary, Alberta, Canada

Correspondence:
Dr. Marc Strous
Department of Geoscience
University of Calgary
Campus Drive 7200 NW
Calgary, T2N 1N4, Alberta, Canada
mstrous@ucalgary,ca

Acknowledgements

Abstract

Sandy coastal sediments are global hotspots for microbial mineralization of organic matter and denitrification. These sediments are characterized by advective porewater flow, tidal cycling and an active and complex microbial community. Metagenomic sequencing of microbial communities sampled from such sediments showed that potential sulfur oxidizing *Gammaproteobacteria* and members of the enigmatic BD1-5/SN-2 candidate *phylum* were abundant *in situ* (>10 % and ~2 % respectively). By mimicking the dynamic oxic/anoxic environmental conditions of the sediment in a laboratory chemostat, a simplified microbial community was selected from the more complex inoculum. Metagenomics, proteomics and fluorescence *in situ* hybridization showed that this simplified community contained both potential sulfur oxidizing *Gammaproteobacteria* (at 24±2 % abundance) and a member of the BD1-5/SN-2 candidate *phylum* (at 7±6 % abundance). Despite the abundant supply of organic substrates to the chemostat, proteomic analysis suggested that the selected *Gammaproteobacterium* grew partially autotrophically and performed hydrogen/formate oxidation. The enrichment of a member of the BD1-5/SN-2 candidate *phylum* enabled, for the first time, direct microscopic observation by fluorescent *in situ* hybridization and the experimental validation of the previously predicted translation of the stop codon UGA into glycine.

Introduction

The Wadden Sea along the northern European coast is the largest tidal system worldwide and has been a UNESCO world heritage area since 2009. It receives nutrients, mainly in the form of nitrate, phosphate, and silicate from a large catchment area in northern Europe, stimulating growth of algae and other microorganisms in the surface water. Tidal pumping of this water through the permeable sediments of tidal flats leads to the continuous removal of nutrients by highly active indigenous benthic microbes. For example, the measured *in situ* denitrification rates are very high, up to 60 µmol m^{-2} h^{-1} (Kieskamp *et al.* 1991).

In the past decades the biogeochemistry and the microbial diversity of the Wadden Sea tidal flat have been studied intensively. The microbial community in the upper oxic tidal flat sediments was shown to be dominated by populations of *Gamma-proteobacteria* (Lenk *et al.* 2011) and *Flavobacteria* (Llobet-Brossa *et al.* 1998; Eilers *et al.* 2001). Potential sulfur oxidizing *Gammaproteobacteria* make up for an important part (approx. 39.6 %) of all *Gammaproteobacteria* found on Janssand. Within *Gammaproteobacteria* these bacteria form a large phylogenetic clade that also includes the ubiquitous marine SUP05 cluster (Walsh *et al.* 2009), many bacterial symbionts of marine invertebrates (e.g. Kleiner *et al.* 2012) and some cultivated species such as *Sedimenticola selenatireducens* (Narasingarao & Häggblom 2006), *Dechloromarinus chlorophilus* (Coates & Achenbach 2004) and *Thiotaurens thiomutagens* (Flood 2010). Many of these bacteria are known to be facultative aerobic chemolithoautotrophs that can use inorganic electron donors such as sulfide and hydrogen to perform aerobic respiration and denitrification (Walsh *et al.* 2009; Stewart 2011). The *Flavobacteria* on the other hand are known to be involved in the degradation of macromolecules such as polysaccharides (Kirchman 2002).

To understand the overall function of individual bacterial species in complex natural microbial communities microbiology has traditionally depended on the isolation of target microbes in pure culture. Because such isolation is often unsuccessful, metagenomic genome reconstruction (Tyson *et al.* 2004; Lo *et al.* 2007; Wrighton *et al.* 2012; Castelle *et al.* 2013) and single cell genomics (Kalisky & Quake 2011; Blainey 2013; Rinke *et al.* 2013) have been used to unravel the

metabolism of key community members without their isolation. Both approaches can yield near-complete (Tyson *et al*. 2004; Wrighton *et al*. 2012; Baker *et al*. 2010) and, in some cases, complete genomes (e.g. Albertsen *et al.* 2013, Castelle *et al.* 2013, Rinke *et al.* 2013). However, sequencing methods alone lack the ability to probe metabolic function and provide limited insight into microbial interactions.

Both with conventional pure cultures and single cell methods, the target microorganism is, literally, isolated from its natural context and it is often difficult to understand the ecological niche of the isolated microorganism. For this, it would be ideal to study the organism in the context of its natural habitat. Because of the dynamics and complexity of natural communities this is generally not straightforward.

Engineering of a simplified natural ecosystem in the laboratory can enable the study of uncultivated bacteria in the context of a simplified microbial community (e.g. Belnap *et al*. 2009, Kartal & Strous 2008). Such a simplified microbial community will assemble spontaneously from a more complex inoculum by natural selection under the conditions applied. In combination with metagenomic genome reconstruction, proteomic and metabolomic approaches this allows for characterization of overall community metabolic function and interactions, and provides a route to unravel the contribution of each of the individual members. Once an engineered system is available, key environmental factors that define the ecological niche of selected populations can be identified by manipulating the applied conditions. Such manipulation is not possible when natural ecosystems are studied directly.

To achieve a significant substrate turnover at low, near *in situ* substrate concentrations, habitat engineering depends on continuous culture cultivation, e.g. a chemostat. The low substrate concentration favors the selection of K-strategists ("oligotrophs") relative to r-strategists ("microbial weeds"; Andrews & Harris 1986). Further, in a continuous culture the applied conditions are stable (or dynamic, if desired) and reproducible for an indefinite amount of time.

In the present study we simulated the environmental conditions of a tidal flat sediment in a simplified form. The most significant difference between the simulated and the natural environment was that in the natural habitat, microbes grow as thin biofilms on sand grains, whereas in the simulated habitat, cells grew in suspension. Nitrate and nitrite were supplied as the main electron acceptors and oxygen was supplied twice daily, mimicking tidal cycling. The carbon and energy source consisted of a mixture of glucose, amino acids and acetate, in a ratio similar to decaying biomass, the main carbon and energy source *in situ*. After 23 days the resulting community was shown to be dominated by representatives of phylogenetic clades also detected in the tidal flat sediments, including a population of potential sulfur oxidizing *Gammaproteobacteria* and a member of the enigmatic bacterial BD1-5/SN-2 clade, which lacks cultivated representatives and was previously predicted to translate the stop codon UGA into glycine. Proteomic analysis of the simplified microbial community enabled the experimental validation of this prediction.

Material & Methods

Sampling and inoculation

Sediment samples were taken from an intertidal flat in the central German Wadden Sea known as "Janssand" located south of the Eastern Friesian Island Spiekeroog (N: 053° 44.151' / E: 007° 41.945'). For direct metagenomic sequencing one sample was collected on October 24, 2009 (0-5 cm depth), and three samples on March 23 2010 (0-2 cm depth). To assess small-scale differences, the three March samples were taken from locations approximately 3 m apart. To analyse sequencing bias, two of the three March samples were pooled, and the pool was divided into two samples, named Mar10/1a and Mar10/1b, to be sequenced in separate sequencing runs. Sediment samples were stored at -20° C in 50 mL falcon tubes. For inoculation sampling was conducted at low tide in May 2011 (15 °C, overcast). Using a trowel, sediment from the upper 2 cm layer of Janssand was shovelled into a cooling box and kept on ice during its 5 h transport to the lab. It was diluted with artificially prepared seawater (33.4 g L^{-1} salt, Red Sea, Somerset, UK; in Milli-Q water) in a ratio of 1:1 and mixed for 3 min using a

drilling machine (PSB 850-2 RE, 850 Watt, Bosch, Stuttgart, Germany) with a cement mixer. The turbid, sand free supernatant (the "cell extract") was filled in 800 mL portions into 1 L transfusion bottles (Ochs Glasgerätebau, Bovenden/Lenglern, Germany) and pH was set to 8.1-8.4 with 1 M NaOH solution. The cell extract was made anoxic by alternately applying vacuum to 0.3 bar and argon to 1.2 bar, 3 times each. Each bottle was supplemented with $NaNO_3$ stock solution to reach a final concentration of 0.1 mM serving as electron acceptor. 50 mg L^{-1} cycloheximide (AppliChem, Darmstadt, Germany) was added and the cell extract was incubated at 4 °C for 24 h to kill predatory eukaryotic organisms. After that, the cell extract was used for inoculation of the continuous culture

Denitrification rates before and after sediment extraction

For a direct comparison, denitrification rates were determined for fresh sediment and cell extract (the inoculum, see above). Batch incubations were conducted as described by Würgler Hansen *et al.* 2000 with minor modifications: Fresh sediment samples and cell extract (see above) were incubated in triplicates in 400 mL nearly gas tight plastic bags (manufactured by sealing from transparent 3-layer PA/PE tubular bags BST-090; Rolf Bayer Vakuumverpackung GmbH, Veitsbronn, Germany). Manufactured glass windings with flat flanges and flanged sockets (Ochs Glasgerätebau) served as openings, tightened with GL14 screw caps with holes (Ochs Glasgerätebau), and closed with natural rubber stoppers (D10, Ochs Glasgerätebau). Air exclusion was achieved by additionally using NBR o-rings (Franz Gottwald GmbH & Co KG, Bremen, Germany) and viton flat gaskets (Rubber BV, Hilversum, The Netherlands). After filling the bags with either 400 mL cell extract or with a mix of sediment and sea water (ratio 1:1) they were sealed with a bag sealer (Polystar 110 GE, Rische + Herfurth GmbH, Hamburg, Germany). The sealed bags were amended with $Na^{15}NO_3$ (0.2 mM final concentration, Sigma Aldrich, Munich, Germany) and with NH_4Cl (1 mM final concentration; to prevent NO_3 consumption *via* assimilation; Roth, Karlsruhe, Germany), manually mixed, and incubated in the dark at room temperature. Sampling of the bags was conducted directly after filling and 4 more times in 90 min intervals. Without leaving a headspace the liquid samples were filled into 3.9 mL soda glass vials (Exetainer®, Labco Ltd., Lampeter Ceredigion, UK)

pre-filled with $HgCl_2$ (saturated in final volume; Roth) to stop all biological activity. A 1.5 mL helium headspace was set to the vials. Headspace samples were analyzed with a GC/IRMS (Fisons VG Optima, MasCom, Bremen, Germany) to determine the N-isotopic composition of N_2 gas produced during the incubations.

Species richness before and after sediment extraction.

Automated ribosomal intergenic spacer analysis (ARISA) was performed to quantify the loss of species during sediment extraction. DNA extraction prior to ARISA was according to Zhou *et al*. 1996. The ARISA procedure was conducted as described by Ramette (2009).

Medium composition

The cultures were continuously supplied with anoxic, sterile liquid medium. Composition of the medium is shown in Table II-1. Nitrate (1 mM) and nitrite (20 mM) served as electron acceptors while a defined mixture of different carbon compounds (30.5 C-mM) was supplied as electron donor. The nutrient concentration in the medium was increased within the first 14 days of the incubation from 5 mM nitrite (stable nitrite:nitrate:carbon ratio as mentioned in Table II-1) up to 20 mM. However, nutrient concentrations in the culture were generally in the low micromolar range because the medium entered the chemostat dropwise.

Table II-1 I Medium composition for continuous cultures.

Substance class	Substance	Concentration	Supplier
	Red Sea Salt	33.4 g L^{-1}	Red Sea
NUTRIENTS		**mM**	
	NaNO$_3$	1	Roth
	NaNO$_2$	20	Roth
	Carbon (see below)	30.5	-
TRACE ELEMENTS		**µM**	
	KH$_2$PO$_4$	500	Roth
	FeSO$_4$*7H$_2$O	10.755	Roth
	CuCl$_2$	1.256	Roth
	ZnCl$_2$	0.051	Roth
	MnCl$_2$*4H$_2$O	0.051	AppliChem
	H$_3$BO$_3$	0.097	Roth
	CoCl$_2$*6H$_2$O	0.05	Roth
	NiCl$_2$*6H$_2$O	0.011	Roth
	Na$_2$MoO$_4$*2H$_2$O	0.011	Roth
	AlCl$_3$*6H$_2$O	0.01	AppliChem
CARBON COMPOUNDS		**% Carbon**	
	Glucose	44.1	AppliChem
	Acetate	7.6	AppliChem
	Amino acids (see below)	48.3	-
AMINO ACIDS		**% Carbon**	-
	Glutamic acid	10.7	AppliChem
	Aspartic acid	11.7	Roth
	Alanine	8.5	AppliChem
	Serine	4.6	AppliChem
	Tyrosine	8.9	AppliChem
	Histidine	1.4	AppliChem
	Methionine	2.4	AppliChem

Actual substrate concentrations inside the chemostat were much lower because the influent medium was diluted dropwise into the culture vessel.

Continuous cultivation

The custom culture vessel (Ochs Glasgerätebau) consisted of a so-called "Kluyver flask" (an inverted Erlenmeyer with a sintered glass disk inserted in its neck) fixed inside a beaker. The bottom of the Erlenmeyer was removed and replaced by a lid with standard Teflon Schott connectors for inserting sensors and tubing. See Figure II-1. The flask content was mixed by recycling gas (4 L min^{-1}; 620S peristaltic pump, Watson Marlow, Wilmington, MA, USA) from the headspace of the flask *via* the beaker and the sintered glass disk into the culture. Recycled gas was continuously amended by 10 mL min^{-1} argon with a mass flow controller (Alicat Scientific, Tucson, AZ, USA). Part of the gas left the culture *via* a water lock to maintain a constant overpressure of 13±5 mbar. The liquid volume of the culture was 2.8 L and the gas volume of the combined headspace and beaker was 3.08 L.

Medium was pumped into the vessel continuously (0.7 mL min^{-1}, dilution rate 0.36 day^{-1}) *via* a glass back growth buffer (Fig. II-1) to prevent colonization of the medium supply tubing by the enriched bacteria. Culture liquid was continuously removed by a second peristaltic pump controlled by a weight sensor (load cell, mounted underneath the culture) to keep the volume constant. The pH was automatically controlled to 8.0±0.05 with 0.2 M NaOH and 0.25 M HCl solutions. To simulate the periodically aerobic/anaerobic conditions associated with a tidal system, from day 9-28 pure oxygen (0.2 mL min^{-1}) was added for 120 min every 12 hours. The gas recycling of the cultivation vessel led to a rapid exchange of oxygen from the gas to the liquid. The temperature was controlled at 25 °C with a resistive heater controlled by a temperature sensor (see below).
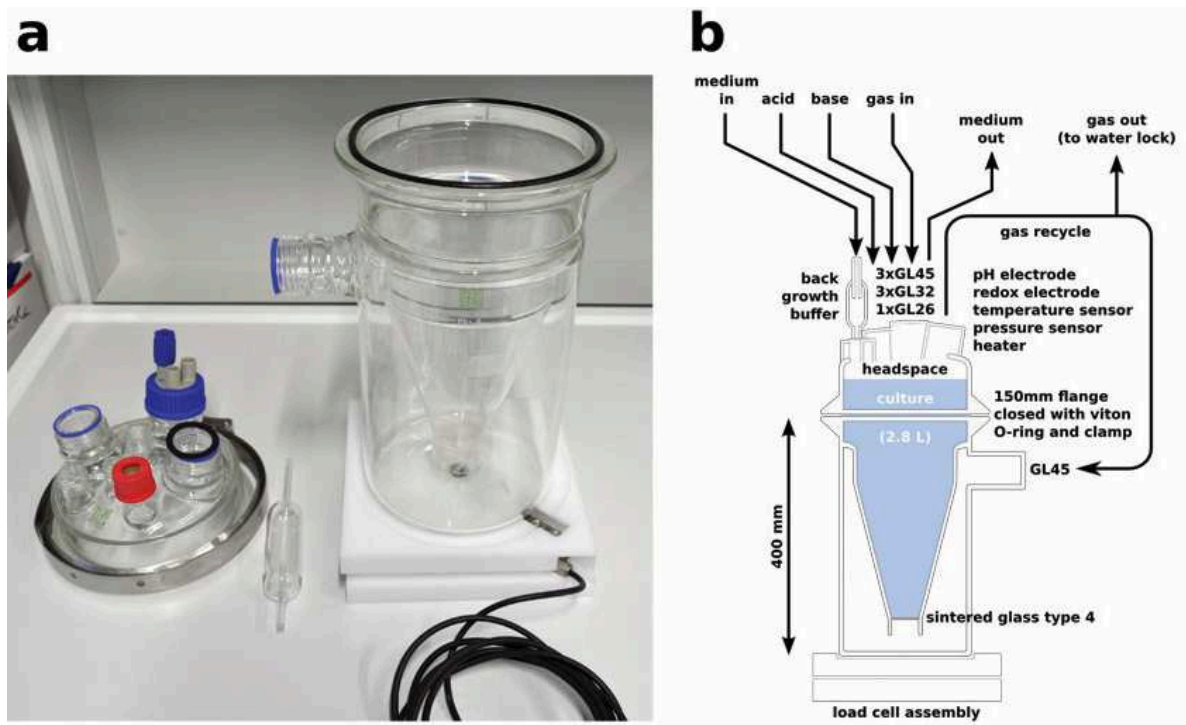
Figure II-1 I The chemostat vessel used. All additions and withdrawals were made from the top of the vessel. The vessel was mixed by recycling gas from the headspace to the bottom *via* a sintered glass filter. The culture volume was measured *via* the vessel weight by a load cell mounted underneath the vessel. Medium was added *via* a glass back growth buffer to prevent contamination of the influent medium tubing.

Both the heater and the temperature sensor were mounted into NMR tubes with help of a thermal compound (Amasan Wärmeleitpaste T12, Jürgen Armack GmbH, Norderstedt, Germany) so that no metal contacted the culture fluid directly.

The continuous culture was controlled by an embedded National Instruments compactRIO real time power PC (cRIO-9014, 128 Mb RAM, 2 GB disc on chip non-volatile storage) combined with an 8-slot Virtex-5 FPGA chassis (cRIO-9112) that interfaced the input/output modules. Seven modules were added to the chassis and they consisted of: (1) a 16 channel (differential) 16 bit 10 V 250 kS/s analogue to digital converter (NI9205), (2) an 8 channel 16 bit 20 mA 200 kS/s analogue to digital converter (NI9203), (3) a 4 channel 24 bit 60 V 100 S/s analogue to digital converter (NI9219), (4) a 16 channel 16 bit 10 V 25 kS/s digital to analogue converter (NI9264), (5) a 32 channel 5 V TTL digital input/output module (NI9403), (6) a high speed (1 μs response time) 8 channel 24 V digital output module (NI9474) and (7) an 8 channel 60 V digital relay module (NI9485). These components were built into a standard 19 inch rack together with a power supply and the electronics for signal conditioning (see below). Sensor signals were acquired and digitized by the analogue to digital converters. Process control (proportional-integral, integration time 2 min) was implemented in hardware logic on the Virtex-5 FPGA and was completely independent of the microprocessor unit for maximum operational stability. Control actions were converted to analogue signals by the digital to analogue converters or *via* pulse width modulation to the digital output modules. The real time microprocessor unit was only used for data logging on the solid-state hard disc, communication (ethernet, TCP/IP) with an external computer, and for creating dynamic conditions inside the culture vessel in an automated way.

The pH was measured with combined pH electrodes (HA405-DPA-SC-S8/120; Mettler Toledo, Giessen, Germany). Redox potential was measured with Mettler Toledo redox electrodes (Pt4805-60-PA-S8/120). The signals of both redox and pH electrodes were conditioned with Picos 1201PH conditioners. These conditioners converted the signal into a 4-20 mA format and interfaced with the NI9203 module of the controller. The overpressure in the headspace of the culture vessels was measured with PA-23S pressure transducers (Comer, Bologna, Italy) that also provided a 4-20 mA signal for the same module. The liquid volume inside the culture vessel was measured gravitometrically with a load cell (LSH-10kg-C3, combined error <0.017 %, AE sensors, Dordrecht, the Netherlands) underneath the culture vessel. The signal was conditioned with an ICA3 signal conditioner

(same supplier, output signal 0-10 V, digitized by module NI9205) built inside the 19 inch rack. Temperature was measured with temperature sensors consisting of two 45016 SP20725 thermistors (YSI, Yellow Springs, Ohio, USA) per sensor. Both thermistors were part of a Wheatstone bridge and measurement noise was filtered and the signal was digitized by a custom analogue to digital converter as previously described (Wu *et al.*, 2012). The resulting bitstream interfaced with module NI9403. With this setup the accuracy of the temperature measurement was around 1-10 µK. For less precise but more flexible measurements a pt1000 sensor was also used. The chemical composition of the headspace gas inside the culture was monitored periodically with a GAM400 on-line mass spectrometer (InProcess, Bremen, Germany) during a complete tidal cycle.

Pumping of fluids (medium, effluent, alkaline and acid solutions for pH control) was performed with OEM peristaltic variable speed pumps (IS3124A) and controllers (IS3186) obtained from Ismatec (Wertheim, Germany) interfacing with module NI9264. Four of these pumps and controllers were built into a single 19 inch rack. Gases were supplied with mass flow controllers (Alicat Scientific) controlled by module NI9264. The feedback signal from the controllers (the actual gas flow) was acquired with module NI9205. Heating was performed with custom cartridge resistors (4 mm diameter, 60 mm long, 20 W at 24 V) obtained from Türk Hillinger (Tuttlingen, Germany). These interfaced (with pulse width modulation at 20 kHz) with module NI9474.

Wet chemical analyses

Nitrite was measured spectrophotometrically according to Bendschneider & Robinson 1952. Nitrate concentrations were calculated by subtraction of nitrite concentrations (see above) from the total NO_x concentration determined with an NO/NO_x analyzer (2 µM detection limit; CLD 66 S, Eco Physics, Munich, Germany). Samples were injected into the reaction chamber connected upstream to the analyzer. At 90 °C with acid VCl_3 (0.1 M), both NO_3^- and NO_2^-, were reduced to NO which was measured by the chemiluminescence detector. Ammonia in the culture liquid was measured according to Solorzano (1969). Protein was measured spectrophotometrically according to Lowry *et al.* (1951).

DNA sequencing for metagenomics

For the sediment metagenome 500 ng of purified DNA per sample were used for the preparation of sequencing libraries according to the "Rapid Library Preparation Method Manual" (October 2009/Rev. January 2010) provided by Roche (Mannheim, Germany). Two GS FLX Titanium sequencing runs were performed with each of the four libraries loaded on half a sequencing picotiterplate. Samples Oct09 and Mar10/1a were sequenced in the same run and sample Mar10/1b was sequenced together with sample Mar10/2.

For the chemostat metagenome, 1 µg of purified DNA per sample was used for the preparation of sequencing libraries according to the "Rapid Library Preparation Method Manual" (May 2011) for GS FLX+ Series provided by Roche. The sample was sequenced on a quarter picotiterplate of a GS FLX+ Titanium sequencing run. Compared to the previous version (Titanium chemistry), the obtained reads were long (average read length 671 nt) but fewer reads were obtained (168 169/quarter plate). IlluminaMiSeq sequencing was carried out as follows: 2 µg of purified DNA were used for generating a barcoded PCR-free TruSeq® illumina sequencing library according to the "TruSeq® DNA PCR-Free Sample Preparation Guide" for a median insert size of 550 bp. The library was sequenced on 3 % of a flow cell in a paired end run (2x255 bases) on a MiSeq instrument. 315,246 read pairs were sequenced, yielding approx. 160 MB sequencing output after quality trimming

In silico computational procedures

The metagenomic reads of the *in situ* metagenomes were screened for fragments of 16S rDNA genes with six Hidden Markov Models (Eddy 2011) trained with forward and reverse-complemented eukaryotic, archaeal and bacterial reference 16S rDNA genes from the SILVA database, release 12 (http://www.arb-silva.de/). Out of 2,511,392 reads (average length 394 bp), 862 reads encoding a partial 16S rDNA gene were so identified. These fragments were classified with BLASTn (Camacho *et al.* 2009) and the SILVA database.

Assembly of the metagenomic reads from the chemostat was carried out (after filtering the reads for quality and removing tags) with the GS De Novo Assembler 2.8 (454 Life Sciences, Branford, CT, USA) using the default settings for genomic

DNA. Assembled contigs were binned based on tetranucleotide compositions combined with Interpolated Markov Models (IMMs) with the Metawatt binner (Strous *et al.* 2012); briefly, four bins were created based on IMMs trained with tetranucleotide bins "high_bin_0", "low_bin_1", "low_bin_2" and "low_bin_4". After that the contigs in "low_bin_2" were separated into two bins with a sequencing coverage cutoff of 7x. 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. Next, redundant sequence information per bin was identified by BLASTn (Camacho *et al.* 2009; "internal overlap" in Table II-3). Transfer-RNAs were identified with Aragorn (Laslett & Canback 2004). Genome completeness was estimated for each bin by representation of 139 conserved genes as described by Campbell *et al.* (2013). The contigs of each bin were annotated separately with Prokka (http://vicbioinformatics.com). Prediction of the alternate genetic code of the BD1-5/SN-2 bacterium was performed as described by Dutilh (2011) and the alternate code was implemented in Prodigal (Hyatt *et al.* 2010) to enable open reading frame (ORF) prediction and annotation. 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). Alignment of 16S rRNA gene sequences was performed with MAFFT (Katoh *et al.* 2002; high accuracy options --maxiterate 1000 --localpair) and a phylogenetic tree was calculated by approximate maximum likelihood with FastTree2 (Price *et al.* 2010) after applying a 50 % conservation filter.

Proteomics

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) (Wiśniewski *et al.* 2009). To 82 µL of sample in an Eppendorf tube was added 30 µL of HPLC grade water, 30 µL of 10 % SDS, and 8 µL of 1 M DTT. 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 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 g for 10 min. Protein digestion was carried out first for 4 h at 37 ℃ using trypsin (Promega) in 1:20 protease to protein ratio. A second aliquot of trypsin was added following first 4 hours and sample was incubated at 37 ℃ for an overnight digestion. Peptides then were collected in a fresh tube after washing the filter with two washes of 50 mM ammonium bicarbonate and final addition of 0.5 M NaCl and spinning at 14,000 g. The pH of resulting peptides solution was adjusted to <3 by addition of formic acid.

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 H_2O, 5 % AcN, 0.1 % Formic acid) to solvent B (30 % HPLC H_2O, 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, Wobum, 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 min RP gradient from 100 % solvent A to 50 % solvent B, while the last salt pulse used 150 min 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 v2.1 (Tabb *et al.* 2007) against a predicted protein database (28,627 sequences with bin E translated thrice: UGA as STOP codon, UGA coding for glycine, UGA coding for tryptophan) constructed from metagenome assembly, along with common contaminants (44 sequences) and reverse sequences. A second search was performed using MyriMatch v2.1 against a predicted database same as before, with the only difference being that bin E was translated 20 times with UGA coding for all twenty amino acids as well as UGA acting as a STOP codon. 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 (Ma *et al.* 2009). (For more information on search settings see Supplementary Tab. 5). For all bins, two replicate samples were used to calculate average peptide coverages for every predicted and annotated ORF and, finally, average peptide coverages for specific metabolic pathways.

Catalyzed Amplified Reporter Deposition Fluorescence in situ Hybridization

Catalyzed Amplified Reporter Deposition Fluorescence *in situ* Hybridization (CARD-FISH) was conducted according to Thiele *et al.* 2011 with minor modifications. The probes applied in FISH targeted the most dominant classes and *phyla*, respectively, detected in the enrichment (Tab. II-2) The novel oligonucleotide probe BD1207 targeting the 16S rRNA of contig01521 (BD1-5/SN-2) was designed using the probe design tool of ARB and the SILVA SSU Ref database. Initial hybridization with the newly designed probe was performed at low stringency using a formamide concentration of 0 and 10 % in order to check for positive signals of the targeted population in the bioreactor. Signals of probe BD1207 were considered to be significant as cells were detected uniformly over the filter and counts exceed signals of the negative control probe NON338. The hybridization stringency of the novel probe BD1207 was optimized

by a series of hybridizations at increasing formamide concentrations (10-60 % in steps of 5 %) following determination of signal cut-off (i.e. decrease in signal intensity). The optimal formamide concentration (here 40 %) was the highest concentration before signal cut-off occurred.

Table II-2 I Oligonucleotide probes used for FISH analyses in this study.

Probe	FA (%)	Target	Sequence (5' → 3')	Reference
BD1207	40	BD1-5 member	AGCCCCAGACGTAAAAGC	this study
Gam42a	35	*Gammaproteobacteria*	GCCTTCCCACATCGTTT	Manz *et al.* 1992
Bet42a unl.	35	*Betaproteobacteria*	GCCTTCCCACTTCGTTT	Manz *et al.* 1992
Alf968	35	*Alphaproteobacteria*	GGTAAGGTTCTGCGCGTT	Neef 1997
Eub338	35	Eubacteria	GCTGCCTCCCGTAGGAGT	Amann *et al.* 1990
Eub338-II	35	Eubacteria	GCAGCCACCCGTAGGTGT	Daims *et al.* 1999
Eub338-III	35	Eubacteria	GCTGCCACCCGTAGGTGT	Daims *et al.* 1999
Non338	35	Negative control	ACTCCTACGGGAGGCAGC	Wallner *et al.*1993

unl = unlabeled (competitor to Gam42a); FA = formamide concentration (v/v) in the hybridization buffer

Data submission

The four metagenomes obtained directly from the sediment were submitted to the sequence read archive (http://trace.ncbi.nlm.nih.gov/Traces/sra/) under the bioproject PRJNA174601 (Accession: SRP015924). The sample accession no. are SRS365699, SRS365698, SRS365700 and SRS365701. The 454 and Illumina sequencing data sets obtained from the chemostat were submitted to the sequence read archive (http://www.ncbi.nlm.nih.gov/Traces/sra/), and the assembled contigs were submitted as metagenome sequencing project to the whole genome shotgun submission portal (https://submit.ncbi.nlm.nih.gov/subs/wgs/). Both reads and contigs are accessible under the BioProject PRJNA209200 (Accession: SRP026254), and the BioSample SAMN02211837 (Accession: SRS451311). The Whole Genome Shotgun project has been submitted to the DDBJ/EMBL/GenBank databases under accession No. ATLU00000000. The version described in this paper is version ATLU01000000.

Results

After extracting the cells from sediment samples collected from the tidal flat, the potential denitrification rate and community composition of the cell suspension was determined and compared to the original sediment before extraction. It was found that 30 % of the potential denitrification rate (8.4 µmol $l^{-1} d^{-1}$ vs 28.7 µmol $l^{-1} d^{-1}$) and 41 % of the OTUs (40 out of 97) were recovered, as shown by incubation with $^{15}NO_3^-$ and ARISA, respectively.

The cell suspension was incubated under dynamically oxic/anoxic conditions favoring aerobic respiration and denitrification. It was continuously supplied with medium containing glucose, amino acids, and acetate as carbon sources (see Tab. II-1). The composition of carbon sources was close to the composition of decaying biomass (in terms of its monomers), the prevalent carbon and energy source *in situ*.

The tidal oxic/anoxic cycling was clearly visible in the signal of the redox electrode (Fig. II-2). During oxygen supply a slight decrease in pH was observed. Over time, pressure decreased slowly from 15 to 9 mbar caused by evaporation of water from the water lock which prevented back-flow of atmospheric gases into the culture. Intermittent steep pressure decreases (Fig. II-2) resulted from sampling and maintenance operations. Nitrate and nitrite were consumed almost completely and responded to tidal cycling, as shown for day 15 (inset). Accumulation of nitrite and nitrate during tidal cycling attenuated with time. Apparently, the microbial community adapted to the periodic oxygen supply, leading to less nitrite accumulation.

Protein concentrations were measured to determine the overall biomass growth yield. The fraction of assimilated carbon ranged from 19 to 25 % of carbon delivered. Ammonia was produced by the culture and its concentration (mM) was consistent with the ammonification of the supplied amino acids.
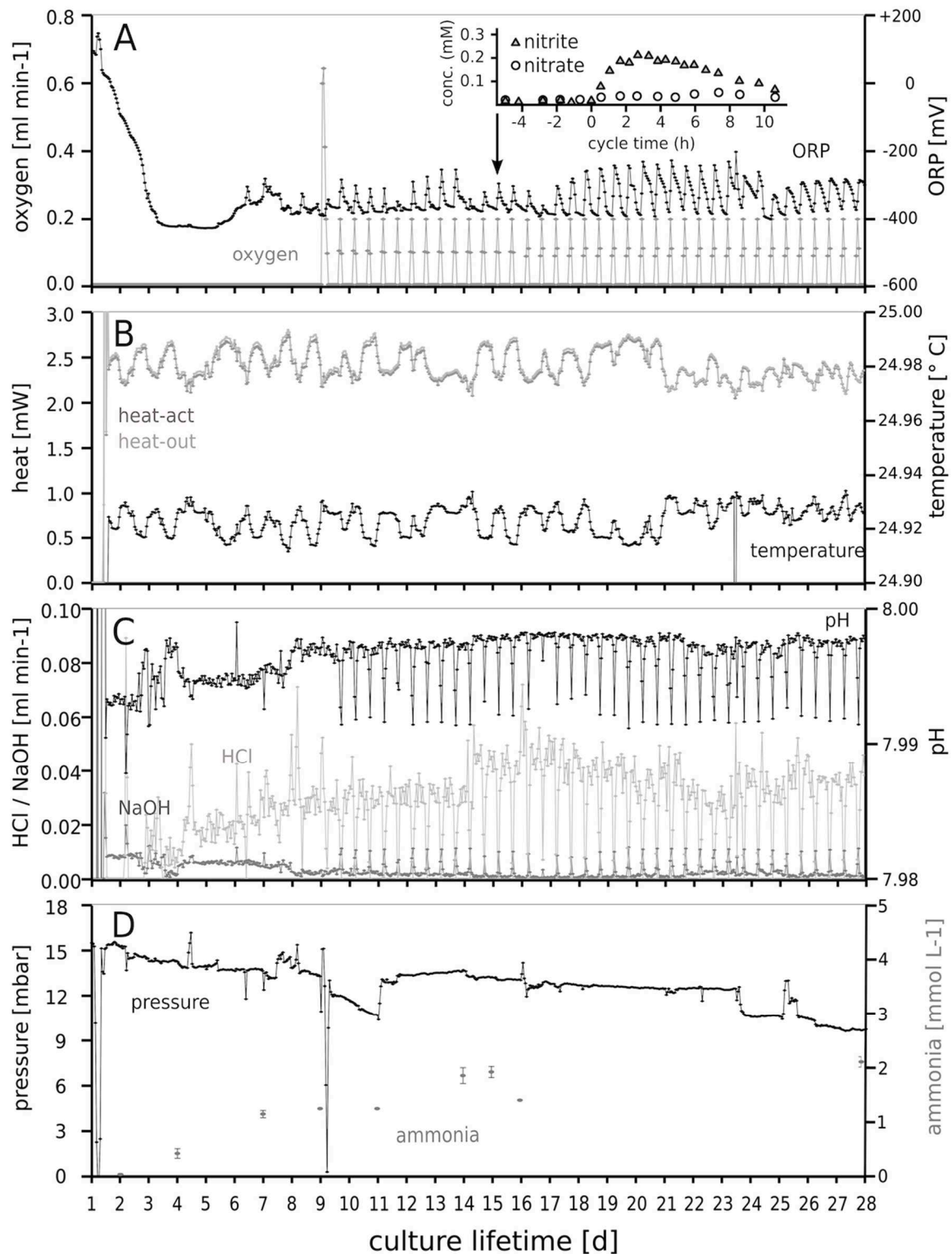
Figure II-2 I Oxygen dosage (A), temperature and heater power (B) and pH control (C) control enabling dynamic "tidal" conditions for selection of the microbial community in the chemostat during 28 days. The response of the culture to the intermittent oxygen supply is clearly visible in the redox sensor (A, ORP*) response. The maintenance of several mbar overpressure (D) indicated the absence of undesired air leaks. The ammonium concentration in the culture vessel (measured off-line) is also shown in panel (E). The response of nitrite and nitrate concentrations to tidal cycling is shown in the inset of panel (A) for day 15.

After 14 days of stable oxic/anoxic cycling (23 days after inoculation) the enriched microbial community was characterized by metagenomic sequencing and proteomic analysis. The N50 contig length of the assembly was 4.1 kb at 5.8x sequencing coverage and in total 13.2 Mb of unique sequence data was assembled. Three full-length 16S rRNA genes were detected in the *de novo* assembly as well as several fragments that were assigned to *Roseobacter* populations; these were combined into 2 additional full-length genes by alignment to reference sequences of closely related bacteria. In parallel, iterative read mapping yielded two full length 16S rRNA genes that were >97 % identical to two of the assembled ones. By binning of contigs based on tetranucleotide frequencies five bins were generated, each with a distinct phylogenetic profile that aligned well with the recovered 16S rRNA gene sequences. Figure II-3 visualizes the position of each bin on the GC *vs.* coverage plot of the assembly as well as the per-bin phylogenetic profile calculated. Figure II-4 shows a phylogenetic tree of the most important 16S rRNA genes recovered and their assignment to the bins. Based on read mapping, the binned populations were estimated to make up approximately 93 % of the overall community.

Sequencing coverage (x)

Percent GC (%)

Bin E (BD1-5, 3.3 Mb, 11% abundance)

N50 1.6kb; 5x coverage; 116/4900 hits
- Bacillales (6.9x@4.0e-16)
- Flavobacteriales (6.0x@3.0e-07)
- Clostridiales (6.6x@2.0e-13)
- Rifle_ACD_49 (5.0x@1.0e-15)
- Rifle_ACD_3 (7.9x@2.0e-33)
- Lactobacillales (5.4x@2.0e-09)
- Ignavibacteriales (11.0x@1.0e-05)
- Rifle_ACD_4 (9.1x@5.0e-10)

76 tRNA's

Bin D (Gammaproteobacteria I.S., 4.0 Mb, 22% abundance)

N50 42kb; 9x coverage; 232/8275 hits
- Ferrimonas balearica (9.6x@6.0e-44)
- Allochromatium vinosum (9.8x@1.0e-46)
- Dechlorosoma suillum (9.3x@5.0e-35)
- Alcanivorax borkumensi (9.3x@4.0e-36)
- Comamonas testosteroni (9.5x@6.0e-19)
- Hahella chejuensis (10.1x@5.0e-10)
- Deinococcus deserti (9.8x@2.0e-18)
- Thiocystis violascens (9.8x@5.0e-20)

50 tRNA's

**Bin C
5 Mb;
13% ab.**
63 tRNA's

Bin B (Rhodobacteracaea, 3.5 Mb, 22% abundance)

N50 20kb; 10x coverage; 469/6697 hits
- Phaeobacter gallaecien (9.6x@4.0e-61)
- Ruegeria sp. (10.0x@4.0e-70)
- Maritimibacter alkaliphil. (10.0x@1.0e-55)
- Roseobacter denitrific. (10.0x@6.0e-49)
- Ketogulonicigenium vul. (9.6x@3.0e-32)
- Rhodobacter capsulatus (10.1x@6.0e-24)
- Paracoccus denitrific. (9.5x@8.0e-38)
- Rifle_ACD_54 bacteria (9.5x@4.0e-21)

38 tRNA's

Bin A (Maritimibacter, 4.0 Mb, 26% abundance)

N50 120kb; 11x coverage; 1934/7729 hits
- Maritimibacter alkalip... (11.9x@2.0e-10)
- Actinoplanes (12.1x@6.0e-14)
- Polymorphum gilvum SL0... (10.6x@7.0e-118)

43 tRNA's

Figure II-3 I GC *vs.* coverage plot showing scattering of the contigs into distinct "clouds", each associated with a different bin. Bins were obtained independent of coverage data with a combination of tetranucleotide compositional analysis and interpolated markov modelling. The distribution of best BLAST hits over reference organisms is shown for four of the five bins. For C, the distribution was very similar to that of bin B. Because no phylogenetically close reference organisms were present in the database for Bin D and E, many contigs of these bins did not give a BLAST hit.

98

0.02 changes per site (1416 positions)

Bin C (contig02701, 3043, 8427, 9063; 8.0x coverage; FISH Alf968)
FJ716885 uncultured bacterium
EF123337 uncultured bacterium
JN874120 uncultured bacterium
CP000376 *Ruegeria sp.* TM1040
AY771774 *Phaeobacter gallaeciensis*
M59063 *Roseobacter denitrificans* OCh 114
ABXE01000006 Rhodobacteraceae bacterium HTCC2083
HM116846 uncultured bacterium
Bin B (contig01530, 2479; 9.7x coverage; FISH Alf968)
FJ869041 Rhodobacterales bacterium CB1005
EU799058 uncultured bacterium
AAMT01000002 *Maritimibacter alkaliphilus*
HM128047 uncultured bacterium
HM127060 uncultured bacterium
Bin A (contig00031 + emirge; 5.2x coverage; FISH Alf968)
CP000264 *Jannaschia sp.* CCS1
CP000830 *Dinoroseobacter shibae*
CP002568 *Polymorphum gilvum*

Alphaproteobacteria

AF432145 *Sedimenticola selenatireducens*
AF170359 *Dechloromarinus chlorophilus*
Bin D (contig00384; 7.4x coverage; FISH Gam42a)
AB597528 uncultured bacterium
JN882289 *Thiotaurens thiomutagens*
HQ191061 Janssand sediment clone
HQ191002 Janssand sediment clone
Escarpia and other symbionts
Anodontia and other symbionts
FN293060 *Thiocystis violacea*
DQ062160 *Allochromatium vinosum*
HQ191037 Janssand sediment clone
Janssand sediment clones
FM165437 *Lamellibrachia sp.* endosymbiont
DQ660820 Endosymbiont of Ridgeia
Loripes and other symbionts
Janssand sediment clones
EU709866 Thioalkalivibrio sp. ALN1
HQ191094.1 Janssand sediment clone
Janssand sediment clones
Janssand sediment clones
Janssand sediment clones
Janssand sediment clones
AJ620496 *Olavius algarvensis* Gamma symbiont
HQ191052.1 Janssand sediment clone
SUP05
Janssand sediment clones
Janssand sediment clones
HQ191087 Janssand sediment clone
HQ191053 Janssand sediment clone
X74689 Vibrio aestuarianus
JF691588 Photobacterium sp. DSM 24471
HQ191057 Janssand sediment clone
DQ778094 Ferrimonas senticii
Janssand sediment clones

Potential sulfur oxidizing Gammaproteobacteria

Gammaproteobacteria

FJ792297 uncultured bacterium
AB476267 uncultured bacterium
Bin E (contig01521 + emirge; 4.5x coverage; FISH BD1207)
JN662058 uncultured bacterium
HM128187 uncultured bacterium
Rifle metagenomes
Uncultured bacteria

BD1-5/SN-2

CP001696 *Methanocaldococcus fervens*
AB603516 *Methanocaldococcus jannaschii*

Figure II-4 *(on the previous page)* **I** Phylogenetic analysis of the full-length ribosomal 16S rRNA genes reconstructed from the metagenome reads sampled after 23 days of selection (blue). Closed circles indicate bootstrap values of >95 %, half open circles indicate bootstrap values of >90 %. Potential sulfur oxidizing *Gammaproteobacteria* previously detected by Lenk *et al.* (2011) are shown in purple.

Table II-3 summarizes the taxonomic assignment of the bins, estimated genome sizes and other properties. The most abundant population in the culture (corresponding to bin A) belonged to the genus *Maritimibacter* (*Alphaproteobacteria*). Bins B and C corresponded to two different *Roseobacter* populations bin D to a potential sulfur oxidizing *Gammaproteobacterium* and bin E to a member of the enigmatic bacterial BD1-5/SN-2 clade which lacks cultivated representatives. Based on the presence of conserved genes, each of the bins apparently contained a nearly complete genome sequence of the associated population.

The abundance of the selected populations in the sediments was evaluated after screening four metagenomes sampled directly from the sediment for 16S rDNA genes and classification of the obtained 16S rDNA fragments. 104 out of 862 were classified as potential sulfur oxidizing *Gammaproteobacteria* (eight of these were >97 % identical to the selected population, bin D), 35 as *Rhodobacterales* (ten of these were >97 % identical to the selected populations, bin A-C) and 16 as BD1-5/SN-2 *candidate phylum* (see Tab. II-4). For the latter reads the highest sequence identity to the selected population (bin E) was 93 %.

Table II-3 I Characteristics and metabolism of the binnable populations at t=23 days .

Bin		A	B	C	D	E
TAXONOMIC CLADE		*Maritimi-bacter*	*Roseo-bacter-Clade*	*Roseo-bacter-Clade*	*Gamma-proteo-bacteria I.S.*	**BD1-5/ SN-2**
Abundance (binning)	(%)	25.9	21.7	12.5	22.3	11.2
Abundance (proteome)	(%)	32.9	25.9	13.2	25.2	2.8
Abundance (*in situ*)	(%)	0.1	2.1	2.1	12.1	1.9
GC content	(%)	62.3	55.9	56.9	50.2	33.2
Bin size	(Mb)	3.7	3.5	5.0	4.0	3.3
Internal Overlap	(%)	2.7	1	12.2	3.2	37.2
Number of tRNAs	(#)	43	38	63	50	76
Genome completeness	(#/#)	122/139	133/139	105/139	135/139	109/139
Estimated genome size	(Mb)	3.6	3.5	4.4	3.9	2.1
Number of contigs	(#)	163	429	4597	248	2747
N50 contig length	(kb)	120	20	1.4	42	1.6
EXPRESSED SUBSYSTEMS		**# Expressed proteins detected (peptide coverage)**				
Ribosomal proteins (64)	(#)	10 (25±5x)	12 (21±7x)	4 (18±9x)	48 (33±6x)	27 (27±12x)
Cell division & growth (30)	(#)	23 (48±9x)	23 (40±7x)	14 (38±10x)	25 (38±8x)	12 (21±14x)
tRNA metabolism (34)	(#)	27 (21±4x)	26 (20±5x)	12 (18±9x)	28 (16±4x)	6 (10±5x)
F0F1 ATP synthase (7)	(#)	7 (46±10x)	7 (45±5x)	2 (15±1x)	6 (42±4x)	2 (23±1x)
Complex I (11)	(#)	8 (37±6x)	5 (35±3x)	8 (24±11x)	6 (21±6x)	n.d.
Complex IV (4)	(#)	3 (39±9x)	3 (37±4x)	3 (26±14x)	2 (43±5x)	n.d.
Oxygen stress (11)	(#)	6 (30±13x)	5 (22±10)	4 (40±8)	10 (46±7x)	6 (18±16x)
Nitrate reductase (3)	(#)	n.d.	1 (49±3x)	1 (48±2x)	3 (46±6x)	n.d.
Nitrite reductase (1)	(#)	1 (44±24x)	1 (44±18x)	n.d.	1 (29±32x)	n.d.
Nitric oxide reductase (1)	(#)	1 (44±0x)	1 (44±4x)	n.d.	1 (37±8x)	n.d.
Nitrous oxide reductase (1)	(#)	1 (58±3x)	1 (49±1x)	1 (60±13x)	1 (66±1x)	n.d.
Sulfide oxidation (13)	(#)	1 (51±10x)	n.d.	n.d.	13 (36±12x)	n.d.
Hydrogen oxidation (4)	(#)	n.d.	n.d.	n.d.	3 (27±3x)	n.d.
Formate oxidation (5)	(#)	n.d.	n.d.	n.d.	5 (26±5x)	n.d.
CO dehydrogenase (3)	(#)	3 (49±16x)	n.d.	3 (40±4x)	n.d.	n.d.
Calvin Cycle (3)	(#)	n.d.	n.d.	n.d.	3 (7±1x)	n.d.
Citric acid cycle (23)	(#)	16 (39±7x)	15 (35±5x)	13 (36±8x)	17 (33±5x)	n.d.
Sugar metabolism (24)	(#)	18 (44±7x)	14 (35±5x)	9 (28±9x)	11 (29±4x)	n.d.
Amino acid metabolism (44)	(#)	30 (28±7x)	32 (26±11x)	26 (26±9x)	30 (26±8x)	4 (11±8x)
TRANSPORTERS		**# Expressed proteins detected (peptide coverage)**				
Sugars (9)	(#)	6 (28±7x)	3 (32±7x)	6 (26±5x)	2 (15±16x)	n.d.
Aminoacids (7)	(#)	5 (34±11x)	4 (59±5x)	3 (19±4x)	3 (46±16x)	n.d.
Di/tricarboxylates (4)	(#)	4 (40±14x)	4 (42±10x)	3 (31±15x)	2 (16±17x)	n.d.
Glycine-betaine (4)	(#)	2 (36±5x)	1 (46±11x)	2 (30±3x)	1 (44±1x)	n.d.
Oligopeptides (3)	(#)	2 (31±5x)	2 (32±16x)	2 (33±6x)	1 (19±6x)	n.d.
Purines (1)	(#)	1 (55±16x)	1 (65±2x)	1 (61±1x)	n.d.	n.d.
Acetate (1)	(#)	n.d.	n.d.	n.d.	1 (13±1x)	n.d.
Urea (1)	(#)	n.d.	1 (42±5x)	1 (30±4x)	0	n.d.
Transporters total (29)	(#)	25	23	24	13	0

n.d. = not detected

Table II-4 I Comparison of population abundances for the selected microbial community (based on metagenomics/FISH) and the sediment community composition *in situ (*based on sequencing reads encoding 16S rDNA genes).

Taxon	Abundance *in situ* (%)	Abundance in chemostat (%)
Gammaproteobacteria	24.6*	19-25
Flavobacteria	10.2	n.d.
Cyanobacteria	10.1	n.d.
Deltaproteobacteria	9.0	n.d.
Eukaryoata	5.7	n.d.
Planctomycetes	4.8	n.d.
Actinobacteria	4.1	n.d.
Alphaproteobacteria	3.9	47-72
Acidobacteria	3.7	n.d.
Sphingobacteria	3.1	n.d.
Verrucomicrobia	2.8	n.d.
Cytophagia	1.9	n.d.
BD1-5/SN-2	1.9	1.4-11.
Chloroflexi	1.4	n.d.
Others	12.8	n.d.

* 12.1 % were affiliated with potential sulfur oxidizing Gammaproteobacteria

It is known that at least some bacteria of the BD1-5/SN-2 clade and related bacteria (*Gracilibacteria* and clade *SN1*) are characterized by the use of an alternate genetic code (Wrighton *et al.* 2012; Campbell *et al.* 2013; Rinke *et al.* 2013). This is a very rare feature among prokaryotes and we first tested the likelihood that the enriched representative of this *phylum* also made use of a different code *in silico*. Indeed, the analysis of conserved gene sequences of bin E (Dutilh *et al.* 2011) suggested that the stop codon UGA was translated as glycine (Fig. II-5). To confirm the use of the alternate genetic code for this population, bin E was translated three times: UGA as stop, as tryptophan and as glycine. Proteomics measurements yielded a total of 27,449 non-redundant peptides from the predicted protein database with 1,056 peptides contributed by the BD1-5/SN-2 population (Supplementary Tables 1,3,4). After applying a stringent cut-off of ±10 ppm mass accuracy on the resultant peptide-spectrum matches for BD1-5/SN-2, 820 peptides survived. Further interrogation of these peptides for their uniqueness within the three versions of BD1-5/SN-2 predicted proteome revealed that 97 of the peptides were unique to the BD1-5/SN-2 UGA-Gly database compared to just 3 peptides that were unique to the BD1-5/SN-2 UGA-Trp database (Supplementary Tab. 2). No peptides were found to be unique for the UGA Stop database. The remaining set of peptides was present in more

than one version of the bin E database, rendering them not definitive for inferring codon usage. However, the occurrence of a large number of unique peptides corresponding to glycine versions of the database suggests that UGA preferentially translates to glycine in this species.

We further investigated whether the possible incidental translation of UGA as tryptophan was biologically real or an artifact of our method. Therefore, sequences of bin E were *in silico* translated with UGA coding for every standard amino acid and a new search was performed using previous database supplemented with these additional proteomes. Results were filtered at different stringencies (5-10 ppm mass accuracy on MS1 and a minimum of 2 filtered spectra). At the highest stringency, translation to tryptophan was still detected for a single peptide (compared to 40 for glycine). Interestingly, a single peptide was also matched to proteins from UGA coding for alanine, lysine and aspartate proteomes (Supplementary Tab. 2).

The five bins were annotated as individual provisional genome sequences that defined the metabolic potential of each of the corresponding populations. The proteomic analyses could now be used to infer which substrates might be used by the different populations. Good peptide coverage was obtained for proteins associated with each of the five bins, despite apparent differences in abundances of the populations. Total peptide counts for each bin agreed well with the abundances for each population estimated by metagenome analyses, except for the BD1-5/SN-2 population of bin E (Tab. II-3).

The expression of 27 ribosomal proteins and 12 proteins involved in cell division and growth was detected for the BD1-5/SN-2 population (bin E) and provided strong evidence for growth. However, we found very little evidence for most aspects of metabolism. Indeed, despite the fact that bin E was predicted to contain most of the sequence information present in the genome of this population (109/139 conserved proteins present, comparable to the *Roseobacter* in bin C, see Table II-3), the enzymes of the citric acid cycle, glycolysis, and lipid biosynthesis were almost completely missing.
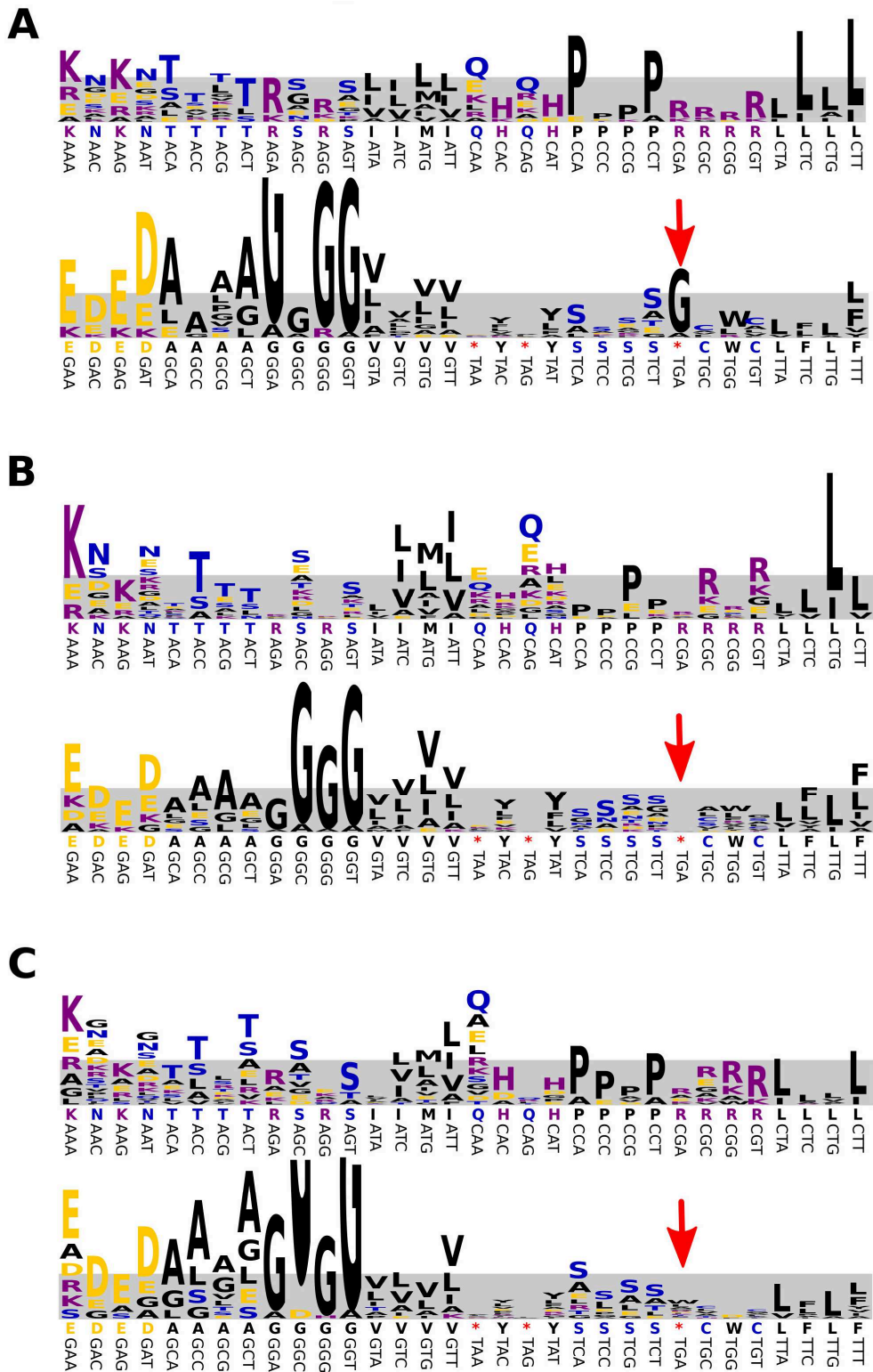
Figure II-5 I Prediction of the genetic code used by the BD1-5/SN-2 population. The results are shown for Bin E (A), E. coli (B) and Mycoplasma (C). Directly above each codon, the canonical translation is shown. The frequency of amino acids observed in conserved positions of conserved proteins is visualized as a WebLogo, where the height of each letter is a measure for the observed frequency (most frequent amino acid shown at the top). The frequencies were normalized to the expected occurrence of the codon predicted from the percentage GC (shaded area). The UGA stop codon (red arrow) was correctly predicted for *E. coli* (stop) and *Mycoplasma* (tryptophan, W) and was predicted to be translated as glycine for the enriched BD1-5/SN-2 bacterium.

104

The partitioning of metabolism over the different populations was inferred from the proteome. Table II-3 shows the average peptide coverages and detected proteins for distinct subsystems for each of the bins and Supplementary Table 6 provides the data on all proteins detected. Proteomic analysis suggested that all populations except BD1-5/SN-2 (bin E) were competing for oxygen, whereas the denitrification pathway may have been characterized by a combination of competition and cross-feeding as can be seen from different steps of denitrification apparently performed by four different populations (bin A-D, Tab. II-3). The expression of transporters, the enzymes involved in glycolysis, the citric acid cycle and respiratory complex I suggested that these four populations were also competing with each other for the supplied carbon substrates. The three *Rhodobacterales* (bin A-C) presumably used the substrates as energy and carbon source. In addition, the *Maritimibacter* population (bin A) may also have used sulfide as additional energy source, as shown by the expression of a sulfide dehydrogenase. The activity of some genes involved in the reversed citric acid cycle in the *Gammaproteobacterium* (bin D) suggested that this population may have mainly used the supplied organic molecules as a carbon source and not as an energy source; a partially reversed citric acid cycle is an indication for the interconversion of amino acids. It may have obtained energy for growth by oxidizing sulfide to sulfate, formate to carbon dioxide, and hydrogen to water. The expression of the Calvin cycle enzymes was also significant, indicating that it may have grown partially autotrophically. Based on the genomic and proteomic evidence, sulfide, hydrogen and formate did not appear to be produced by any of the major populations presented in Table II-3. Their consumption hinted at the presence of other, sulfate reducing and fermentative populations that remained below the detection limit of our metagenome.

The expression of a monofunctional carbon monoxide dehydrogenase (Ni-Fe-CODH) by two of the *Rhodobacterales* (bins A and C) may indicate a presently unknown source of carbon monoxide. Turnover of this molecule is known to be important in many systems where it can be produced by photooxidation (Carpenter *et al.* 2012) but photooxidation could be excluded in our culture because it was always kept in the dark.

Finally, the relative abundances of the major populations as inferred from metagenomic and proteomic analyses were validated experimentally with fluorescence *in situ* hybridization (FISH) and microscopy (Tab. II-2, Tab. II-3, Fig. II-6). The abundance of total *Alphaproteobacteria* (bin A-C) was 46.9 %, of *Gammaproteobacteria* (bin D) 19 % and of BD1-5/SN-2 (bin E) 1.4 %. These numbers are consistent with the estimates derived from the metagenomic and proteomic data. The BD1-5/SN-2 cells were small and coccoid with a diameter of 0.3-0.5 µm. The cells were mainly free living but association to other cells was also frequently observed (Fig. II-6).
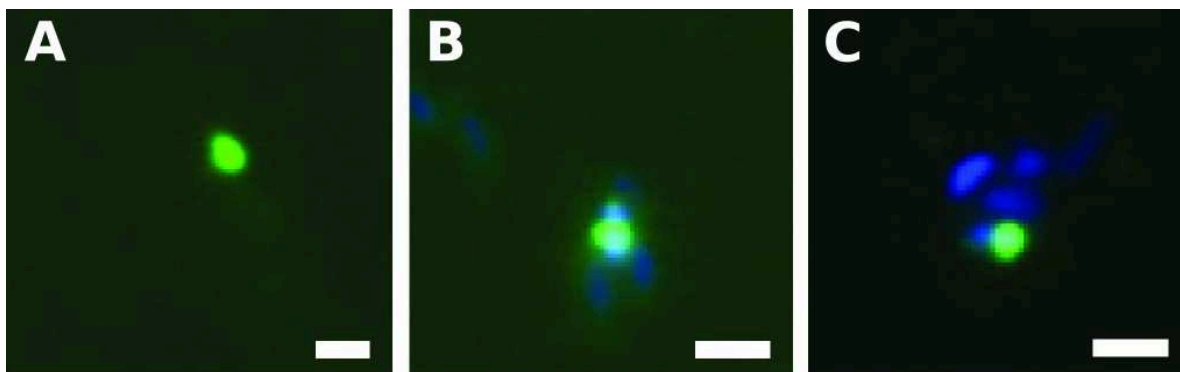
Figure II-6 I Fluorescence *in situ* hybridization of bacteria of the BD1-5/SN-2 division, showing free living cells (A) and cells attached to other bacteria (B, C). Scale bar = 1 µm.

Discussion

Microbial communities in convective tidal flat sediments are active in the remineralization of organic matter and denitrification (Kieskamp *et al.* 1991). Like many sediments they are characterized by a complex microbial community that defies current ecological approaches, for example to derive function-identity relationships or pinpoint community interactions (Lenk *et al.* 2011). In this study a laboratory chemostat was used to mimic the tidal flat conditions and select for a simplified tidal flat microbial community. The simplified community still contained representatives of populations that were abundant *in situ*. For most of these populations, the ecological functions and interactions with other community members could be provisionally resolved with proteomics. Ultimately, the functions as inferred from the proteome should be validated experimentally (e.g. isotope labeling, enzyme assays). However, the time needed for the experimental logistics and computational analyses of the metagenomic data was too long to enable the experimental follow-up required for further validation.

Although the extraction of microbes from the sediment led to a loss of microbial activity and induced changes in the microbial community composition, a detectable fraction of the *in situ* activity and community richness was preserved. Because we applied a selective force after the extraction procedure, representative extraction was not a prerequisite for our experiment as long as sufficient biodiversity and activity were preserved to allow for meaningful selection afterwards.

Thus, even though the conditions in the chemostat were very different from those in the sediment (e.g. the absence of sand), the *in situ* abundance of the selected populations was detectable. Apparently, the chemostat was still sufficiently close to the natural environment to select for some populations that were also successful *in situ*. The low concentrations of the substrates (micromolar range) in the chemostat might be one of the explanations for this success, because selection of r-strategists (Andrews & Harris 1986) could be prevented. Low substrate concentrations would be expected to select for a community of K-strategists; heterotrophic, facultatively aerobic denitrifiers. Indeed, after 23 days, the microbial community was dominated by three competing *Alphaproteobacteria* (bin A-C) that performed aerobic respiration and denitrification coupled to the

oxidation of organic molecules as the carbon and energy source; two of these were members of the *Roseobacter* clade, the most abundant 16S rRNA gene lineage in marine ecosystems, and one population was related to *Maritimibacter alkaliphilus*, isolated from the Western Sargasso Sea (Atlantic Ocean) *via* high throughput culturing approaches (Lee *et al.* 2007; Thrash *et al.* 2010).

Interestingly, a population of potential sulfur oxidizing *Gammaproteobacteria* (bin D) was co-enriched with the *Alphaproteobacteria*. Proteomic analysis suggested that this population had a partially chemolithoautotrophic metabolism and used sulfide, formate and hydrogen as electron donors. Bacteria belonging to this clade of *Gammaproteobacteria* are very common at our sampling site (Lenk *et al.* 2011), in the oceans in general (e.g. SUP05; Walsh *et al.* 2009), and have also frequently been encountered as symbionts of marine invertebrates (e.g. Kleiner *et al.* 2012). The enriched organism was most closely related to the isolates *Sedimenticola selenatireducens* (Narasingarao & Häggblom 2006), *Thiotaurens thiomutagens* (Flood 2010), and *Dechloromarinus chlorophilus* (Coates & Achenbach 2004; Clark *et al.* 2013). Its 16S rDNA sequence was 94 % identical to a sequence previously detected in Janssand sediments (HQ191061, Lenk *et al.* 2011) and 88 % identical to the SUP05 16S rDNA genes. The active expression of a complete sulfide oxidation pathway (including flavocytochrome c sulfide dehydrogenase, dissimilatory sulfite reductase, adenylsulfate reductase, sulfate adenyl transferase) by the potential sulfur oxidizing *Gammaproteobacterium* in the chemostat suggested the activity of a so called "cryptic" sulfur cycle, whereby sulfate reduction and sulfide re-oxidation are so tightly coupled that sulfide does not accumulate (Canfield *et al.* 2010). However, no sulfide was detected and no evidence for the presence of sulfate reducing bacteria was found. Therefore, it is perhaps more likely that the sulfide oxidation pathway was expressed constitutively, without an active function in the chemostat enrichment. The activity of the Calvin Cycle (e.g. expression of ribulose bisphosphate carboxylase, phosporibulokinase) suggested a (partially) autotrophic lifestyle. Even though organic compounds (glucose, aminoacids) were continuously supplied to the chemostat, microbial competition for these substrates led to low actual substrate concentrations in the chemostat. It is possible that the selected population of *Gammaproteobacteria* also made use of the energy conserved from formate and

hydrogen oxidation to assimilate CO_2 as an additional carbon source. This was apparent from the active expression of formate dehydrogenase and a nickel/iron uptake hydrogenase (see also Tab. II-3). These physiological interpretations where compared to what is known for related, cultivated bacteria: A provisional genome sequence is available for *Dechloromarinus chlorophilus (*Clark *et al.* 2013*)* and the genome of this bacterium also encodes genes for formate dehydrogenase, a nickel/iron uptake hydrogenase, key Calvin cycle enzymes (ribulose bisphosphate carboxylase and phosphoribulokinase) but only a single gene of the sulfide oxidation pathway (*dsrB*). For *T. thiomutagens* a genome sequence is not available but the physiology of this organism is consistent with the proteomic results presented here. Both the activity of the Calvin cycle and sulfur oxidation were previously shown to be detectable for potential sulfur oxidizing *Gamma-proteobacteria* in different marine habitats (Lavik *et al.* 2009; Walsh *et al.* 2009; Stewart 2011; Canfield *et al.* 2010).

Candidate division BD1-5/SN-2 and the closely related candidate *phyla SR1* are very common components of microbial communities in very different habitats such as the human gut, the oral cavity (Campbell *et al.* 2013), subsurface aquifers (Wrighton *et al.* 2012) and marine sediments. At our sampling site almost 2 % of all 16S rRNA genes identified in 4 different metagenomes belonged to this division. Members of BD1-5/SN-2 have not been cultivated so far but a fermentative, possibly sulfur reducing lifestyle has been inferred from genomes reconstructed from metagenomic data (Wrighton *et al*. 2012) and single cell genomes (Campbell *et al*. 2013). Their abundance in the community selected here (bin E) was between 1.4 % and 5 %, as shown by FISH/proteomics and metagenomics, respectively. The relatively short contigs in bin E and the significant degree of internal overlap in this bin suggested that the enriched population was not clonal; even though only a single 16S rRNA gene sequence was recovered (assembly and iterative read mapping produced identical sequences). The crossassembly of multiple, closely related populations in bin E led to a considerable degree of redundancy and an overestimation of the genome size, which is probably still significantly lower than the 2.1 Mb estimated in Table II-3.

Both *in silico* analysis and proteomics showed very strong evidence for the use of an alternate genetic code by this bacterial division. As was previously described for the related division SR1 and Gracilibacteria, the opal stop codon, UGA, was found to be translated as glycine (Campbell *et al*. 2013; Rinke *et al*. 2013). Alternate genetic codes are relatively rare among bacteria, but also translation of UGA as tryptophan is known in multiple organisms (Yamao *et al*. 1985; McCutcheon *et al*. 2009).

In depth investigation of the possible translations of UGA in bin E by proteomics also showed that low levels of "mistranslation" may occur: a single peptide was detected for tryptophan, alanine, aspartate and lysine. Such mistranslations may actually be a prerequisite for the evolution of alternate genetic codes as the one reported here.

The BD1-5/SN-2 genomic dataset in bin E encodes very little recognizable metabolic capacity. Very few genes were detected that encoded enzymes involved in respiration. Genes encoding enzymes involved in the citric acid cycle, glycolysis, amino acid metabolism, and lipid metabolism were almost completely absent, a finding that parallels the prior results of Wrighton *et al*. (2012) and Rinke *et al.* (2013). The only enzyme complex that was clearly expressed was the F0F1 ATP synthase. The apparent lack of encoded metabolic capacity could not easily be explained by annotation problems because the genes encoding cell biological processes (DNA replication, transcription, translation and cell division) were detected without problems (and were also actively expressed). Based on the lack of metabolism, a highly syntrophic or parasitic lifestyle of the enriched BD1-5/SN-2 bacterium seems to be the only possible explanation for these findings. However, fluorescence *in situ* hybridization provided only limited evidence for the association of BD1-5/SN-2 with other cells (Fig. II-6); most cells appeared to be free living. The identification of the ecological niche of the microbes that constitute the BD1-5/SN-2 *phylum* will be an exciting avenue for future investigations.

The relative abundances of potential sulfur oxidizing *Gammaproteobacteria* (bin D) and BD1-5/SN-2 (bin E) in the selected microbial community were close to the *in situ* abundances (Tab. II-4). Other populations that were abundant *in situ* were not selected: *Flavobacteria*, *Eukaryota*, *Cyanobacteria* and *Deltaproteobacteria*. *Flavobacteria* are known as polymer degraders (Kirchman, 2002) and their selection was not expected because the substrates were supplied as monomers. *Cyanobacteria* were excluded because the incubation was performed in the dark and *Eukaryota* were removed from the inoculum by the addition of cycloheximide. *Deltaproteobacteria* (e.g. sulfate reducers) might be more successful *in situ* than in the incubation because of the presence of oxygen and nitrate gradients *in situ* leading to anoxic micro-niches, which were absent in the incubation. As a consequence, most of the supplied substrates were consumed by *Alphaproteobacteria* instead and these came to dominate the selected community.

In conclusion, in this study we engineered specific aspects of the marine tidal sediment habitat in a laboratory continuous culture incubation. In this incubation, the environmental conditions selected for specific populations of the natural sediment community and allowed us to study these populations in the context of a simplified microbial community. Metagenomic and proteomic approaches provided the first experimental evidence for the translation of the stop codon UGA as glycine by a member of the BD1-5/SN-2 *phylum* and also provided hints about the niche partitioning of *Alphaproteobacteria* and potential sulfur oxidizing *Gamma-proteobacteria* with regard to carbon metabolism. In future studies, similar approaches could be used to address key problems in microbial ecology, such as the cryptic sulfur cycle, community dynamics resulting from viral predation, antagonistic interactions between bacteria, cross feeding and competition. Such ecological phenomena cannot be studied with isolated strains and although direct observation of natural ecosystems may also yield results, that approach is much more challenging because of the higher complexity and the lack of experimental control.

Author contributions

AH and EH performed sampling and cultivation with support from JG and MS. AH performed FISH with support from SL. HT performed DNA sequencing and assembly. BK, AH and TH performed the benchmarking of the sediment extraction. Controllers were designed and tested by MS, KM, RW and VM and assembled by HO. RS and RH performed proteomic analyses. The manuscript was written by AH and MS with support from all other authors.

References

Albertsen M, Hugenholtz P, Skarshewski A, Nielsen KL, Tyson GW, Nielsen PH. (2013). Genome sequences of rare, uncultured bacteria obtained by differential coverage binning of multiple metagenomes. *Nat Biotechnol* 31:533-538.

Amann R, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA. (1990). Combination of 16S rRNA-Targeted Oligonucleotide Probes with Flow Cytometry for Analyzing Mixed Microbial Populations. *Appl Environ Microbiol* 56:1919-1925.

Andrews JH and Harris RF. (1986). r- and K-selection and microbial ecology. *Adv Microb Ecol* 9: 99-147.

Baker BJ, Comolli L, Dick G, Hauser L, Hyatt D, Dill BD *et al.* (2010). Enigmatic, ultra-small uncultivated Archaea. *Proc Nat Acad Sci U S A* 107:8806-8811.

Belnap CP, Pan C, VerBerkmoes NC, Power ME, Samatova NF, Carver RL *et al.* (2009). Cultivation and quantitative proteomic analyses of acidophilic microbial communities. *ISME J* 4:520–530.

Bendschneider K and Robinson RJ. (1952). A new spectrophotometric method for the determination of nitrite in sea water. *J Mar Res* 11:87-96.

Blainey PC. (2013). The future is now: single-cell genomics of bacteria and archaea. FEMS *Microbiol Rev* 37:407-427.

Campbell JH, O´Donoghue P, Campbell AG, Schwientek P, Sczyrba A, Woyke T *et al.* (2013). UGA is an additional glycine codon in uncultured SR1 bacteria from the human microbiota. *Proc Nat Acad Sci U S A* 110:5540-5545.

Canfield DE, Stewart FJ, Thamdrup B, De Brabandere L, Dalsgaard T, Delong EF *et al.* (2010). A cryptic sulfur cycle in oxygen-minimum-zone waters off the Chilean coast. *Science* 330:1375-1378.

Carpenter LJ, Archer SD, Beale R. (2012). Ocean-atmosphere trace gas exchange. *Chem Soc Rev* 41:6473-6506.

Castelle CJ, Hug LA, Wrighton KC, Thomas BC, Williams KH, Wu D *et al.* (2013). Extraordinary phylogenetic diversity and metabolic versatility in aquifer sediment. *Nat Commun* 4:2120.

Clark IC, Melnyk RA, Engelbrektson A, Coates JD (2013) Structure and evolution of chlorate reduction composite transposons. mBio 4(•):e00379-13. doi:10.1128/mBio.00379-13.

Coates JD and Achenbach LA. (2004). Microbial perchlorate reduction: rocket-fuelled metabolism. *Nature Rev Microbiol* 2:569-580.

Daims H, Bruehl A, Amann R, Schleifer KH, Wagner M. (1999). The Domain-specific Probe EUB338 is Insufficient for the Detection of all Bacteria: Development and Evaluation of a more Comprehensive Probe Set. *Syst Appl Microbiol* 22:434-444.

Dutilh BE, Jurgelenaite R, Szklarczyk R, van Hijum SAFT, Harhangi HR, Schmid M *et al.* (2011). FACIL: Fast and accurate genetic code inference and logo. *BMC Bioinformatics* 27:1929-33.

Eddy SR. (2011). Accelerated profile HMM searches. *PLoSComputBiol* 7:e1002195+.

Eilers H, Pernthaler J, Peplies J, Gloeckner FO, Gerdts G, Amann R. (2001). Isolation of novel pelagic bacteria from the German bight and their seasonal contributions to surface picoplankton. *Appl Environ Microbiol* 67:5134–5142.

Flood BE. (2010). The utilization of important derivatives (TMA & Thiotaurine) of eukaryotic osmolytes by novel strains of eubacteria. //gradworks.umi.com/34/34/3434417.html; publication number 3434417.

Hyatt D, Chen GL, Locascio PF, Land ML, Larimer FW, Hauser LJ. (2010). Prodigal: prokaryotic gene recognition and translation initiation site identification. *BMC Bioinformatics* 11:1-11.

Kalisky T and Quake SR. (2011). Single-cell genomics. *Nat Methods* 8:311-314.

Kartal B and Strous M. (2008). Methods to study consortia and mixed cultures. In: Zengler, K (ed.) Accessing Uncultivated Microorganisms, ASM Press: Washington, pp 205-219.

Kieskamp WMLL, Epping W, Helder W. (1991). Seasonal variation in denitrification rates and nitrous oxide fluxes in intertidal sediments of the western Wadden Sea. *Mar Ecol Prog Ser* 72:145-151.

Kirchman D. (2002). The ecology of *Cytophaga-Flavobacteria* in aquatic environments. *FEMS Microbiol Ecol* 39:91–100.

Kleiner M, Wentrup C, Lott C, Teeling H, Wetzel S, Young J *et al.* (2012). Metaproteomics of a gutless marine worm and its symbiotic microbial community reveal unusual pathways for carbon and energy use. *Proc Nat Acad Sci U S A* 109:E1173-E1182.

Katoh K, Misawa K, Kuma KI, Miyata T. (2002). MAFFT: a novel method for rapid multiple sequence alignment based on fast fourier transform. Nucleic Acids Res 30:3059-3066.

Langmead B and Salzberg SL. (2012). Fast gapped-read alignment with bowtie 2. Nat Methods 9:357-359.

Laslett D and Canback B. (2004). ARAGORN, a program to detect tRNA genes and tmRNA genes in nucleotide sequences. Nucleic Acids Res32:11-16.

Lavik G, Stührmann T, Brüchert V, Van der Plas A, Mohrholz V, Lam P *et al.* (2009). Detoxification of sulphidic African shelf waters by blooming chemolithotrophs. *Nature* 457:581-585.

Lee K, Choo YJJ, Giovannoni SJ, Cho JCC: (2007). *Maritimibacter alkaliphilus* gen. nov., sp. nov., a genome-sequenced marine bacterium of the *Roseobacter* clade in the order *Rhodobacterales*. *Int J Syst Evol Microbiol* 57:1653-1658.

Lenk S, Arnds J, Zerjatke K, Musat N, Amann R, Mußmann M. (2011). Novel groups of *Gammaproteobacteria* catalyse sulfur oxidation and carbon fixation in a coastal, intertidal sediment. *Environ Microbiol* 13:758-774.

Llobet-Brossa E, Rosselló-Mora R, Amann R. (1998). Microbial community composition of Wadden Sea sediments as revealed by fluorescence *in situ* hybridization. *Appl Environ Microbiol* 64: 2691–2696.

Lo I, Denef VJ, VerBerkmoes NC, Shah MB, Goltsman D, DiBartolo G *et al.* (2007). Strain-resolved community proteomics reveals recombining genomes of acidophilic bacteria. *Nature* 446:537-541.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. (1951). Protein measurement with the Folin-phenol reagent. *J Biol Chem,*193:265-275.

Manz W, Amann R, Ludwig W, Wagner M, Schleifer KH. (1992). Phylogenetic oligodeoxynucleotide probes for the major subclasses of *proteobacteria*-problems and solutions. *Syst Appl Microbiol* 15:593-600.

Ma ZQ, Dasari S, Chambers MC, Litton MD, Sobecki SM, Zimmerman LJ, Halvey PJ, Schilling B, Drake PM, Gibson BW, Tabb DL. (2009). IDPicker 2.0: Improved protein assembly with high discrimination peptide identification filtering. J Proteome Res 8:3872-81.

McCutcheon JP, McDonald BR, Moran NA. (2009). Origin of an Alternative Genetic Code in the Extremely Small and GC–Rich Genome of a Bacterial Symbiont. *PLoS Genet* 5:1-11.

Miller CS, Baker BJ, Thomas BC, Singer SW, Banfield JF. (2011). EMIRGE: reconstruction of full-length ribosomal genes from microbial community short read sequencing data. *Genome Biol* 12:R44.

Narasingarao P and Häggblom MM. (2006). Sedimenticola selenatireducens, gen. nov., sp. nov., an anaerobic selenate-respiring bacterium isolated from estuarine sediment. *Syst Appl Microbiol* 29:382-388.

Neef A. (1997). PhD thesis. Technische Universitaet Muenchen, Munich, Germany.

Price MN, Dehal PS, Arkin AP (2010). FastTree 2 – approximately Maximum-Likelihood trees for large alignments. *PLoS ONE* 5:e9490+.

Rapid Library Preparation Method Manual (May 2011): http://454.com/downloads/my454/documentation/gs-flx-plus/Rapid-Library-Preparation-Method-Manual_XLPlus_May2011.pdf.

Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P *et al*. (2013). *Nucleic Acids Res* 41:D590-D596.

Ramette A. (2009). Quantitative community fingerprinting methods for estimating the abundance of operational taxonomic units in natural microbial communities. *Appl Environ Microbiol* 75:2495-2505.

Rinke C, Schwientek P, Sczyrba A, Ivanova NN, Anderson IJ, Cheng JF, Darling A, Malfatti S, Swan BK, Gies EA, Dodsworth JA, Hedlund BP, Tsiamis G, Sievert SM, Liu WT, Eisen JA, Hallam SJ, Kyrpides NC, Stepanauskas R, Rubin EM, Hugenholtz P, Woyke T (2013). Insights into the phylogeny and coding potential of microbial dark matter. *Nature advance online publication* (7459), 431-437.

Solorzano L. (1969). Determination of ammonia in natural waters by the phenol hypochlorite method. *Limnol Oceanogr* 14:799-801.

Stewart FJ. (2011). Dissimilatory sulfur cycling in oxygen minimum zones: an emerging metagenomics perspective. *Biochem Soc Trans* 39:1859-1863.

Strous M, Kraft B, Bisdorf R, Tegetmeyer HE. (2012). The binning of metagenomic contigs for microbial physiology of mixed cultures. *Front Microbiol* 3, article 410.

Tabb DL, Fernando CG, Chambers MC. (2007). MyriMatch: highly accurate tandem mass spectral peptide identification by multivariate hypergeometric analysis. *J Proteome Res* 6:654-61.

Thrash JC, Cho JC, Ferriera S, Johnson J, Vergin KL, Giovannoni SJ. (2010). Genome sequences of *Pelagibaca bermudensis* HTCC2601^T and *Maritimibacter alkaliphilus* HTCC2654^T, the type strains of two marine *Roseobacter* genera. *J Bacteriol* 192:5552-5553.

Thiele S, Fuchs BM and Amann RI (2011) Identification of Microorganisms Using the Ribosomal RNA Approach and Fluorescence *In Situ* Hybridization. In: Peter Wilderer (ed.) Treatise on Water Science, vol. 3, pp. 171–189 Oxford: Academic Press.

Tyson GW, Chapman J, Hugenholtz P, Allen EE, Ram RJ, Richardson PM *et al.* (2004). Community structure and metabolism through reconstruction of microbial genomes from the environment. *Nature* 428:37-43.

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

Walsh DA, Zaikova E, Howes CG, Song YC, Wright JJ, Tringe SG *et al.* (2009). Metagenome of a versatile chemolithoautotroph from expanding oceanic dead zones. *Science* 326:578-582.

Wrighton KC, Thomas BC, Sharon I, Miller CS, Castelle CJ, Ver Berkmoes NC *et al.* (2012). Fermentation, hydrogen, and sulfur metabolism in multiple uncultivated bacterial *phyla*. *Science* 337:1661-1665.

Wu R, Huijsing JH, Makinwa KAA. (2012). A 21b ±40mV range read-out IC for bridge transducers. *J Solid-State Circuits* 47:2152–2163.

Würgler Hansen J, Thamdrup B, Jørgensen BB. (2000). Anoxic incubation of sediment in gas-tight plastic bags: a method for biogeochemical process studies. *Mar Ecol Prog Ser* 208:273–282.

Yamao F, Muto A, Kawauchi Y, Iwami M, Iwagami S, Azumi Y and Osawa S (1985) UGA is read as tryptophan in Mycoplasma capricolum. *Proc Natl Acad Sci U S A.* 82(8): 2306–2309.

Zhou J, Bruns MA, Tiedje JM. (1996). DNA recovery from Soils of Diverse Composition. *Appl Environ Microbiol* 62:316-322.

Supplementary Material

Due to the large sizes of the supplementary tables (>100,000 rows) this data is not presented in the context of this thesis.

The Supplementary Material for this publication can be found online at: www.frontiersin.org/journal/10.3389/fmicb.2014.00231/abstract

Publication III

Title:

Competition between thermodynamically unsorted redox processes limits productivity of microbial ecosystems

Authors:

Jianwei Chen, Anna Hanke, Halina E. Tegetmeyer, Ines Kattelmann, Ritin Sharma, Emmo Hamann, Theresa Hargesheimer, Beate Kraft, Sabine Lenk, Jeanine S. Geelhoed, Robert L. Hettich, Marc Strous

Status:

Submitted to *Nature*

Own contribution:

AH planned and conducted preliminary experiments and provided training and supervision in continuous cultivation for the final experiments.

Competition between thermodynamically unsorted redox processes limits productivity of microbial ecosystems

Jianwei Chen[1,2], Anna Hanke[1], Halina E. Tegetmeyer[1,3], Ines Kattelmann[3], Ritin Sharma[4,5], Emmo Hamann[1,2], Theresa Hargesheimer[1], Beate Kraft[1,6], Sabine Lenk[1], Jeanine S. Geelhoed[1,7], Robert L. Hettich[4,5], and Marc Strous[1,2,3]

[1]Max Planck Institute for Marine Microbiology, Celsiusstraße 1, D-28359 Bremen, Germany

[2]Department of Geoscience, University of Calgary, T2N1N4, Alberta, Canada

[3]Institute for Genome Research and Systems Biology, Center for Biotechnology, Bielefeld University, Universitätsstraße 27, D-33615 Bielefeld, Germany☐

[4]Chemical Science Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee, TN 37831, USA

[5]UT-ORNL Graduate School of Genome Science and Technology, University of Tennessee, 15 Knoxville, USA

[6]Dept. of Organismic & Evolutionary Biology, Harvard University, Cambridge, MA, USA

[7]NIOZ Royal Netherlands Institute for Sea Research, Yerseke, The Netherlands

Correspondence: mstrous@ucalgary.ca

Abstract

Sorting of redox processes is commonly assumed to optimize the productivity of microbial communities. According to this paradigm, aerobic respiration, denitrification, fermentation and sulphate reduction proceed in a thermodynamically defined order, known as the "microbial redox tower"[1-3]. Recent anomalous observations[4,5] prompted us to address the general validity of the redox tower in controlled laboratory experiments. We subjected aerobic microbial communities sampled from a marine tidal flat to 100 days of natural selection in continuous culture. Redox conditions similar to those of marine oxygen minimum zones and coastal sediments selected for microbial populations that performed aerobic and anaerobic metabolism in parallel in the presence of oxygen, as shown by metagenomics, transcriptomics, proteomics, and stable isotope incubations. Effective oxygen consumption sustained the activity of oxygen-sensitive anaerobic enzymes, thus enabling the coexistence of unsorted redox processes. Genes encoding these anaerobic enzymes were also found to be actively transcribed both in the core of the Peruvian oxygen minimum zone and in an oxic marine sediment. With a stoichiometric model of microbial metabolism, we estimate that the absence of thermodynamic sorting contributes up to 50 % to the total productivity losses of microbial ecosystems. Falsification of the general validity of the redox tower is a major step forward toward understanding of microbial communities.

Main Text

The Wadden Sea is one of the largest intertidal systems of the temperate world[6], to which nutrient import was historically enhanced by human activity[7]. The indigenous microorganisms perform many different reactions in parallel, including aerobic denitrification[4], an anomalous observation in the context of the microbial redox tower. As the starting point for our investigation we sampled the oxic upper 2 cm of an intertidal flat in this ecosystem. Microbial cells were extracted from the sediment and incubated in continuous culture. The culture was provided with a pulse of oxygen two times per day in a mimicked tidal cycle. Artificial seawater containing nitrate (1 mM), nitrite (20 mM), and a mixture of carbon compounds was supplied and removed continuously, so that the actual substrate concentrations in the culture were low (e.g. <5 µM nitrite, <10 µM nitrate) and as close as possible to those experienced by the microorganisms in the natural habitat. The supplied mixture of carbon compounds consisted of glucose (~50 % of the carbon supplied), acetate (~10 %) and seven different amino acids (~40 %). These monomers represented the main classes of molecules set free in approximately the same ratio during the hydrolysis of biomass, the primary carbon and energy source *in situ*.

The culture was run without external interruption for over three months, approximately 37 microbial generations at the dilution rate applied (0.26 day^{-1}). During this time the microbial community was optimized by natural selection. As shown by 16S rRNA gene tag sequencing (Fig. III-1a), microbial populations representing five different phylogenetic clades came to dominate the selected microbial community over the first 50 days. On-going variation in relative abundances during the next 50 days indicated that natural selection did not yield a completely stable community even though the environmental conditions in the culture were stable and nitrite, nitrate, and oxygen were completely consumed at all times, apart from transient responses to diurnal oxygen supply. Relative abundances estimated from 16S tag sequencing were validated with fluorescence *in situ* hybridization (FISH) microscopy on day 83 (Tab. III-1).
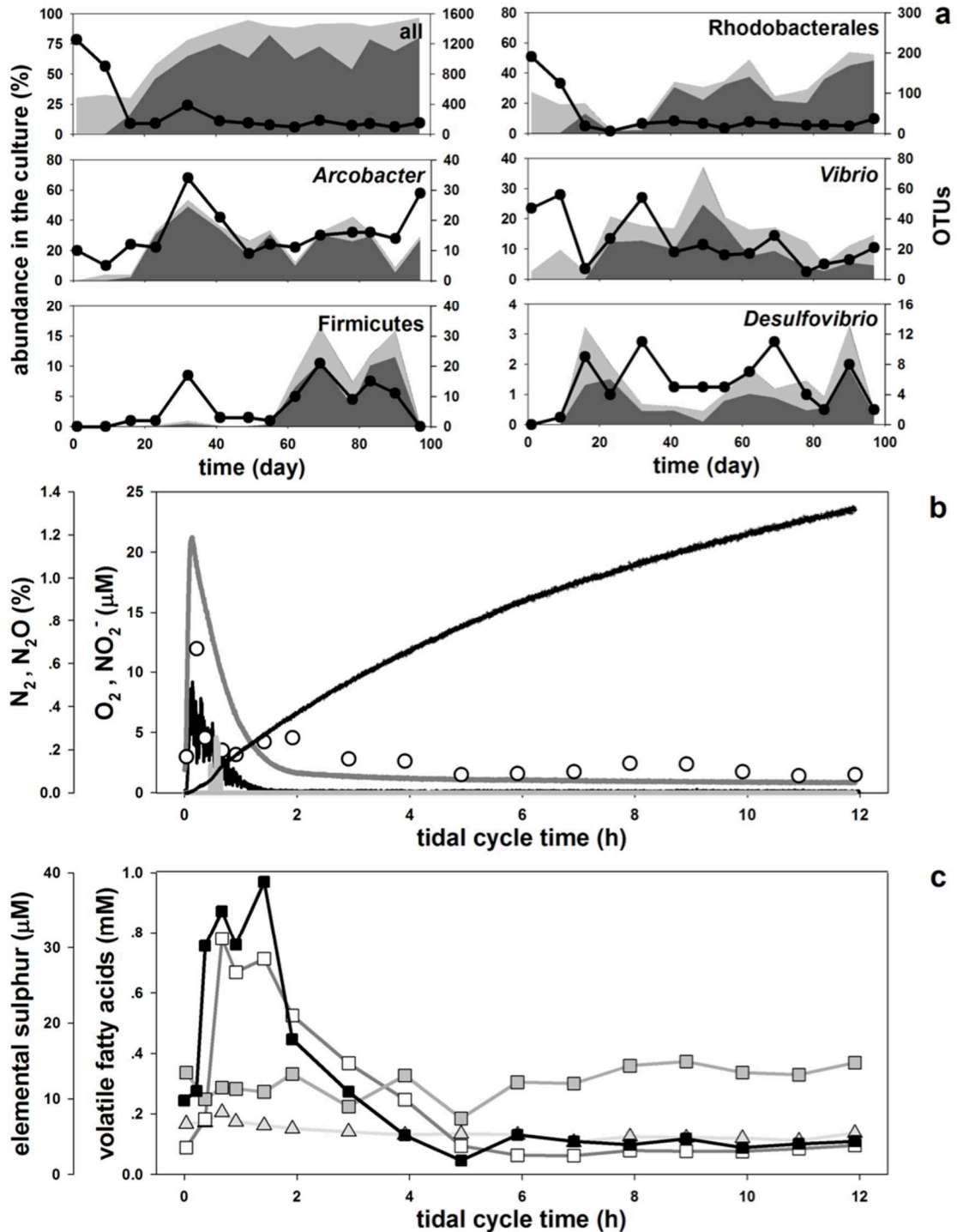
Figure III-1 I Natural selection of a microbial community in continuous culture subjected to tidal cycling. a, Abundances in 16S rRNA tag libraries of the dominant representatives (black areas, 97 % identity cut-off) of the five most abundant clades (grey areas, 92 % identity cut-off) and number of operational taxonomic units (OTUs) within each clade (black circles, 97 % identity cut-off) show selection of clades and of dominant representatives within the clades. **b**, Concentrations of dissolved O_2 (black line showing transient accumulation) and $^{15}NO_2^-$ (circles), and concentrations of O_2 (grey line) $^{30}N_2$ (black line showing continuous accumulation), $^{46}N_2O$ (light grey line) in the recirculating gas during tidal cycling on day 87. **c**, Transient accumulation of formate (open squares), elemental sulphur (solid squares), acetate (grey squares) and succinate (triangles) during the same cycle.

Table III-1 I Abundance estimates and characteristics of metagenomic bins representing the five major clades of the selected microbial community and estimated biomass yields for all clades based on stoichiometric modelling. Bins A-D were obtained directly by assembly and binning. For bins E and F assembly was generally unsuccessful and numbers are shown in parentheses and refer to genome data of closely related species (based on the assembled 16S rRNA genes shown in Figure III-3) that were also used as a template for mapping the transcriptome reads.

Bin	Total	A	B	C	D	(E)	(F)
Affiliation	-	Rhodo-bacter-ales	Arco-bacter	Arco-bacter	Vibrio-nales	Desulfo-vibrio salexi-gens	Firmi-cutes
Genome size (Mb)	17.9	3.86	3.29	2.38	5.19	(4.29)	(13.8)
Number of contigs (#)	20,071	295	2,230	4,260	6,844	(1)	(499)
N50 contig length (kb)	0.93	19.7	2.9	0.65	1.1	4.29	73.9
GC content (%)	35.4	57.5	26.6	29.1	43.2	47.1	33.7
Number of CSCGs (#)*	438	135	123	92	154	(132)	(291)
Number of tRNAs (#)	165	41	38	12	77	(89)	(137)
Metawatt bin	-	L0,L1	L2,L6**	L5**	L3,L4	-	-
Abundances in culture (%)							
16S rRNA sequencing (days 83,90,97, 95% identity threshold)	93 ± 4	49 ± 8	23 ± 13	0.9 ± 0.5	10 ± 5	1.5 ± 1.5	9 ± 8
DNA (day 83)	58.7	34.7	14.4	0.7	4.9	0.5	3.6
DNA (day 90)	61.3	29.0	7.9	5.4	12.9	1.2	4.8
DNA (day 97)	65.6	41.9	14.3	2.7	6.2	0.5	0.0
RNA1 (oxic, day 87)#	82.8	24.4	24.8	8.3	8.4	2.9	7.8
RNA2 (anoxic, day 87)#	80.4	23.4	15.4	12.4	10.2	4.6	8.2
RNA3 (anoxic, suspended,day 84)#	84.3	21.1	7.0	1.8	32.7	3.3	5.4
RNA4 (oxic, suspended, day 84)#	88.1	25.3	18.0	3.5	17.4	4.3	5.9
DNA (normalized, average)	100	57 ± 10	20 ± 6	5 ± 4	13 ± 7	1.2 ± 0.7	5 ± 4
RNA (normalized, average)	100	28 ± 2	20 ± 9	8 ± 6	21 ± 13	5 ± 1	8 ± 2
Proteome (day 83)	100	47 ± 0.4	23 ± 0.2	4 ± 0.0	8 ± 0.1	0.1 ± 0	18 ± 0.1
FISH (day 83)	100	64 ± 7	13 ± 2		16 ± 2	3.8 ± 0.4	2 ± 0.2
Experimental averages%	100	50 ± 13	17 ± 7		14 ± 5	2 ± 2	9 ± 6
Model predictions	100	48 ± 7	23 ± 3		18 ± 3	1 ± 0.2	10 ± 2
Yield estimates (% of carbon assimilated)							
Experimental (day 49-83)&	37 ± 8						
Model predictions	51 ± 8	37 ± 5	25 ± 4		11 ± 2	N/A	16 ± 2
Model predictions (pure culture)%	60						
Biomass protein content (% w/w)							
Experimental (day 49-83)	32 ± 9						
Model predictions%	37						

*) Number of Conserved Single Copy Genes detected (out of a set of 139). Numbers higher than 139 may indicate the presence of DNA originating from more than a single population in the bin. Numbers lower than 139 indicate the provisional genome sequence associated with the bin may be incomplete.

**) Cross binning between bins B and C was corrected with the GC vs. coverage plot.

#) Transcriptomes of day 87 were obtained directly from the culture; samples of day 84 were obtained from secondary anaerobic and aerobic batch incubations with filtered biomass from the culture (suspended cells).

&) Based on measurement of protein and particulate organic carbon concentration measurements in the culture.

%) Averages of estimates based on DNA, RNA, proteomes, 16S rRNA tag sequencing and FISH.

N/A, not applicable.

The overall communal metabolism of the selected microbial community was determined by monitoring concentrations of carbon, nitrogen, and sulphur compounds during tidal cycling (Fig. III-1b). To more precisely quantify nitrogen conversions, we supplied medium with ^{15}N-labelled nitrite during these experiments and measured the gaseous products with real time mass spectrometry. The dissolved oxygen concentration of the culture liquid was monitored on line with an optical oxygen probe. After oxygen was supplied at the start of a tidal cycle, transient accumulation of the denitrification intermediate nitrous oxide (^{46}N$_2$O) was observed, but nitrogen (^{30}N$_2$) was always the main product of denitrification. Apparently, denitrification to N$_2$ proceeded even in the presence of oxygen, as was reported for the intertidal flat itself[4].

Interestingly, tidal cycling also resulted in the transient accumulation of formate (0.8 mM) and elemental sulphur (30 μM) during the oxic periods (Fig. III-1c). This was counter-intuitive because thermodynamic sorting according to the microbial redox tower should not result in the accumulation of reduced products in the presence of excess oxygen. The production of formate and succinate suggested that fermentative microorganisms were present and that these organisms were active also during oxic periods. The presence of reduced sulphur could only be explained by the presence and activity of sulphate reducing bacteria. Indeed, also the tag sequencing results provided strong evidence for the presence of populations of potentially fermentative *Firmicutes* and of a population closely related to *Desulfovibrio salexigens,* a known sulphate reducer (Fig. III-1a).

To determine the ecological roles of the identified populations, DNA was extracted on days 83, 90, 97 and subjected to next generation sequencing. The sequencing reads were assembled and binned (Fig. III-2, Tab. III-1), resulting in a provisional whole genome sequence for the dominant representative of three of the five major clades shown in Figure III-1a. Two different populations of *Arcobacter* were assembled (bins B and C). For each clade, the expression of the predicted genes was determined with proteomics and the effect of the tidal cycling on protein expression was analysed with transcriptomics. For all bins, a complete 16S rRNA gene could be assembled that was nearly identical to the 16S tag sequences of the dominant representatives of each clade shown in Figure III-1a (Fig. III-3).
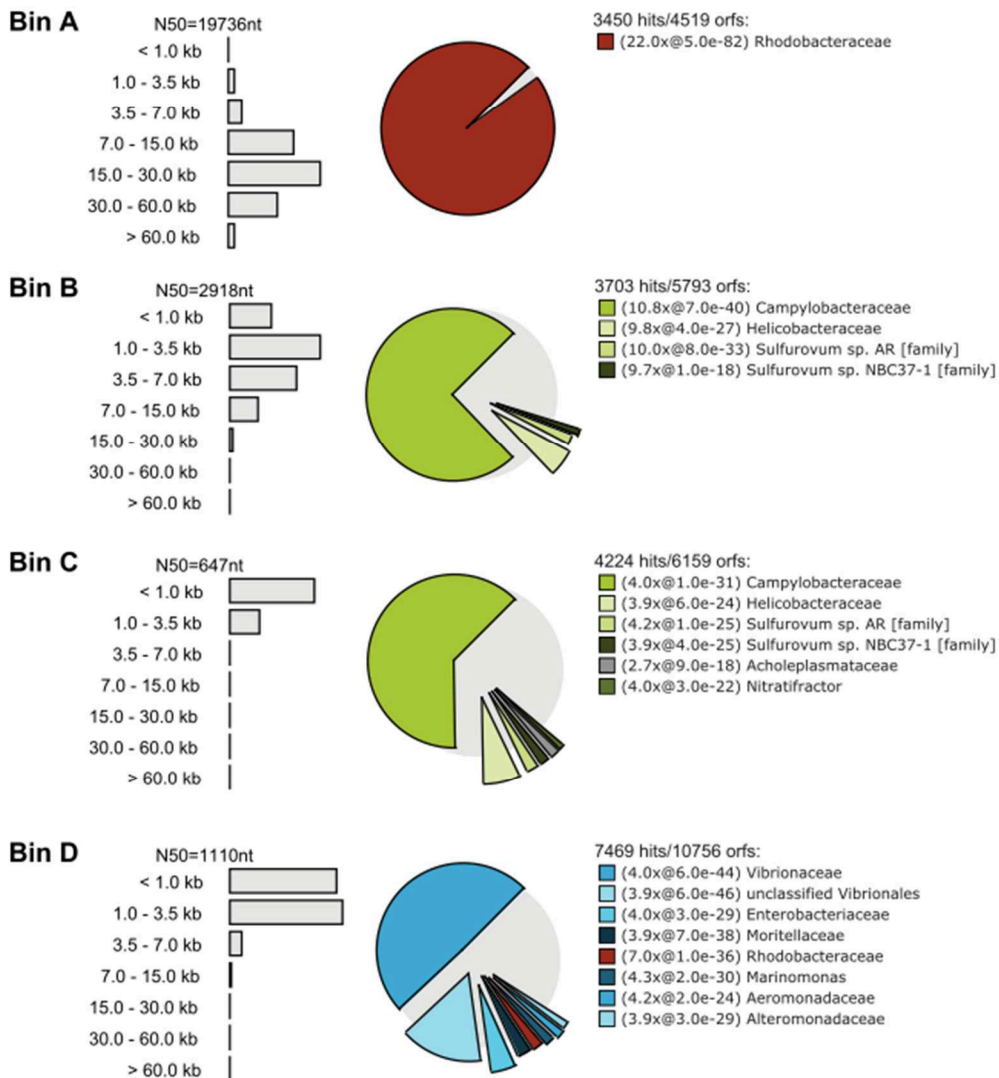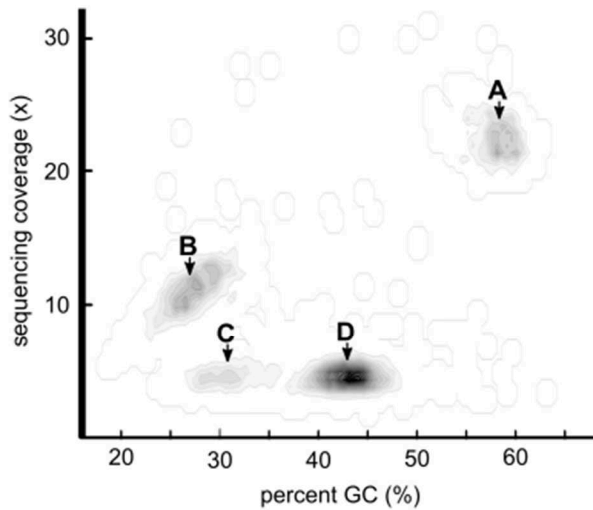
Figure III-2 I Scattering of the contigs of the four assembled and binnable populations on a GC *vs.* sequencing coverage plot and phylogenetic profiles for each of the bins. For each bin, the length distribution of contigs is shown and the pie diagrams show the phylogenetic distribution of BLAST hits of all predicted open reading frames for each bin.

The detected peptides and transcripts suggested that the supplied carbon sources were fermented by the *Vibrio* and *Firmicutes* populations (bins D and F, Fig. III-4). These populations apparently converted glucose and the amino acids alanine and serine into formate, acetate and hydrogen, as shown by elevated transcriptional activities of glycolysis, alanine dehydrogenase, hydrogen-evolving hydrogenases and pyruvate-formate-lyase, a glycyl radical enzyme extremely sensitive to oxygen. Because of the high diversity within the *Vibrio* clade (Fig. III-1a), assembly was less successful for this clade and presence and activity of key genes was validated by BLASTing raw sequencing reads directly to a reference genome (*Vibrio tubiashii*). Gene activities in bins D and F were consistent with the fermentation of the other supplied amino acids into succinate, which was also detected experimentally in the culture liquid (Fig. III-1c). At the same time, the *Vibrio* population respired oxygen with a *bd*-type terminal oxidase and potentially converted nitrate to nitrite and nitrous oxide to N_2. The expression of these enzymes may have served two functions: to protect the oxygen-sensitive pyruvate-formate-lyase and to dispose of excess reducing equivalents during fermentation.

The *Desulfovibrio* population (bin E) also actively expressed a *bd*-type terminal oxidase. The transient accumulation of formate during periods of oxygen excess (Fig. III-1b) suggested that with its *bd*-type terminal oxidase, the formate-producing *Vibrio* population outcompeted the formate-consuming *Arcobacter* populations (bins B and C) for oxygen. The *Rhodobacterales* (bin A) and *Arcobacter* populations apparently performed aerobic respiration and denitrification in parallel; both made use of a *cbb₃*-type terminal oxidase. *Rhodobacterales* apparently converted nitrite into nitrous oxide and *Arcobacter* was only capable of nitrate and nitrous oxide reduction. Gene activities suggested fermentation products such as dicarboxylates (e.g. succinate), acetate, formate and hydrogen were used as electron donors (Fig. III-4) but at least the *Rhodobacterales* population also appeared to compete for glucose. The *Arcobacter* populations could apparently only oxidise sulphide to elemental sulphur, which accumulated in the medium and as a film on the glass walls of the culture vessel.
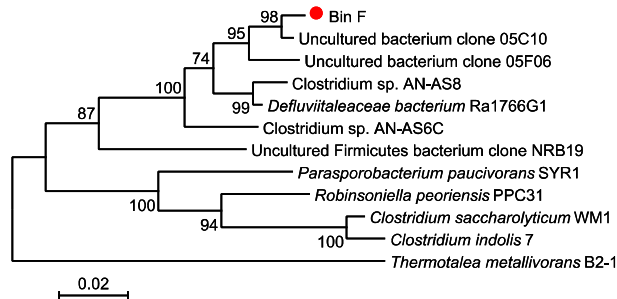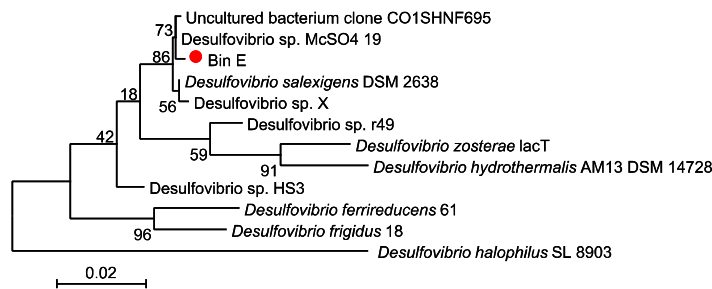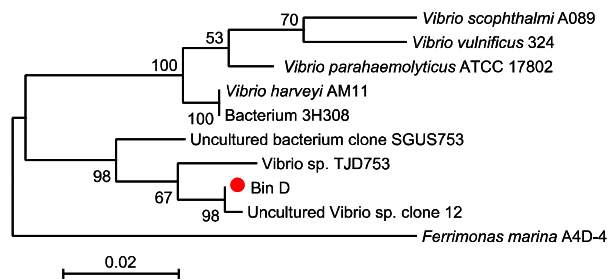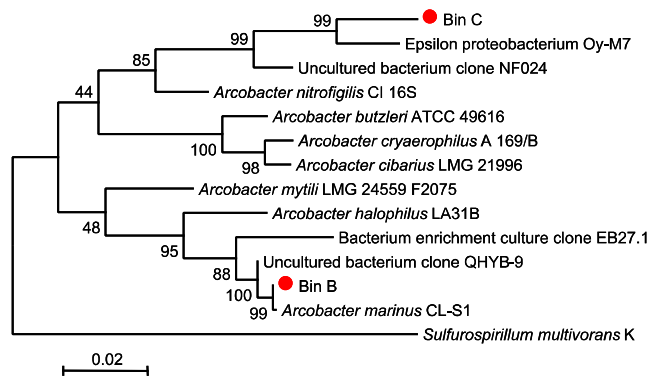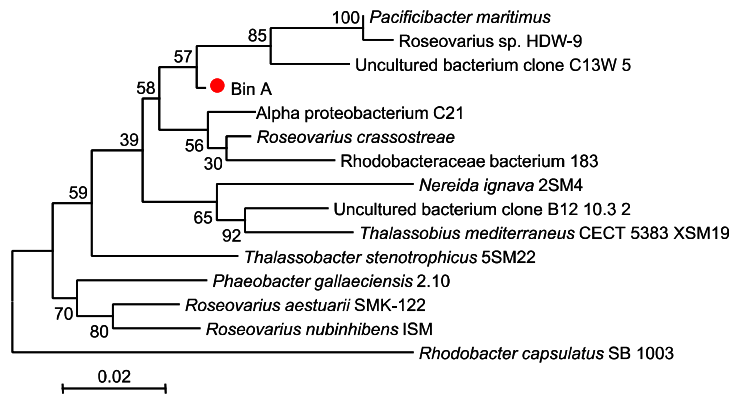
Figure III-3 I Phylogenetic affiliation of each of the dominant representatives of the five major clades, based on phylogenetic analysis of 16S rRNA genes. Bin identifiers are shown for each gene.

All populations naturally selected, except the *Firmicutes*, followed the same ecological strategy: They performed aerobic and anaerobic metabolism simultaneously; effectively consuming oxygen, enabling the activity of oxygen sensitive enzyme systems such as pyruvate-formate-lyase and nitrous oxide reductase. However, a simple mass balance over a microbial cell shows that a perfectly suspended bacterial cell cannot effectively deplete oxygen by respiration, unless the oxygen concentration is in the low nanomolar range. At oxygen concentrations above 25 µM (10 % air saturation), micro-aggregates of at least 11-24 µm would be necessary to completely consume the oxygen in the immediate surroundings of the cells, a prerequisite for anaerobic metabolism.

	A	DE	B,C	F	
cytochrome oxidase (bd)	-	4.1	0.5	0.1	-
cytochrome oxidase (cbb3)	3.6	-	-	5.9	-
nitrate reductase	0.1	0.3	0.4	4.8	-
nitrite reductase	7.1	-	-	-	-
nitrous oxide reductase	1.4	0.1	-	4.6	-
sugar uptake	2.1	1.3	-	-	12.0
dicarboxylate uptake	2.6	-	-	2.6	-
acetate activation	1.4	-	0.1	2.9	-
pyruvate formate lyase	-	1.8	0.1	-	9.6
sulfide dehydrogenase	-	-	-	1.1	-
dissimilatory sulfite reductase	-	-	1.3	-	-
formate dehydrogenase	0.3	-	0.3	18.3	-
hydrogenase	0.4	0.1	2.1	1.6	1.5
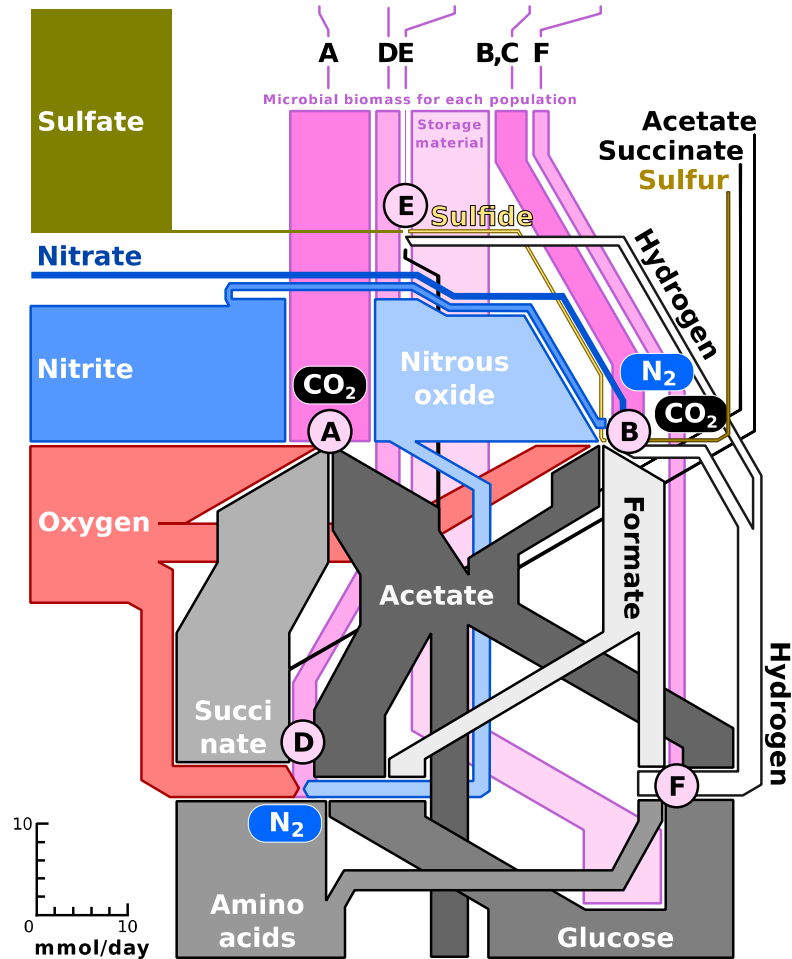
Figure III-4 I Unravelling of communal metabolism based on gene activities, protein expression and mathematical modelling. The metabolic network was calculated with a stoichiometric model of communal metabolism based on the transcriptomic and proteomic analyses presented in the Supplementary Information. Each population (bins A-F) is shown as a node (pink circles) in the network, with arrows showing the conversion of substrates into products by each population. The conversion of the supplied carbon and energy sources is shown from bottom to top and the conversion of the supplied electron acceptors is shown from left to right. Pink arrows show the amount of biomass produced for each population (Tab. III-1) and the amount of storage materials produced, which was not assigned to any specific population. Production of the end-products nitrogen and carbon dioxide is indicated for each node and the experimentally determined amounts of remaining acetate, succinate and elemental sulphur are indicated. Width of all arrows is proportional to the calculated elemental flows (mmol C, O, N, S day^{-1}, horizontal and vertical scale-bar in lower left corner). The table shows transcriptome-derived gene activities of key metabolic modules for the six abundant clades (bins A-F). Bold numbers indicate that expression was detected experimentally by proteomics. Red and blue numbers indicate up regulation in the presence and absence of oxygen respectively.

Microscopic investigation showed that the selected cells formed such micro-aggregates (Fig. III-5a). To further investigate whether these micro-aggregates were essential for the activity of oxygen-sensitive enzyme systems, the culture was filtered through a 5 µm membrane filter and the filtrate, containing only suspended bacterial cells (Fig. III-5b), was incubated with the carbon substrates and ^{15}N-labelled nitrite, at different oxygen concentrations. Transcriptomic analysis showed that among the five most abundant clades (Fig. III-1a) the *Arcobacter* populations were ~2x less abundant in the cell suspensions, whereas the *Vibrio* populations were ~3x more abundant and other clades showed no difference (Tab. III-1). With suspended cells, complete denitrification was no longer observed in the presence of oxygen and was completely absent at oxygen concentrations between 25 µM and 125 µM (Fig. III-5c). Interestingly, above 125 µM, aerobic denitrification was again observed but with nitrous oxide rather than nitrogen as the end-product.

This unexpected result could be explained as follows: Firstly, the simultaneous transcription and expression of the *cbb$_3$* terminal oxidase and the nitrate, nitrite, nitric oxide and nitrous oxide reductases indicated that these enzyme complexes were competing for electrons in a branched respiratory chain, as was previously described[8]. Indeed, it was shown that in the presence of nitrate, the oxygen uptake rate was reduced (Fig. III-5d). Secondly, the *cbb$_3$* terminal oxidase usually sustains micro-aerophilic metabolism[9] so it might be inhibited at high oxygen concentrations. Indeed such inhibition was observed here (Fig. III-5e). Thus, at high oxygen concentrations (above 0.15 mM), the denitrification enzymes became the prevalent electron sink of the respiratory chain. Finally, because nitrous oxide reductase is the only intrinsically oxygen sensitive enzyme of the denitrification pathway, nitrous oxide was the end product of denitrification at high oxygen concentrations.
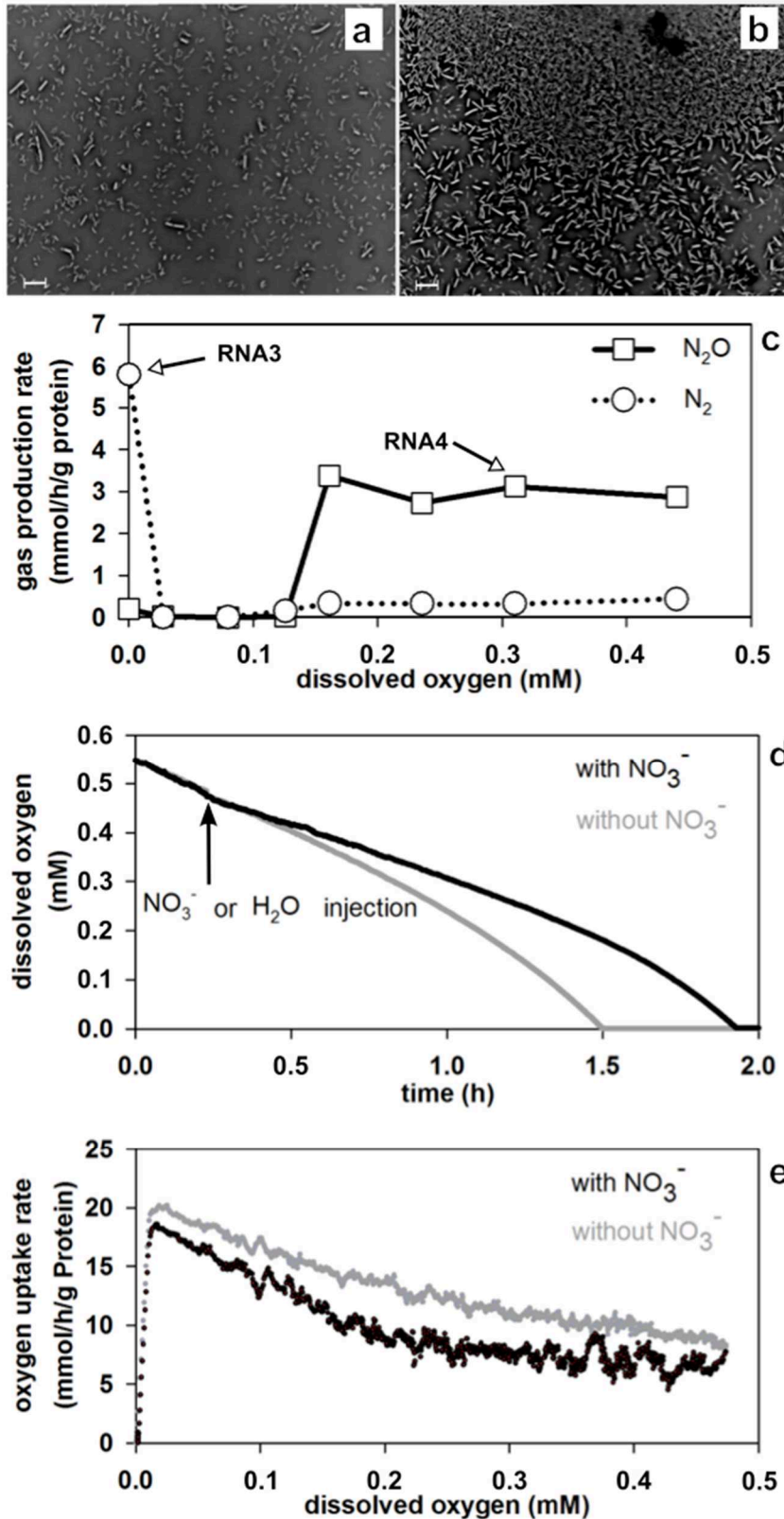
Figure III-5 I Aerobic denitrification by suspended bacteria. **a**, **a**ggregated cells sampled from the culture. **b**, suspended cells obtained by filtration. **c**, production of nitrogen (circles) and nitrous oxide (squares) as a function of oxygen concentration. **d**, inhibition of oxygen consumption after injection of 0.8 mM nitrate (arrow, black line) compared to control (grey line), **e**, inhibition of oxygen consumption as a function of oxygen concentration in the presence (black) and absence (grey) of nitrate. Scale bar 5 μm.

132

Together, our results show that the parallel occurrence of aerobic and anaerobic processes within a single cell combined with the production of micro-aggregates was a successful ecological strategy that led to a loss of thermodynamic sorting. In theory this could compromise the overall productivity of a microbial ecosystem, mainly because fermenting bacteria hydrolyse carbon-carbon bonds that need to be reformed during assimilation by respiratory bacteria. Indeed the experimentally determined growth yields were low and decreased from 0.44±0.03 to 0.32±0.02 C-mol assimilated/C-mol consumed during natural selection from day 49 to 83. For comparison, growth yields of up to ~0.7 C-mol assimilated/C-mol consumed have been reported for pure cultures oxidizing glucose with oxygen or nitrate[10]. Yields as low as 0.3 C-mol/C-mol have also been reported for denitrifying cultures oxidizing acetate[11] but these values are not relevant to the present study, because in our case glucose and amino acids were the main electron donors and aerobic respiration was as important as denitrification (Fig. III-4). The observed low growth yields were reproduced in two additional continuous culture experiments performed with identical conditions for the same amount of time (100 days, 0.22±0.06 C-mol assimilated/C-mol consumed). The potential productivity loss was quantified by mathematical modelling using metabolic constraints that are conserved across the domains of life. Given a known electron donor and acceptor pair and known carbon sources for growth, the growth yield of a bacterium can be predicted with reasonable accuracy (15 %)[9]. We used the experimentally determined gene expression patterns and the observed substrate turnover rates to mathematically estimate the relative abundances of the populations of the five major clades, the productivity of the community as a whole, and the theoretical productivity for a thermodynamically sorted scenario. Production of intracellular storage materials in the form of glycogen was added to the model, because without storage materials the experimentally observed oxygen consumption could not be explained. The experimentally determined protein content of the cells was low, only 32±9 % w/w, compared to 50-70 % observed for pure cultures without storage materials and supported the presence of storage materials. The model predicted a productivity of 0.51±0.08 C-mol assimilated/C-mol consumed, significantly higher what was measured experimentally (0.37±0.08).

The relative abundances of the five major selected clades were consistent with the experimentally resolved values (Tab. III-1 and Supplementary Information). The model predicted a productivity of 0.60±0.09 C-mol/ C-mol for the thermodynamically sorted case, indicating that the lack of thermodynamic sorting explained approximately 50 % of the difference between the observed low productivity of the selected microbial community and the higher produc-tivity of pure cultures. Other dissipative processes such as motility and viral predation likely also contributed to the low productivity (Supplementary Tab. S2-S7).

Finally, we investigated whether the dissipative ecological system that resulted from natural selection in the laboratory might also occur in nature. For this, we re-inspected available metagenomes and metatranscriptomes from the Peruvian oxygen minimum zone[12] and our sampling site, an intertidal flat of the Wadden Sea (Fig. III-6), and recorded the abundance and transcriptional activity of genes encoding key anaerobic dissipative enzymes. In the continuous culture, the ratio of genes encoding pyruvate-formate-lyase and *bd* terminal oxidases to genes encoding the heme copper oxidases of complex IV was approximately 1:10. Both in the core of the oxygen minimum zone and in the oxic upper tidal flat sediments, the ratio was above this value, and both genes were found to be actively transcribed. This indicated that also *in situ* aerobic and anaerobic metabolism proceed in parallel, as suggested by previous studies[4,5]. Phylogenetic analysis of the genes detected in nature showed that in both habitats fermentation was mainly performed by polymer degrading *Flavobacteria*[13] rather than marine *Vibrionales* that were selected by the supply of monomers.
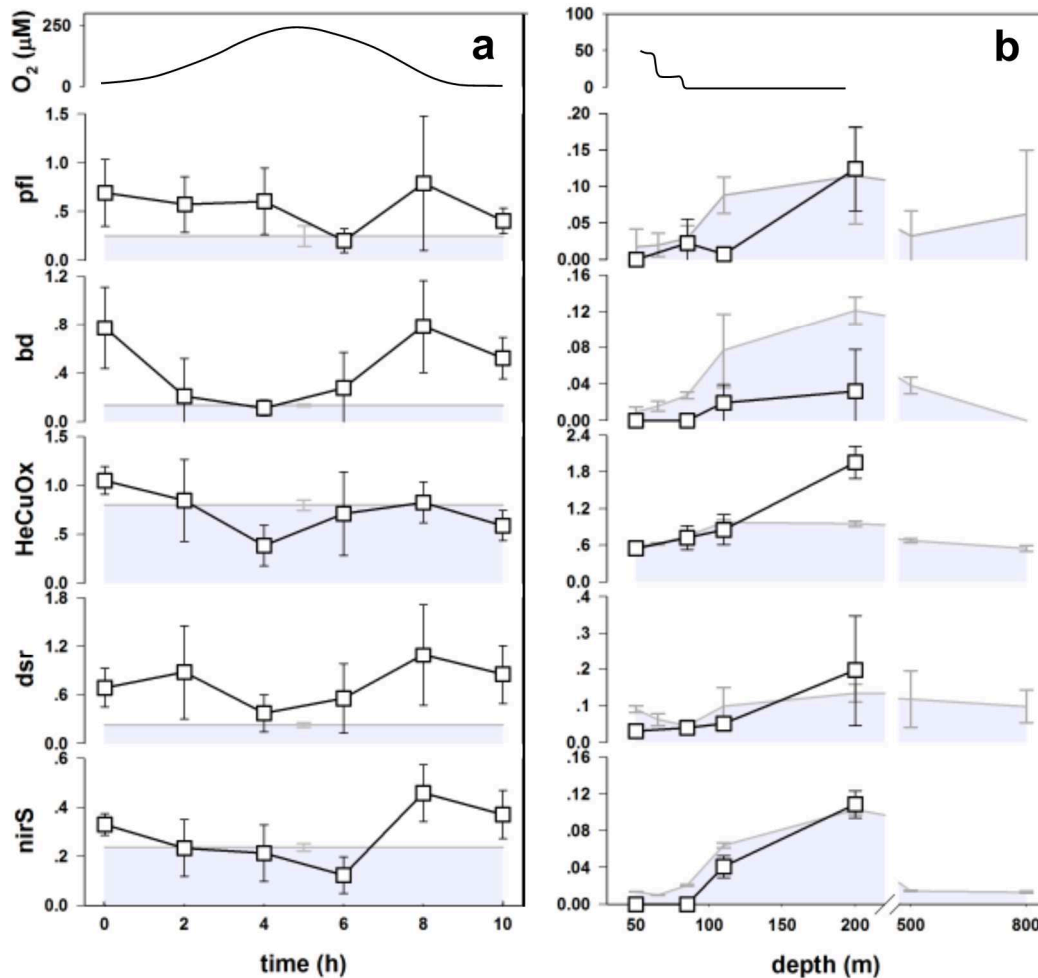
Figure III-6 I Abundance (shaded areas) and activity (lines) of anaerobic genes in the environment. **a**, oxic marine sediment used as inoculum for the continuous cultures. **b**, Peruvian oxygen minimum zone[9a]. *pfl*, pyruvate-formate-lyase, *bd*, *bd*-type terminal oxidase, HeCuOx, heme copper oxidases. *dsr*, dissimilatory sulfate reductase, *nirS*, *cd*-type nitrite reductase. Error bars indicate standard deviations.

A lack of thermodynamic sorting in the form of aerobic fermentation was previously observed for pure cultures of bacteria, industrial yeast production, and cancer cells. In all these cases, the cells experience high substrate concentrations not relevant to the natural environment. Our study shows how low substrate concentrations can also select for microbial communities that perform unsorted redox processes in parallel. We also show that this leads to a loss of ecosystem productivity and, potentially, nitrous oxide emissions. Productivity is often assumed to be optimized by natural selection and to be positively correlated to biological diversity. This hypothesis was falsified; natural selection led to the coexistence of aerobic and anaerobic metabolism without thermodynamic sorting, at the price of reduced productivity.

Material & Methods

Sampling, cell extraction, continuous cultivation, and on-line mass spectrometry.

Sediment samples for incubations were taken from an intertidal sand flat (low tide) in the central German Wadden Sea (N53°44.151', E007°41.943') on November 1st, 2011 and bacteria were extracted from the sediments and cultivated in continuous culture as previously described[14]. The filter-sterile (0.2 µm, Sartopore MidiCaps, Sartorius, Göttingen, Germany) medium contained (mM): $NaNO_2$ 20.0, $NaNO_3$ 1.0, $FeSO_4$ 0.01, KH_2PO_4 0.50, $CuCl_2$ 0.0013, glucose 6.0, acetate 3.1, glutamate 1.1, aspartate 1.5, alanine 1.5, serine 0.80, tyrosine 0.51, histidine 0.12 and methionine 0.25, 33.4 g L^{-1} Red Sea Salt (Aquaristic.net) and 0.2 mL L^{-1} trace element solution[11] was added. Pure oxygen was supplied at 20 mL/min for 5 min, once every 12 h (one tidal cycle). Off gas was led to a GAM 400 mass spectrometer (In Process Instruments, Bremen, Germany) for on-line detection of O_2, Ar, N_2 and N_2O after replacing the nitrite in the medium with its ^{15}N-labelled form.

Incubation of suspended cells

Incubations were performed in the dark in 200 mL serum flasks stirred at 300 rpm with a magnetic stirrer bar and fitted with optical oxygen sensors (FSO2-4, Pyroscience, Aachen, Germany). A final concentration of 1.0 mM ^{15}N-nitrite or nitrate and 0.30 g HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) were added to the medium described above. The oxygen concentration was set by adjusting the O_2/Ar ratio in the headspace, and validated mass spectrometrically (GAM 400, In Process Instrument, Bremen, Germany). The cells sampled from the culture were injected through a 5 µm syringe filter (pore). Gas and liquid samples were taken once every 20-30 min for 3 h. In control experiments for nitrate inhibition of aerobic respiration, water was injected instead of nitrate.

Chemical analysis

Presence or absence of nitrite and nitrate was determined with Quantofix test strips (0-80 mg L^{-1} NO$_2^-$, 0-500 mg L^{-1} NO$_3^-$, Merck, Germany). Nitrite, ammonium and protein and sulphur concentrations were quantified as previously described[15-17]. Fatty acids were quantified with high performance liquid chromatography (HPLC)[18]. Dissolved oxygen was measured using an optical oxygen meter (FSO2-4, Pyro-Science, Germany). Particulate organic carbon was determined with cuvette-tests (Hach Lange GmbH, Düsseldorf, Germany) in a Thermostat LT200 and a DR3900 photometer (Hach Lange GmbH, Düsseldorf, Germany). Protein content of cells was calculated from protein and particulate organic carbon measurements.

16S rRNA gene tag sequencing

For 16S rRNA gene tag sequencing, primers S-D-Arch-0519-a-S-15 and S-D-Bact-0785-b-A-18 covering the v4 region[19] were used and extended with MIDs of 7 to 10 bp in length for multiplexing. PCR was performed using 25-125 ng template DNA and the Phusion® High-Fidelity PCR Master Mix (Finnzymes) with the following protocol: 30 sec initial denaturation (98 °C), 30 cycles of 10 sec denaturation (98 °C), 30 sec primer annealing (66 °C) and 12 sec extension (72 °C), and a final extension step at 72 °C for 10 min. The amplicons (approx. 300 bp in length) were purified and pooled, an end repair step was performed, adapters for PGM sequencing were ligated and the resulting ion torrent libraries were sequenced on the PGM platform using 400 bp read chemistry. A total of 14 samples taken at different time points were sequenced yielding 729,581 reads. After demultiplexing and filtering with trimmomatic[20] (quality >20) and mothur[21] (bdiffs=1, pdiffs=2, minlength=250, maxlength=350, maxambig=0, maxhomop=8), 349,170 reads remained. These were classified to the assembled 16S rRNA gene sequences, one for each bin, with usearch[22] at 92 % and 97 % identity thresholds. Numbers of Operational Taxonomic Units (OTUs) were determined for each sample and each clade by clustering with usearch at 97 % identity.

Next generation sequencing, proteomics and in silico procedures

RNA samples were fixed immediately with RNAlater® (Ambion). 2 mL culture were used to extract DNA or RNA[23,24]. The isolated DNA and RNA were sequenced with the Ion Personal Genome Machine® (PGM™) System (Life Technologies) on 316 and 318 chips respectively. The combined reads of all three DNA samples were assembled from the sff-files generated by the Torrent Suite software 2.0.1 with the GS De Novo Assembler 2.6 (454 Life Sciences, Branford, CT, default settings for genomic DNA). To recover 16S rRNA genes, an additional assembly was performed[25]. Briefly, the cDNA option was selected during sff-files loading, the 'Minimum overlap identity' was set to 99 %, and 'Extend low depth overlaps' as well as 'Reads limited to one contig' were selected. Contigs were binned as previously described[14,26] and yielded 4 bins representing 3 of the 5 abundant clades. Detection and phylogeny of 16S rRNA genes was performed as previously described[14]. Abundances of all clades were estimated based on sequenced DNA and RNA by mapping sequencing reads to the assembled contigs with BBMap (http://sourceforge.net/projects/bbmap/, k 13, minid 0.6). For the two clades that assembled poorly, genomes of closely related bacteria were used as the template for mapping (Genbank CP001649 and BioProject PRJNA246767/Bin A). The assembled contigs were annotated separately for each bin with Prokka[27]. Transcriptional per-gene activities were computed for all predicted open reading frames (ORFs) from the mapped transcriptomes by dividing (number of reads mapped to ORF/ORF length) by (total number reads mapped to all ORFs in bin/total length of all ORFs in bin). This way, a transcriptional activity of 1.0 corresponded to the average transcriptional activity. For Bin D (*Vibrionales*), less conserved regions of some key genes were assembled incompletely. For those genes, gene completeness was validated independent of assembly, by performing BLASTx of all reads (both transcriptomes and metagenomes) against the genome of a related reference organism (*Vibrio tubiashii*). RNA was extracted from the culture at 2 different time points during tidal cycling shown in Fig. III-1, RNA1 at 0.3 h, at the peak of the oxygen profile and RNA2 at 2 h, the base of the oxygen profile, and from anoxic (RNA3) and oxic (RNA4) incubations of filtered cells shown in Fig. III-5, at 0.0mM and 0.3 mM O_2, respectively.

138

Proteomic analyses of two technical replicates were performed as previously described[14]. Population abundances estimated from the proteomic results were estimated by counting unambiguously identified peptides for each bin. Sequencing reads from metagenomes and transcriptomes from the Peruvian Oxygen Minimum Zone[12] (www.ncbi.nlm.nih.gov/Traces/sra/?study=SRP003331) and the Janssand tidal flat (Short Read Archive SRP021900) were translated in six frames and scanned for key functional genes with Hidden Markov Models constructed from aligned reference amino acid sequences, one for each gene (*pflAB*, *bdI*, *bdII*, the heme copper oxidase superfamily, *dsrAB*, *nirS* and *rpoBC*) with hmmscan (version 3.0) with an e-value cut off of $1e^{-10}$. Read counts for each functional gene were normalized against counts for *rpoBC*. Gene abundances were inferred from metagenomes, gene activities from transcriptomes.

Mass balancing of substrate consumption and supply for suspended cells and micro-aggregates

The oxygen flux *J* towards a single cell or aggregate of cells is:

$$J = 4\pi DR ([O_2]_{bulk} - [O_2]_{cell}) \qquad (2)$$

With *D*, the diffusion coefficient (10^{-9} m^2 s^{-1}), *R*, the radius of a static boundary layer surrounding the cell or aggregate ($>10^{-5}$ m, the Kologorov scale in turbulent systems), *[O_2]_{bulk}*, the oxygen concentration as measured in the liquid and *[O_2]_{cell}*, the concentration of oxygen at the cell or aggregate surface. When the oxygen is depleted at the cell surface (*[O_2]_{cell}* = 0), the oxygen uptake rate of the cell or aggregate should be equal to *J*. The average oxygen uptake rate of the cells in the continuous culture was at most 20 mmol g^{-1} protein h^{-1} (Fig. III-5e), or, depending on the cell diameter, at most 25 fmol cell^{-1} day^{-1} for spherical cells 1 µm wide.

Fluorescence in situ hybridization (FISH)

Fluoresence *in situ* hybridization ((MONO-)FISH) and catalysed reporter deposition-FISH (CARD-FISH) were performed as previously described[28,29]. The probes and formamide concentrations used are shown in Table III-2.

Table III-2 I Probes and formamide concentrations used for fluorescence *in situ* hybridization (FISH). FISH was performed both with and without catalyzed reporter deposition.

Probe	Sequence (5′→3′)	Target organisms	FA (%)	Ref
EUB338 I[2]	GCTGCCTCCCGTAGGAGT	Most Bacteria	35[1,2]	Amann *et al.*, 1990
NON338	ACTCCTACGGGAGGCAGC	Control	35[1,2]	Wallner *et al.*, 1993
GRB	GTCAGTATCGAGCCAGTGAG	*Rhodobacter*	25[1]	Eilers *et al.*, 2000
GV	AGGCCACAACCTCCAAGTAG	*Vibrio*	30[1]	Eilers *et al.*, 2000
ARC94	TGCGCCACTTAGCTGACA	*Arcobacter*	25[2]	Snaidr *et al.*, 1997
ARC1430	TTAGCATCCCCGCTTCGA	*Arcobacter*	25[2]	Snaidr *et al.*, 1997
DSV698 (& competitor)	GTT CCT CCA GAT ATC TAC GG (GTT CCT CCA GAT ATC TAC GC)	Some *Desulfovibrio*, *Bilophila wadsworthia*, *Lawsonia intracellularis*	50[1]	Manz *et al.*, 1998
CLO864	TTCCTCCTAATATCTACGCA	*Clostridia*	30[1]	Kraft *et al.*, 2014
ALT1413	TTT GCA TCC CAC TCC CAT	*Alteromonas, Colwellia*	40[2]	Eilers *et al.*, 2000
PS440LP	CCCTTCCTCCCAACTT	Pseudomonads	35[2]	Lenaerts *et al.*, 2007
PSA184	CCC CTT TGG TCC GTA GAC	*Pseudoalteromonas, Colwellia*	40[2]	Eilers *et al.*, 2000

[1]*Formamide concentration in the CARD-FISH hybridization buffer*

[2]*Formamide concentration in the MONO-FISH hybridization buffer*

Stoichiometric modelling of metabolism

Nineteen chemical reactions were defined that described the major chemical reactions performed by the five clades dominating the culture as inferred from the transcriptomes and proteomes. Bin A was modelled to perform five chemical reactions, bins B and C seven, bin D four, and bins E and F one (Supplementary

Tab. S11). Production of glycogen (storage material) was added as a final reaction that was not attributed to any of the clades because it was unknown how much individual clades contributed to storage materials. Each reaction is the sum of a catabolic reaction and an anabolic reaction and is shown on a single row of the table. The table shows the stoichiometric factor for each compound (listed on the first row), with consumption as a negative value and production as a positive value. The reactions were calculated from the Gibbs free energy change of the catabolic reaction, the anabolic reaction and the estimated energy dissipated during anabolism as previously described[9]. Gibbs free energy changes were calculated at the environmental conditions prevailing in the culture. Energy dissipated in respiratory catabolism was corrected based on the degree of coupling known from biochemical characterization of the relevant enzyme complexes – the enzyme complexes of denitrification and the *bd*-type terminal oxidases translocate fewer protons than the complexes of the canonical aerobic respiratory chain. For each reaction, elemental and charge balances are shown in Supplementary Tab. S11 columns AG – AM. Rates were then estimated for each of the nineteen reactions so that the overall conversion for each compound matched the experimental values. The widths of the arrows in Figure III-4 are based on these rates. Significant differences between the model predictions and the experimental mass balances are shown in red. For each clade, the estimated overall biomass yield (C-mol assimilated/C-mol consumed) calculated from the individual reactions is shown in bold in column E. The predicted community composition is shown in column G (bold, %). These values have relative standard deviations of 15 % that are not shown in the table. The predicted overall yield for the community as a whole was 0.51 C-mol assimilated/C-mol converted, higher than the experimentally determined value (0.38 C-mol assimilated/C-mol converted). For the thermodynamically sorted case, the matrix could not be solved. Therefore, two scenarios were calculated, in one case the electron donors were balanced, in the other the electron acceptors and the average value for these two scenarios (0.60 C-mol assimilated/C-mol converted) was used to calculate the productivity loss attributed to the absence of thermodynamic sorting (~15 %), which explained half of the difference between the productivity observed for our community (0.38 C-mol/C-mol) and the productivity reported for pure cultures (0.7 C-mol/C-mol).

Author contributions

M.S., J.C. and A.H. designed the experiments. J.C., E.H. A.H. performed the sediment sampling in the tidal flat and the continuous cultivation with input from J.S.G., T.H. and B.K. J.C. performed on-line measurements, aggregate free incubations, the chemical analysis and data processing and FISH (with input from S.L.). H.T. and I.K. performed metagenomic/transcriptomic sequencing and assembly; R.S. and R.L.H. performed proteomics. M.S. and J.C. performed *in silico* analysis and modelling. M.S. and J.C. wrote the manuscript with input from all co-authors.

Author information

References

1   **Fenchel**, T. M. & Jorgensen, B. B. Detritus food chains of aquatic ecosystems: the role of bacteria. *Adv Microb Ecol* **1**, 1-58 (1977).

2   **Gujer**, W., Henze, M., Mino, T. & van Loosdrecht, M. Activated sludge model No. 3. *Water Sci Technol* **39**, 183-193 (1999).

3   **Madigan**, M. T., Martinko, J. M., Stahl, D. A. & Clark, D. P. *Brock biology of microorganisms*. 13th edn, (Benjamin-Cummings, 2011).

4   **Gao**, H. *et al.* Aerobic denitrification in permeable Wadden Sea sediments. *ISME J* **4**, 417-426 (2010).

5   **Canfield**, D. E. *et al.* A cryptic sulfur cycle in oxygen-minimum-zone waters off the Chilean coast. *Science* **330**, 1375-1378 (2010).

6   **Reise**, K. *et al.* The Wadden Sea - a universally outstanding tidal wetland. *Wadden Sea Ecosystem* **9**, 7-24 (2010).

7   **Wolff**, W. J., Bakker, J. P., Laursen, K. & Reise, K. The Wadden Sea quality status report - synthesis report 2010. *Wadden Sea Ecosystem* **29**, 25-74 (2010).

8   **Zumft**, W. G. Cell biology and molecular basis of denitrification. *Microbiol Mol Biol Rev* **61**, 533-616 (1997).

9   **Preisig**, O., *et al.* A high-affinity cbb3-type cytochrome oxidase terminates the symbiosis-specific respiratory chain of bradyrhizobium japonicum. *J Bac* **178**, 1532-153.(1996).

10   **Heijnen**, J. J., Van Loosdrecht, M. C. M., Tijhuis, L. A black box mathematical model to calculate auto- and heterotrophic biomass yields based on Gibbs energy dissipation. *Biotechnol Bioeng* **40**, 1139-1154 (1992).

11   **Strohm**, T. O., Griffin, B., Zumft, W. G. & Schink, B. Growth yields in bacterial denitrification and nitrate ammonification. *Appl Environ Microbiol* **73**, 1420-1424 (2007).

12   **Stewart**, F.J., Ulloa, O., DeLong, E.F. Microbial metatranscriptomics in a permanent marine oxygen minimum zone. *Environ. Microbiol.* **14,** 23-40 (2012).

13   **Kirchman**, D. L. The ecology of *Cytophaga-Flavobacteria* in aquatic environments. *FEMS Microbiol Ecol* **39**, 91-100 (2002).

14   **Hanke**, A. *et al.* Recoding of the stop codon UGA to glycine by a BD1-5/SN-2 bacterium and niche partitioning between *Alpha-* and *Gammaproteobacteria* in a tidal sediment microbial community naturally selected in a laboratory chemostat. *Front Microbiol* **5**, 231. doi: 10.3389/fmicb.2014.00231 (2014).

15   **Eck**, G.-R. v. Physiological and chemical tests for drinking water. *NEN 1056, IV-2, Nederlands Normalisatie Institut Rijswijk* (1966).

16   **Lowry**, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**, 265-275 (1951).

17   **Kamyshny**, A., Ekeltchik, I., Gun, J. & Lev, O. Method for the determination of inorganic polysulfide distribution in aquatic systems. *Anal Chem* **78**, 2631-2639 (2006).

18   **Albert**, D. B. & Martens, C. S. Determination of low-molecular-weight organic acid concentrations in seawater and pore-water samples *via* HPLC. *Mar Chem* **56**, 27-37 (1997).

19   **Klindworth**, A. *et al.* Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res* **41**, e1 (2013).

20   **Bolger**, A. M., Lohse, M. & Usadel, B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* (2014).

21   **Schloss**, P. D. *et al.* Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* **75**, 7537-7541 (2009).

22   **Edgar**, R. C. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **26**, 2460-2461 (2010).

23   **Zhou**, J. Z., Bruns, M. A. & Tiedje, J. M. DNA recovery from soils of diverse composition. *Appl Environ Microb* **62**, 316-322 (1996).

24   **Smith**, C. J., Nedwell, D. B., Dong, L. F. & Osborn, A. M. Diversity and abundance of nitrate reductase genes (narG and napA), nitrite reductase genes (nirS and nrfA), and their transcripts in estuarine sediments. *Appl Environ Microb* **73**, 3612-3622 (2007).

25   **Fan**, L., McElroy, K. & Thomas, T. Reconstruction of ribosomal RNA genes from metagenomic data. *PLoS ONE* **7**, e39948 (2012).

26   **Strous**, M., Kraft, B., Bisdorf, R. & Tegetmeyer, H. E. The binning of metagenomic contigs for microbial physiology of mixed cultures. *Front Microbiol* **3**, 410 (2012).

27   **Seemann** T. PROKKA: Rapid Prokaryotic Genome Annotation. *Bioinformatics*, March 18 [Epub] (2014)

28   **Llobet**-**Brossa**, E., Rosselló-Mora, R. & Amann, R. Microbial community composition of Wadden Sea sediments as revealed by fluorescence *in situ* hybridization. *Appl Environ Microb* **64**, 2691-2696 (1998).

29   **Pernthaler**, A., Pernthaler, J. & Amann, R. *Sensitive multi-color fluorescence in situ hybridization for the identification of environmental microorganisms.* 711-726 (Kluwer Academic Publishers, 2004).

Supplementary Material

Due to the large sizes of the supplementary tables (>35,000 rows) this data is not presented in the context of this thesis.

Supplementary Information for this publication can be found under
http://www.mpi-bremen.de/Page8392.html

Publication IV

Title:

Temperature as a factor for natural selection on marine denitrifying bacterial communities in continuous cultures

Authors:

Anna Hanke, Jasmine Berg, Theresa Hargesheimer, Halina E. Tegetmeyer, Christine E. Sharp, Marc Strous

Status:

In preparation

Own contribution:

AH planned and conducted the cultivation experiments with help of JB, determined, analyzed, and visualized the nutrient data, and wrote the manuscript with help of all coauthors.

Temperature as a factor for natural selection on marine denitrifying bacterial communities in continuous cultures

Anna Hanke[1], Jasmine Berg[1], Theresa Hargesheimer[1], Halina E. Tegetmeyer[1,2], Christine E. Sharp[2], Marc Strous[1,2,3]

[1]Max Planck Institute for Marine Microbiology, Celsiusstraße 1, D-28359 Bremen, Germany

[2]Department of Geoscience, University of Calgary, T2N1N4, Alberta, Canada

[3]Institute for Genome Research and Systems Biology, Center for Biotechnology, Bielefeld University, Universitätsstraße 27, D-33615 Bielefeld, Germany

Correspondence: mstrous@ucalgary.ca

Acknowledgements

Abstract

Thermodynamic sorting of redox processes and oxygen sensitivity of certain enzymes responsible for microorganism anaerobic metabolic conversions are factors that explain the spatial separation of anaerobic and aerobic processes. However, long-term continuous cultures of marine denitrifying bacteria at 10 °C and 25 °C, showed that, thermodynamically unsorted, fermenting and denitrifying bacteria co-evolved in single cultures. Moreover, temperature exerted a substantial effect on the denitrifying and fermenting capabilities of the enriched organisms. This was illustrated by an increased demand for electron donors needed for the exhaustive reduction of nitrite at lower temperatures. This correlated with the higher abundance of fermenters in low temperature cultures. After several months, denitrification activity in the low (10 °C) temperature culture collapsed entirely, while cultures at high temperature (25 °C) sustained the partitioning between fermenting and denitrifying organisms. This supports our conclusion that temperature had a strong selective force on the enriched denitrifying population. Therefore, fermentation outcompeted denitrification for carbon sources at temperatures as low as 10 °C. Metagenomic analyses of the organisms enriched at 10 C° illustrate the effect of low temperatures, presumably disabling the enriched bacterial community to partition both metabolic activities among its different members. The results show that at a cell level fermentation and denitrification occurred simultaneously at low temperatures with fermentation in favor over denitrification. Higher incubation temperatures on the other hand, led to the development of different species specialized for either one of the metabolic pathways and enabled the community to sustain fermentation and denitrification in parallel.

Introduction

Nitrogen is a key element with respect to species compositions, diversity and dynamics of many terrestrial, freshwater and marine ecosystems (Vitousek *et al.* 1997). Human influence on the cycling of nitrogen, mostly *via* industrial N-fixation and fossil fuel combustion (Vitousek *et al.* 1997, Gruber & Galloway 2008) has increased dramatically since pre-industrial times (Hanke & Strous 2010). The implication of nitrogen for humans is reflected in both its use in form of agricultural fertilizer (Vitousek *et al.* 1997) and its involvement in the formation of nitrogen oxides like nitrous oxide (N_2O) and nitric oxide (NO), which act as greenhouse gases and ozone depleting substances (Carpenter *et al.* 2012), reviewed by (Byrnes 1990).

Naturally, nitrogen cycling is mainly mediated by microorganisms involved in biogeochemical transformations such as denitrification and nitrification (Hanke & Strous 2010). Denitrification, the stepwise reduction of nitrate (NO_3^-) *via* nitrite (NO_2^-), nitric oxide (NO), and nitrous oxide (N_2O) to dinitrogen (N_2), acts on the removal of fixed nitrogen species, consequently supporting wastewater treatment (Tallec *et al.* 2006, Osaka *et al.* 2008). However, under certain circumstances denitrification promotes global warming by release of NO and N_2O (Crutzen 1970, Carpenter *et al.* 2012). Until the year 2100, a 1.4-5.8 °C rise in mean global surface temperature relative to the year 1990 has been estimated (Cubasch *et al.* 2013). Global warming in turn can reinforce denitrification. Data from ice cores link historical temperature rising events with increases of atmospheric N_2O concentrations due to increased biological activities in soils and oceans (Smith 1997). Denitrification is promoted by temperature increase in different ways: for example, increasing temperature normally accelerates biochemical reactions up to certain thresholds (Thomann & Mueller 1987). Moreover, it decreases the solubility of oxygen in water and simultaneously promotes respiration rather than photosynthesis causing depletion of dissolved oxygen in aquatic ecosystems (Seiser & Walz 1925) (Allen *et al.* 2005). As denitrification is a predominantly anaerobic process its rates are enhanced by a temperature-mediated drop in oxygen availability (Veraart *et al.* 2011). Further, temperature rise may affect biochemical reactions preceding and competing with denitrification, either directly

or *via* decreasing the dissolved oxygen concentration. In freshwater ecosystems this leads to an overall acceleration of denitrification (Veraart *et al.* 2011).

To investigate the long-term effect of high *vs.* low temperatures on a denitrifying microbial community, microbes from North Sea sediment were repeatedly incubated in parallel continuous culture setups. Incubations were carried out in the dark, anaerobically with nitrate and nitrite as sole electron acceptors, and at 10 °C and 25 °C, respectively. The composition and the metabolic activity of the cultures were determined by 16S rDNA sequencing, metagenomics, and transcriptomics. Retrieved culture community compositions were compared to microbial diversity mentioned in two previous studies investigating the influence of temperature on microbial consortia (Fuhrman *et al.* 2008, Sharp *et al.* 2014). Our results indicate a reproducible parallel selection of denitrifying and fermenting organisms in single cultures as could be observed previously (Hanke *et al.* 2014, Kraft *et al.* in press). However, the success of these cultures seemed to strongly depend on the incubation temperature. At 10 °C, single cells were presumably able to switch between denitrification and fermentation whereby fermentation had a selective advantage leading to a reduction in denitrification and finally to a collapse of denitrifying activity. At 25 °C, the enriched community seemed to rather consist of species capable of either denitrification or fermentation which enabled them to sustain both metabolisms for the duration of the experiments. Furthermore, the enrichment of an organism closely affiliated with Terasakiella was observed. Genome analyses revealed that this organism encoded a putative nitric oxide dismutase in its genome, which was previously proposed to enable the intra-aerobic oxidation of methane coupled to denitrification (Ettwig *et al.* 2010, Ettwig *et al.* 2012).

Material & Methods

Sampling and inoculation

Sediment samples for inoculating the continuous cultures were taken from the intertidal flat "Janssand" located in the Central German Wadden Sea south of the Eastern Friesian Island Spiekeroog. Sampling was conducted twice in subsequent years to receive inocula for two replicate experiments. First on March 9th 2011 (N: 053°44.153'/E: 007°41.943', 3 °C water temperature, overcast), second on March 6th 2012 (N: 053°44.133'/E: 007°41.989', 8 °C water temperature, sunny). Sampling procedure and preparation was according to (Hanke *et al.* 2014).

Medium composition

Both cultures were continuously supplied with identically prepared anoxic, sterile liquid medium containing a defined mixture of glucose, aminoacids, acetate, nitrite and nitrate. The carbon substrates were provided in a ratio as described in (Hanke *et al.* 2014). Substrate concentrations were gradually increased during the first 2-7 weeks to allow gradual adaptation of the inoculated microbial community and prevent build-up of high nitrite concentrations. After this adaptation period, the concentrations in the influent medium were maintained at 20 mM nitrite, 1 mM nitrate for all cultures, and additionally 28.13 mM carbon for the 25 °C cultures and 37.5 mM carbon for the 10 °C cultures, respectively.

Note that the substrate concentrations in the culture were generally in the low micromolar range as the medium was supplied dropwise to the cultures.

Continuous cultivation

Continuous incubations of the sampled microbial communities lasted 100 and 250 days, respectively, for two independent sets of experiments, and were conducted in custom culture vessels that are described in detail elsewhere (Hanke *et al.* 2014). The cultures were kept anoxic and the temperature was maintained at 10 °C and 25 °C in two parallel incubations for both sets of experiments. All physical and some chemical culture conditions such as temperature, pressure, dilution rate, redox potential, and pH were automatically monitored and controlled

by pumps, weight sensors underneath the culture vessels, sensors and heaters extending into the culture liquid, and finally a computer based control unit, which is described in detail elsewhere (Hanke *et al.* 2014). Temperature was maintained with help of resistive heaters (Hanke *et al.* 2014) and for the 10 °C incubations additionally with a Minichiller-NR (230 V 1~50/60 Hz, -20 °C…+40 °C; Peter Huber Kältemaschinenbau GmbH, Germany) coupled to a cooling finger inserted into the culture vessel. Mixing of the culture liquid was performed by the recirculation of gas from the headspace of the culture to the bottom. The culture volume was kept constant at 2.8 L by continuously feeding fresh medium and simultaneously removing spent culture liquid from the vessel. The dilution rate ranged from 0.15 to 0.7 mL min^{-1}. The pH was automatically controlled at 8.0±0.05 with help of NaOH and HCl, 0.25 M. An overpressure of 13±5 mbar was maintained *via* constantly amending the culture with 10 mL min^{-1} argon and piping spent gas though a water lock. For more details on the cultivation procedure see (Hanke *et al.* 2014).

Batch incubations I

Batch incubations with Janssand sediment for determination of *in situ* denitrification rates were conducted as described previously (Hanke *et al.* 2014).

Batch incubations II

Batch incubations for determining culture fermentation and denitrification rates were conducted in duplicates in sterile 30 mL injection bottles (Ochs Glasgerätebau, Bovenden/ Lenglern, Germany). The bottles were prefilled with 225 µl of a sterile carbon compound stock solution (400 mM; composition see Hanke *et al.* (2014)) and 75 µl sterile HEPES stock solution (3 M initial concentration; Roth, Karlsruhe, Germany). Bottles were made anoxic by alternately applying vacuum down to 0.05 bar and adding Argon up to 1.5 bar, 5 times each. 15 mL culture was added to each incubation by injection. In cases of low biomass content indicated by an optical density (OD) of <0.3, the cultures were concentrated 2 times prior to injection by centrifuging 30 mL culture 5 min at 5,000 x g and 4 °C and resuspending the pelleted cells in 15 mL anoxic artificial seawater (see above). After injecting the culture the bottles were shaken

immediately to homogenize the incubation fluid. Samples taken for later analyses of fermentation products and ammonia were centrifuged 4 min at 14,500 x g and the supernatant was stored at -20 °C until later analyses. Sampling started directly after shaking and was conducted 5 times in 2 h intervals. Bottles were incubated at the respective original culture incubation temperature and were periodically shaken by hand in 30 min intervals.

Batch incubations for denitrification rate determination were conducted in triplicates as described above, with following differences: nitrite stock solution (sodium nitrite, 200 mM Roth, Karlsruhe, Germany), 200 mM) was added to a final concentration of 0.5-0.8 mM. Carbon mixture (glucose, aminoacids, acetate, see above) stock solution (400 mM carbon) was added to 1.0-1.5 mM carbon final concentration. HEPES stock solution (3 M) was added to a final concentration of 10 mM. Culture liquid was injected without pre-concentration. Samples for later nitrite analyses were taken 7 times in 20 min intervals, centrifuged 4 min at 14,500 x g and finally the supernatant was stored at -20 °C until further analyses. Nitrate, nitrite and fermentation products were measured as described below.

Wet chemical analyses

Ammonia, nitrite and protein concentrations in the culture liquid were measured spectrophotometrically according to Solorzano (1969), Bendschneider & Robinson (1952), and Lowry *et al.* (1951), respectively.

Nitrate concentrations were calculated by subtraction of nitrite concentrations from the total NO_x concentration determined with an NO/NO_x analyzer (2 µM detection limit; CLD 66 S, Eco Physics, Munich, Germany). Samples were injected into the reaction chamber connected upstream to the analyzer. At 90 °C, with VCl_3 (0.1 M), both NO_3^- and NO_2^-, were reduced to NO, which was measured by the chemiluminescence detector.

DNA sequencing for metagenomics

Sampling for metagenome sequencing of the 2011 cultures was conducted at t=97 days (25 °C; sample ID "2011/25.1" and "25A", respectively) and t=101 days

154

(10 °C; sample ID "2011/10.1"/"10A", respectively. DNA was extracted from 50 mL of pelleted culture, according to (Zhou *et al.* 1996). For the 2011 metagenomes 1 µg of purified DNA per sample was used for the preparation of whole metagenome shotgun sequencing libraries according to the "Rapid Library Preparation Method Manual" (May 2011) for GS FLX+ Series provided by Roche (Mannheim, Germany). The sample was sequenced on a quarter picotiterplate of a GS FLX+ Titanium sequencing run. 201,559 reads with an average length of 644 bases and 192,432 reads with an average length of 603 bases were obtained for samples "2011/25.1"/"10A" and "2011/10.1"/"25A", respectively.

Samples for metagenomics of the 2012 cultures were taken at t=35, 64, and 86 days Sample IDs (preliminary differing between identifiers of published metagenomic data and figure descriptions) are: "2012/10.1" (and "10B1", respectively), "2012/10.2" ("10B2"), "2012/10.3" ("10B3"), "2012/25.1" ("25B1"), "2012/25.2" ("25B2"), and "2012/25.3" ("25B3"), respectively. Ascending numbers indicate ascending culture age at the time of sampling, compare Figure IV-1. DNA was extracted as for the 2011 samples. For whole metagenome shotgun sequencing, between 2 and 2.5 µg of extracted DNA per sample were mechanically fragmented using Nebulizers (Roche) with 32 psi applied for 6 min, in 500 µl nebulization buffer (Roche). The fragmented DNA was purified using MinElute PCR purification columns (Qiagen, Düsseldorf, Germany) and eluted in 50 µL low TE (Life Technologies, Darmstadt, Germany). The entire eluate was used for the preparation of barcoded PGM™ sequencing libraries with the Ion Xpress™ Plus gDNA Fragment Library Preparation Kit (manual Pub. No 4471989, Rev. M, May 2013) (Life Technologies). Library insert sizes were approx. 200 bp. Libraries were sequenced with the Personal Genome Machine® (PGM™), using the chemistry for 200 bp libraries. Base calling was performed with the Torrent Suite v3.2 software, with default settings. 2,045,060 reads with an average length of 150 bases, 1,768,544 reads with an average length of 136 bases, and 1,746,609 reads with an average length of 148 bases were obtained for samples "2012/10.1" ("10B1"), "2012/10.2" ("10B2"), and "2012/10.3" ("10B3") respectively. According numbers for the 2012/25-samples no. 1-3 ("25B1-3") are as follows: 1,370,288, 2,159,050, and 1,543,063 reads, respectively, with an average length of 154, 158, and 169 bases, respectively.

Sequence reads of each sample were assembled with the Newbler assembler v.2.6 with default settings for genomic DNA assembly for non-paired reads.

RNA sequencing for transcriptomics

Samples for transcriptome analyses were taken at t=78 and t=86 days for the 2012 cultures (10 °C and 25 °C). For RNA preservation, culture samples were centrifuged 3 min at 14,500 x g. Supernatant was discarded and each pellet was resuspended in 300 µl Lifeguard^{TM} Soil Preservation Solution (MO BIO Laboratories, Carlsbad, CA, USA). Samples were stored at -20 °C until further treatment. Total RNA was extracted from 4-10 mL of pelleted culture (stored in Soil Preservation Solution) as follows: The pellet was re-suspended in 1 mL of TRI Reagent® solution (Applied Biosystems, Darmstadt, Germany). 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/sec for 45 sec. After incubation at room temperature (RT) for 5 min, 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 were 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 ice-cold isopropanol were 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 approx. 10 min. The pellet was resuspended in sterile TE buffer (pH 8.0) an incubated on ice for approx. 30 min for complete dissolving. The extracted RNA was treated with DNase (Promega, Mannheim, Germany) and purified using RNeasy MinElute spin columns (Qiagen).

Prior to library preparation for ion torrent sequencing, rRNA was depleted from 3 to 5 µg total purified RNA of each sample using the Ribo-Zero^{TM} rRNA removal Kit (Bacteria) (epicentre, Madison, WI, USA). The rRNA depleted sample was the used for library preparation with the ion total RNA-Seq Kit v2 (Life Technologies) following the protocol for whole transcriptome library preparation. The generated cDNA libraries were sequenced with the Personal Genome Machine® (PGM^{TM}),

using the chemistry for 200 bp libraries. Base calling was performed with the Torrent Suite v3.2 software, with default settings.

Fatty acids determination

Fermentation products (formate, acetate, succinate, butyrate) were analyzed *via* high-performance liquid chromatography (HPLC) after thawing and centrifuging the samples at 14,500 x g and filtering the supernatant through a 4 mm diameter / 0.45 µm pore size nylon syringe filter (Chromatographie Handel Müller, Fridolfing, Germany) into 2 mL CRIMPSNAP-Vials (WICOM, Heppenheim, Germany). Fatty acid analyses were conducted with an HPLC system from Sykam (Eresing, Germany) as previously described in (Bondarev *et al.* 2013).

In silico computational procedures.

Assembly of the metagenomic reads from the chemostat was carried out (after filtering the reads for quality and removing tags) with the GS De Novo Assembler 2.8 (454 Life Sciences, Branford, CT, USA) using the default settings for genomic DNA. On average 85 % of the reads were assembled into contigs. Assembled contigs were binned based on tetranucleotide compositions (Strous *et al.* 2012). Fifteen bins, each with a consistent phylogenetic profile, were identified. 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. Transfer-RNAs were identified with ARAGORN (Laslett & Canback 2004). Genome completeness was estimated for each bin by representation of 139 conserved single copy genes as described by Campbell *et al.* (2013). The contigs of each bin were annotated separately with Prokka (Seemann 2014). 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). Alignment of 16S rRNA gene sequences was performed with MAFFT (Katoh *et al.* 2002); high accuracy options --maxiterate 1000 --localpair) and a phylogenetic tree was calculated by approximate maximum likelihood with FastTree2 (Price *et al.* 2010) after applying

a 25 % conservation filter. Key functional genes (*nirS, nirK, nosZ, pflB*) were detected with Hidden Markov Models, one for each gene, calculated from alignments of representative reference genes. Prior to detection all contigs were translated in six frames without open reading frame prediction. DNA regions potentially encoding a functional gene were analyzed further with BLASTx against the refseq database.

Data submission

The metagenome sequence data is accessible via the NCBI resource platform http://www.ncbi.nlm.nih.gov/bioproject under bioproject PRJNA255460.

The individual sample accession numbers are:
SAMN02919310, SAMN02919311, SAMN02919312, SAMN02919313, SAMN02919314, SAMN02919315, SAMN02919316, SAMN02919317 (http://www.ncbi.nlm.nih.gov/biosample/).

The the assembled contigs were deposited at DDBJ/EMBL/GenBank as Whole Genome Shotgun project under the accessions: not available yet.

Data will be released once referenced data is published.

Results and Discussion

Bacteria were extracted from the upper 2 cm of a tidal flat sediment in the German Bight in two subsequent years, 2011 and 2012. Sampling was performed on March 9 and March 6, respectively. *In situ* surface water temperatures were 3 °C and 8 °C respectively. Mean (1948-2012) annual surface water temperatures in March were ~4 °C. Over this period mean surface water temperatures in the German Bight peaked in August at 16 °C. However, when these sediments were exposed to sun and atmosphere during low tide, much higher sediment temperatures of up to 32 °C have been recorded (CWSS 2008). Seasonal potential denitrification rates of the sediments were measured between October 2009 and July 2010. Denitrification rates in March were ~0.8 µmol N_2 L^{-1} day^{-1} and insensitive to incubation temperature (10-30 °C). The highest denitrification rates were observed in autumn (October). Denitrification rates in autumn were furthermore much more sensitive to temperature, increasing from (2.2±0.6) to (6.1±0.7) µmol N_2 L^{-1} day^{-1} with increasing incubation temperature.

Bacteria extracted from both samples were used to inoculate 2 continuous cultures, one at 10 °C and one at 25 °C. The continuous cultures were maintained anoxically for at least 100 days and were supplied with medium containing nitrite as the main electron acceptor and a mixture of organic carbon compounds as the energy and carbon source. Some nitrate was provided in addition to nitrite, but only a small amount, because the extracted cells rapidly converted the provided nitrate to nitrite, leading to high (>1 mM), potentially toxic, nitrite concentrations in the cultures. The organic carbon mixture contained glucose, amino acids and acetate in a ratio that mimicked the composition of decaying biomass, the main carbon and energy source *in situ*, in terms of its monomers. Initially, the cultures received nitrite and organic carbon in the same C to N ratio (0.8:1 N-mmol:C-mmol for 2011 cultures and 1.1:1 for 2012 cultures). At this ratio, nitrite was the limiting substrate in the 25 °C cultures as shown by its complete conversion to N_2. Carbon compounds were supplied in excess, as shown by the presence of organic compounds in the culture liquid (butyrate 0.313 mM in the 2012-10 ° culture and 0.352 mM in the 2012-25 ° culture on day 9 after inoculation). However, in both 10 °C cultures, the same C:N ratio as applied for the 25 °C cultures led to the

accumulation of nitrite in the culture, indicating that nitrite was not the limiting substrate at 10 °C, despite the availability of sufficient organic compounds in the culture liquid. The accumulation of nitrite could be reversed by addition of more carbon compounds to the medium supplied to the 10 °C cultures or by decreasing the medium supply (dilution) rate of these cultures. However, after some time, nitrite tended to accumulate again and this could only be reversed by adding yet more carbon or by decreasing the dilution rate even further. This led to a lower substrate supply rate and a higher C to N ratio in the 10 °C cultures (Fig IV-1). Eventually, the denitrification rates in the 10 °C cultures collapsed entirely (see below).
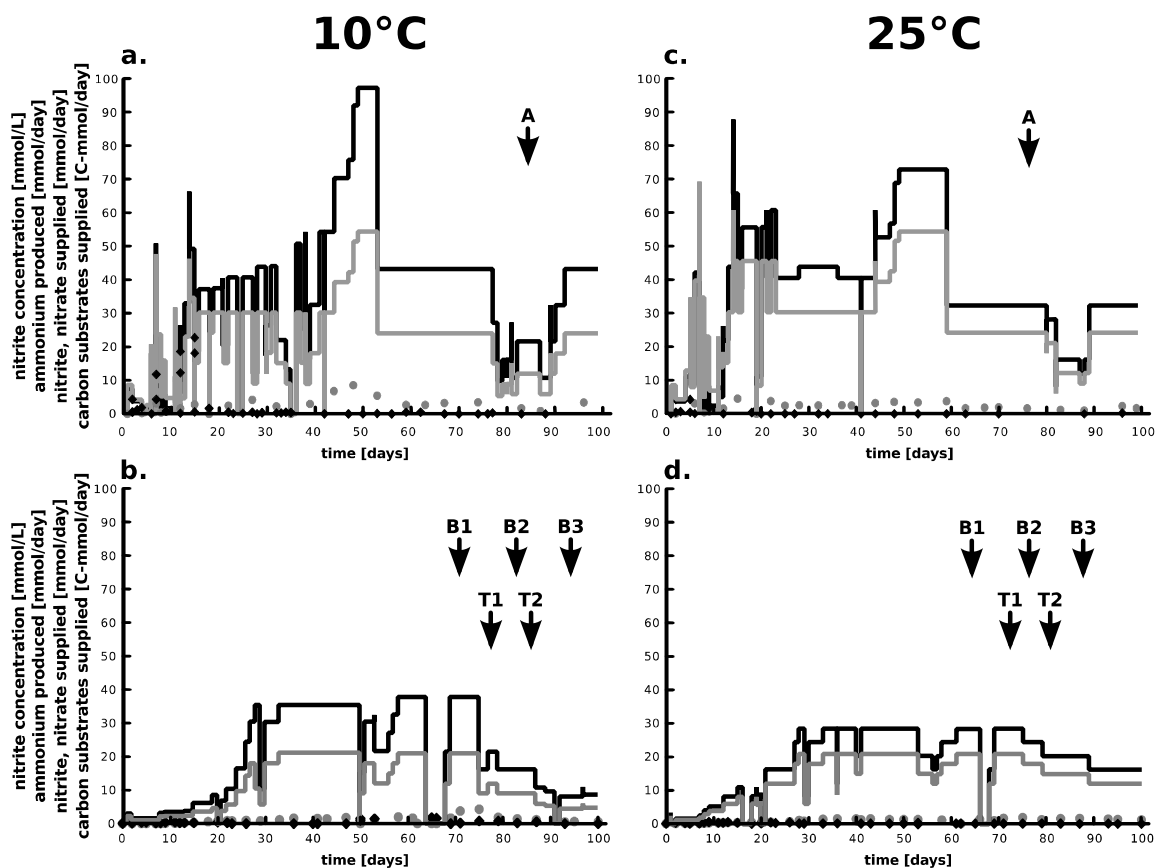
Figure IV-1 I Overall carbon and nitrogen conversions in the four cultures, two at 10 °C (a,b) and two at 25 °C (c,d). Supply of total nitrite and nitrate (grey line) and supply of carbon substrates (black line). Actual concentrations of nitrite (black diamonds) and ammonium (grey circles) measured in the cultures are indicated. Nitrate was always fully converted (detection limit 10 µM). The timing of eight samples used for metagenomic sequencing of DNA (A, B1-3) and RNA (T1-2) are indicated by arrows.

160

To investigate the mechanisms responsible for the malfunctioning of denitrification at 10 °C we performed metagenomic and transcriptomic analyses of the microbial community of all four cultures, at different time points (Fig IV-1, arrows). Pooled and tagged DNA from the 2011 and 2012 samples were sequenced on a 454 sequencer and an Ion Torrent Personal Genome Machine®, respectively. Reads were assembled into contigs and contigs were binned as previously described (Strous *et al.* 2012, Hanke *et al.* 2014). Fifteen bins were obtained, each corresponding to a distinct "blob" on the GC *vs.* coverage plots (Fig. IV-2). Binning results were also supported by a consistent taxonomic assignment of the contigs within each bin (Fig. IV-3). Binning accuracy increases with contig length and different bins were observed to differ in their contig size distributions. Poor assembly, leading to short contigs may have resulted from low sequencing coverage (low abundance) and a higher degree of (micro-)diversity in the microbial populations associated with each bin. For example, bin A appeared to include sequence data of at least two different populations in sample 25B2. One population was very clonal and yielded long contigs that formed a dense blob at 23x sequencing coverage. However, many short contigs belonging to a closely related population (as shown by cross binning and read mapping) formed more disperse blobs at higher sequencing coverages (>30x). In this case, a bin including all the long contigs of the dense blob was selected as the bin representing this group of related populations. As a second example, bins L and M both represented populations affiliated with *Epsilonproteobacteria*, one present in the 2011/25°C culture (bin L, sample 25A) and one present in the 2012/10°C culture (bin M, sample 10B1). The latter was less abundant, leading to a lower sequencing coverage and yielding shorter contigs.
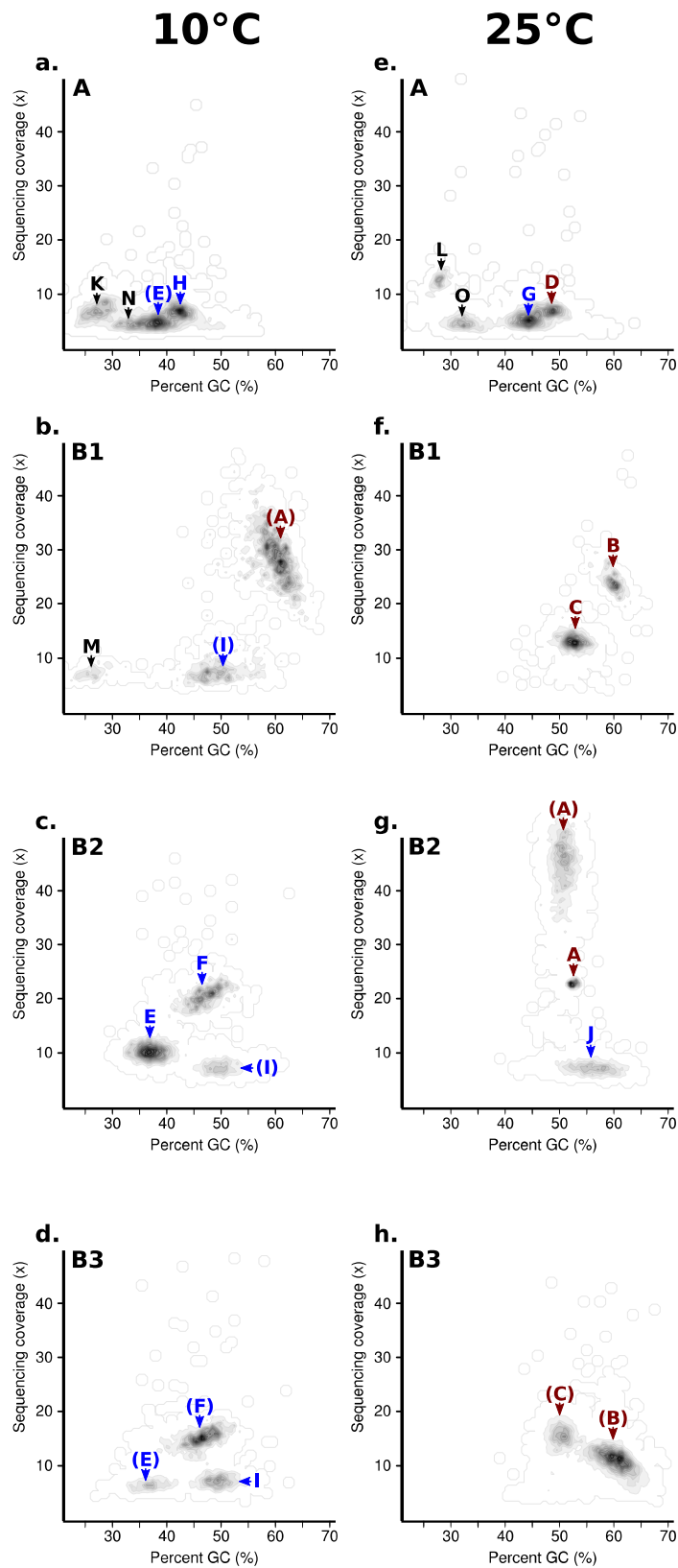
Figure IV-2 I Assignment of bins to "blobs" on the GC *vs.* coverage plot of assembled contigs after binning of the eight metagenomes. For timing of samples A (2011) and B1-3 (2012), see Figure IV-1. Capital letters A-O refer to bin identifiers. A parenthesized letter means that the bin was detected but was assembled better in a different sample.

Bin A

N50=130072nt

< 1.0 kb
1.0 - 3.5 kb
3.5 - 7.0 kb
7.0 - 15.0 kb
15.0 - 30.0 kb
30.0 - 60.0 kb
> 60.0 kb

1777 hits/6323 orfs:
- (13.3x@8.0e-53) Polymorphum gilvum
- (13.8x@5.0e-59) Rhizobiaceae
- (11.3x@4.0e-28) Rhodobacteraceae
- (16.6x@2.0e-48) Phyllobacteriaceae
- (14.6x@2.0e-37) Xanthobacteraceae
- (15.4x@9.0e-45) Brucellaceae
- (16.9x@9.0e-34) Bradyrhizobiaceae

Bin B

N50=20520nt

< 1.0 kb
1.0 - 3.5 kb
3.5 - 7.0 kb
7.0 - 15.0 kb
15.0 - 30.0 kb
30.0 - 60.0 kb
> 60.0 kb

3929 hits/5109 orfs:
- (14.5x@2.0e-57) Rhodobacteraceae

Bin C

N50=5604nt

< 1.0 kb
1.0 - 3.5 kb
3.5 - 7.0 kb
7.0 - 15.0 kb
15.0 - 30.0 kb
30.0 - 60.0 kb
> 60.0 kb

3778 hits/7418 orfs:
- (7.3x@2.0e-33) Hyphomicrobiaceae
- (7.6x@2.0e-31) Rhodobacteraceae
- (7.0x@9.0e-29) Rhizobiaceae
- (6.8x@3.0e-31) Phyllobacteriaceae
- (6.5x@8.0e-29) Polymorphum gilvum
- (7.1x@5.0e-34) Hyphomonadaceae
- (6.4x@1.0e-34) Xanthobacteraceae
- (6.4x@2.0e-30) Brucellaceae

Bin D

N50=10032nt

< 1.0 kb
1.0 - 3.5 kb
3.5 - 7.0 kb
7.0 - 15.0 kb
15.0 - 30.0 kb
30.0 - 60.0 kb
> 60.0 kb

1585 hits/3772 orfs:
- (1.1x@1.0e-68) Rhodospirillaceae
- (1.0x@5.0e-53) Rhodobacteraceae
- (0.9x@3.0e-79) Alteromonadaceae
- (1.1x@6.0e-77) Bradyrhizobiaceae
- (1.0x@5.0e-21) Hyphomonadaceae
- (1.0x@3.0e-61) Magnetococcaceae
- (1.1x@4.0e-30) Hyphomicrobiaceae
- (1.0x@2.0e-45) Comamonadaceae

Bin E

N50=1735nt

< 1.0 kb
1.0 - 3.5 kb
3.5 - 7.0 kb
7.0 - 15.0 kb
15.0 - 30.0 kb
30.0 - 60.0 kb
> 60.0 kb

5898 hits/8539 orfs:
- (5.9x@1.0e-39) Colwelliaceae
- (5.8x@7.0e-34) Alteromonadaceae
- (6.0x@1.0e-32) Pseudoalteromonadaceae
- (8.1x@5.0e-30) Vibrionaceae
- (7.6x@2.0e-37) Psychromonadaceae
- (7.0x@5.0e-35) Marinomonas
- (6.2x@2.0e-30) Shewanellaceae
- (6.6x@1.0e-28) Aeromonadaceae

Bin F

N50=21065nt

< 1.0 kb
1.0 - 3.5 kb
3.5 - 7.0 kb
7.0 - 15.0 kb
15.0 - 30.0 kb
30.0 - 60.0 kb
> 60.0 kb

6096 hits/8886 orfs:
- (12.7x@5.0e-69) Vibrionaceae
- (11.9x@3.0e-29) Enterobacteriaceae
- (10.6x@9.0e-31) Aeromonadaceae
- (4.0x@4.0e-31) Pseudomonadaceae

Bin G

N50=7578nt

< 1.0 kb
1.0 - 3.5 kb
3.5 - 7.0 kb
7.0 - 15.0 kb
15.0 - 30.0 kb
30.0 - 60.0 kb
> 60.0 kb

3901 hits/5705 orfs:
- (1.1x@1.0e-99) Vibrionaceae
- (0.8x@8.0e-122) Colwelliaceae
- (1.3x@4.0e-60) Enterobacteriaceae
- (1.3x@9.0e-37) Alteromonadaceae
- (1.1x@8.0e-101) Psychromonadaceae
- (1.0x@8.0e-81) Marinomonas
- (1.3x@7.0e-46) Aeromonadaceae
- (1.1x@1.0e-108) Rhodobacteraceae

Bin H

N50=3585nt

< 1.0 kb
1.0 - 3.5 kb
3.5 - 7.0 kb
7.0 - 15.0 kb
15.0 - 30.0 kb
30.0 - 60.0 kb
> 60.0 kb

3727 hits/4686 orfs:
- (0.7x@4.0e-81) Vibrionaceae
- (0.8x@4.0e-62) Enterobacteriaceae
- (0.8x@5.0e-56) Acholeplasmataceae
- (0.7x@1.0e-45) Aeromonadaceae
- (0.8x@4.0e-39) Rhodobacteraceae
- (0.5x@2.0e-76) Flavobacteriaceae

Bin I

N50=984nt

< 1.0 kb
1.0 - 3.5 kb
3.5 - 7.0 kb
7.0 - 15.0 kb
15.0 - 30.0 kb
30.0 - 60.0 kb
> 60.0 kb

3466 hits/4806 orfs:
- (3.7x@5.0e-34) Pseudomonadaceae
- (3.9x@7.0e-23) Alteromonadaceae
- (8.9x@2.0e-47) Vibrionaceae
- (3.6x@2.0e-28) Aeromonadaceae
- (3.7x@1.0e-26) Rhodocyclaceae
- (3.6x@3.0e-30) Halomonadaceae
- (4.2x@5.0e-30) Marinomonas
- (3.7x@5.0e-24) Comamonadaceae

Bin J

N50=767nt

< 1.0 kb
1.0 - 3.5 kb
3.5 - 7.0 kb
7.0 - 15.0 kb
15.0 - 30.0 kb
30.0 - 60.0 kb
> 60.0 kb

5050 hits/8468 orfs:
- (3.9x@9.0e-37) Chromatiaceae
- (3.4x@1.0e-28) Rhodobacteraceae
- (4.0x@2.0e-31) Ectothiorhodospiraceae
- (3.9x@5.0e-31) Rhodocyclaceae
- (4.1x@8.0e-26) Hydrogenophilaceae
- (3.9x@3.0e-33) O. algarvensis Gamma 3 symbiont
- (4.0x@1.0e-28) Hahellaceae
- (3.8x@7.0e-30) Candidatus Accumulibacter phosphatis

Bin K

N50=4884nt

< 1.0 kb
1.0 - 3.5 kb
3.5 - 7.0 kb
7.0 - 15.0 kb
15.0 - 30.0 kb
30.0 - 60.0 kb
> 60.0 kb

2607 hits/4666 orfs:
- (1.2x@7.0e-61) Campylobacteraceae
- (1.0x@5.0e-43) Helicobacteraceae
- (1.0x@9.0e-39) Sulfurovum sp. NBC37-1
- (1.1x@2.0e-31) Rhodobacteraceae
- (1.2x@1.0e-46) Halanaerobiaceae

Bin L

N50=28606nt

< 1.0 kb
1.0 - 3.5 kb
3.5 - 7.0 kb
7.0 - 15.0 kb
15.0 - 30.0 kb
30.0 - 60.0 kb
> 60.0 kb

1717 hits/3277 orfs:
- (1.9x@1.0e-84) Campylobacteraceae
- (1.7x@1.0e-84) Helicobacteraceae

Bin M

N50=375nt

< 1.0 kb
1.0 - 3.5 kb
3.5 - 7.0 kb
7.0 - 15.0 kb
15.0 - 30.0 kb
30.0 - 60.0 kb
> 60.0 kb

2160 hits/3258 orfs:
- (3.1x@4.0e-28) Campylobacteraceae
- (3.2x@6.0e-20) Helicobacteraceae
- (3.1x@4.0e-24) Sulfurovum sp. NBC37-1 [family]
- (3.6x@1.0e-19) Flavobacteriaceae

Bin N

N50=2434nt

< 1.0 kb
1.0 - 3.5 kb
3.5 - 7.0 kb
7.0 - 15.0 kb
15.0 - 30.0 kb
30.0 - 60.0 kb
> 60.0 kb

1792 hits/2501 orfs:
- (0.7x@3.0e-59) Helicobacteraceae
- (1.0x@4.0e-55) Campylobacteraceae
- (0.7x@6.0e-51) Sulfurovum sp. NBC37-1
- (0.9x@2.0e-30) Nautiliaceae
- (1.4x@4.0e-15) Aeromonadaceae

Bin O

N50=2236nt

< 1.0 kb
1.0 - 3.5 kb
3.5 - 7.0 kb
7.0 - 15.0 kb
15.0 - 30.0 kb
30.0 - 60.0 kb
> 60.0 kb

1162 hits/2109 orfs:
- (0.7x@2.0e-47) Acholeplasmataceae
- (0.5x@6.0e-61) Flavobacteriaceae
- (0.7x@1.0e-37) Bacillaceae
- (1.0x@1.0e-38) Lachnospiraceae
- (0.7x@5.0e-46) Clostridiaceae
- (0.7x@2.0e-58) Thermoanaerobacteraceae
- (0.7x@4.0e-30) Peptostreptococcaceae
- (0.7x@1.0e-46) Peptococcaceae

Figure IV-3 I Contig length distribution and phylogenetic profile (family level) of each of the 15 bins obtained. Median e values for each family are indicated.

163

The per-sample abundances of the populations associated with the bins were estimated based on sequencing coverage. Previously, it was shown that estimates obtained with this approach correlated well with results from fluorescence *in situ* hybridization (FISH) (Hanke *et al.* 2014). The results are shown in Fig. IV-4a. In three out of four samples, populations affiliated with *Gammaproteobacteria* were more abundant at 10 °C and populations affiliated with *Alphaproteobacteria* were more abundant at 25 °C. Using previously published 16S rRNA tag sequencing data (Fuhrman *et al.* 2008, Sharp *et al.* 2014), we investigated whether this observation was part of a more general trend. However, this did not appear to be the case; no relationship between temperature and relative abundance of *Alpha-* and *Gammaproteobacteria* was observed in data from two studies (Fuhrman *et al.* 2008, Sharp *et al.* 2014) that addressed the effect of temperature on microbial community composition. In some samples (e.g. 10-B1, 10-B3), a lower portion of the reads was assembled and a lower portion of assembled contigs could be binned. This most likely resulted from a higher biodiversity in these samples, leading to less effective assembly and shorter contigs that could not be binned with confidence based on tetranucleotide frequencies.
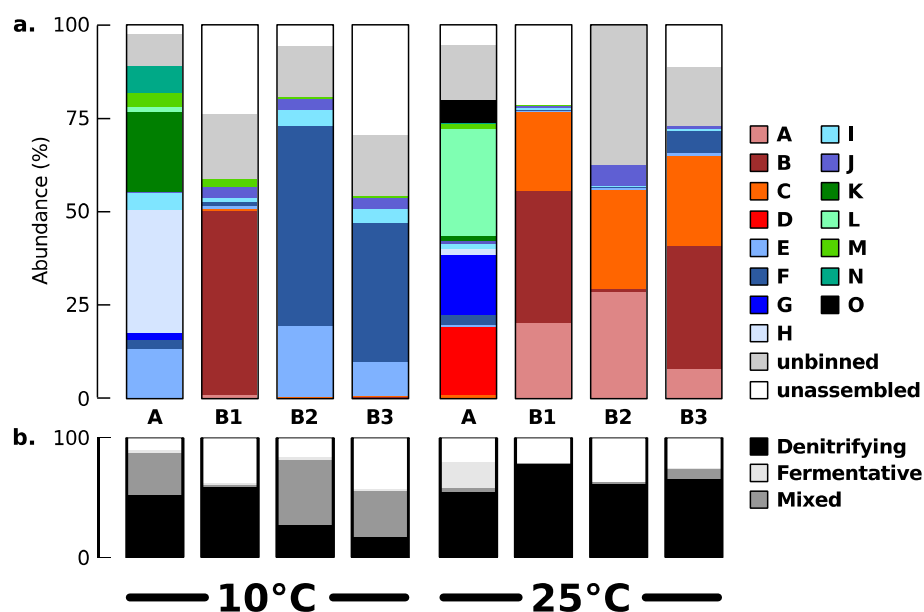
Figure IV-4 I Estimated abundances of the binned populations (Fig. IV-3) and their contributions to denitrification and fermentation. (a) Population abundances in the four cultures at different time points (Fig. IV-1) based on mapping of sequencing reads to the assembled contigs. Populations affiliated with Alpha-, *Gamma-* and *Epsilonproteobacteria* are shown in red/orange, blue and green colors, respectively. (b) Total abundances of populations contributing to denitrification, fermentation and both processes simultaneously (mixed) based on detection of functional genes (Tab. IV-2).

16S ribosomal gene sequences were recovered from the data with two independent approaches: detection in the assembled contigs and by iterative read mapping. In total, 27 sequences were recovered, including a number of incomplete genes (Fig. IV-4). Iterative read mapping yielded 4 genes that were identical to genes detected in the assembly. 16S rDNA genes generally have atypical tetranucleotide frequencies and higher sequencing coverages so they cannot be binned with high confidence. Therefore, 16S rDNA genes were assigned to bins based on high Pearson correlation coefficients of bin and gene abundances over the eight different samples and consistent taxonomic affiliations of bins (Fig. IV-3) and genes (Fig. IV-5). In some cases, it was not possible to conclusively link 16S rRNA genes with bins. For example, the precise affiliations of populations associated with bins A and B (both *Rhodobacterales*), bins F and G (both *Vibrionales/Photobacterium*) and bins L and M (both *Arcobacter*) could not be unambiguously determined.

To unravel the ecological roles of the different populations, the contigs constituting each bin were annotated as a provisional whole genome sequence. First, the degree to which this whole genome sequence was complete was evaluated by detecting a set of 139 conserved single copy genes (CSCG's; Campbell *et al.* (2013)) and transfer RNA genes (Tab. IV-1). It appeared that bin A contained sequence data from multiple (related) populations within *Rhodobacterales*, as shown by the presence of multiple copies of CSCG's. Many other bins also appeared to contain sequence data from multiple related populations and two bins appeared to contain an obviously incomplete whole genome sequence (bins J and M).

Figure IV-5 *(on the next page)* **I** Phylogenetic analysis of assembled 16S rRNA sequences and assignment of these sequences to the bins based on matching phylogenetic profiles (Fig. IV-3) and correlations between 16S rRNA gene abundances and bin abundances (Fig. IV-4). For partial sequences the length is indicated. For each sequence recovered, the sample and contig identifier is indicated. Independent recovery by iterative read mapping is indicated with *.

10-B3/00938 (570 nt)
CP002623 *Roseobacter litoralis*
M59063 *Roseobacter denitrificans*
JN248461 Sulfitobacter sp. RCC 2406
FR685915 uncultured marine bacterium
GU584500 uncultured bacterium
25-A/02150*
HQ203938 uncultured bacterium
AALY01000002 *Roseovarius nubinhibens*
HM116053 uncultured Roseovarius sp.
25-B1/00979 (1086 nt)
GQ274080 uncultured alpha proteobacterium
FJ869041 Rhodobacterales bacterium CB1005
CP000264 Jannaschia sp. CCS1
CP000830 *Dinoroseobacter shibae*
25-B3/07136 (642 nt)
AJ301667 *Maricaulis virginensis*
CP002568 *Polymorphum gilvum*
JF767277 uncultured marine bacterium
25-B3/03328*
AB583774 *Maritalea porphyrae*
AB681571 *Robiginosimarina momoshimaensis*
CP002447 *Mesorhizobium ciceri*
CP001830 *Sinorhizobium meliloti*
EU709017 *Pelagibacterium halotolerans*
FJ716857 uncultured bacterium
25-A/00477
FJ202706 uncultured bacterium
AB680460 *Terasakiella pusilla*

25-B2/06662 (510 bp)
CP003241 Vibrio sp. EJY3
FM204866 *Vibrio parahaemolyticus*
CP001805 Vibrio sp. Ex25
25-B2/02402 (1110 bp)
FM954972 *Vibrio splendidus*
10-B1/01179 (1230 bp)
CP001133 *Vibrio fischeri*
GU223580 Vibrio sp. F74
10-A/02810
10-B2/02894 (991 bp)
FJ467295 Photobacterium sp. QD-1A
25-A/02724 (1045 bp)
DQ660390 Photobacterium sp. JCS290
AJ630157 Photobacterium sp. J15
25-B3/12611*
10-A/02852
CP000083 *Colwellia psychrerythraea*
JN019002 uncultured bacterium
HQ225261 uncultured bacterium
10-B3/01683 (546 bp)
AB162002 *Thalassomonas agarivorans*
25-B2/04479 (706 bp), 25-B2/00381 (556 bp)
AF432145 *Sedimenticola selenatireducens*
HQ697917 Marinobacter sp. enrichment culture clone DF58
25-B1/01135 (617 bp)
CP000514 *Marinobacter aquaeolei*
10-A/04780 (636 bp)
FJ196022 Marinobacter sp. ZS2-30
10-B1/03181 (753 bp)
EU287245 uncultured bacterium
AB330881 *Amphritea japonica*
JF747202 Neptunomonas sp. P10-2-4
JN256092 uncultured bacterium
10-B2/01054*
GQ241352 Pseudomonas sp. C23
HQ403254 uncultured bacterium
25-B3/00667 (1299 bp)
FJ825447 uncultured bacterium
AF110005 Thauera sp. MZ1T

10-A/00625
EU052236 uncultured epsilon proteobacterium
EU512920 *Arcobacter marinus*
CP001999 *Arcobacter nitrofigilis*
10-B3/05009 (528 bp)
AB243994 uncultured bacterium
FJ959747 uncultured bacterium
CP000361 *Arcobacter butzleri*
HQ203962 uncultured bacterium
HM057704 uncultured bacterium
25-A/00564
10-B3/06810 (493 bp)
HQ203797 uncultured bacterium
FJ717122 uncultured bacterium
10-A/01709
L40808 *Sulfurimonas denitrificans*
25-A/01230
AB369171 uncultured bacterium
CU469464 Candidatus "Phytoplasma mali"
JN935890 *Acholeplasma laidlawii*
Flavobacteria

Bin A, B

Bin C

Bin D

Bin H

Bin F, G

Bin E

Bin J

Bin I

Bin K

Bin L, M

Bin N

Bin O

Alphaproteobacteria

Gammaproteobacteria

Epsilon proteo bacteria

Molli cutes

0.02 changes per site (1513 positions)

Table IV-1 I Properties of the 15 bins obtained by tetranucleotide binning of eight metagenomes obtained from the four cultures.

Bin	A	B	C	D	E	F	G	H
Affiliation	Rhodo-bacter-ales	Rhodo-bacter-ales	Mari-talea	Terasa-kiella	Col-wellia	Photo-bacter-ium	Photo-bacter-ium	Vibrio
Size (kb)	5.85	3.34	3.82	3.37	4.13	5.77	3.74	5.21
Number of contigs (#)	2552	558	2076	523	3520	1147	1392	1013
N50 contig length (kb)	71.8	20.5	5.6	10.0	1.7	21	3.6	7.6
GC content (%)	51.6	59.3	52.0	47.7	36.2	47.3	43.4	41.7
Number of CSCGs* (#)	235	113	131	111	111	134	158	163
Number of tRNAs (#)	53	40	32	34	25	54	66	52
Sample/Bin	25-B2/M0	25-B1/L0	25-B1/FD	25-A/L1	10-B2/M4	10-B2/L0	25-A/L2	10-A/L0

Bin	I	J	K	L	M	N	O
Affiliation	Pseudo-monas	Chroma-tiales	Arco-bacter	Arco-bacter	Arco-bacter	Sulfuri-monas	Achole plasma-tales
Size (kb)	2.38	3.30	3.45	2.92	0.96	1.61	1.41
Number of contigs (#)	3141	5349	1095	206	2640	762	704
N50 contig length (kb)	0.97	0.77	4.9	28.6	0.38	2.4	2.2
GC content (%)	48.8	57.0	26.9	27.6	29.0	32.5	32
Number of CSCGs* (#)	118	80	170	113	55	95	96
Number of tRNAs (#)	22	29	40	33	10	23	13
Metawatt bin	10-B3/L3	25-B2/H6	10-A/L1	25-A/L0	10-B1/L5	10-A/L2	25-A/L4

*) Number of Conserved Single Copy Genes detected (out of a set of 139). Numbers higher than 139 indicate the presence of DNA originating from more than a single population in the bin. Numbers lower than 139 indicate the provisional genome sequence associated with the bin may be incomplete.

Unfortunately, annotation was difficult because of the presence of many stop codons within genes of contigs assembled from reads generated on the Ion Torrent Personal Genome Machine®. These (presumably artifactual) stop codons resulted from sequencing errors, and were also previously observed (Kraft *et al.*, in press). Further, correct prediction of open reading frames in short contigs proved to be difficult. However, annotation did still provide some evidence for the presence of genes associated with denitrification in most of the populations. Also, it was apparent that some populations were capable of fermentation *via* pyruvate-formate-lyase. This enzyme converts pyruvate into formate and acetate.

Pyruvate could be produced from glucose, alanine and serine, together accounting for over 50 % of the carbon substrates provided to the culture. Transcriptomics analysis of RNA extracted at two time points in two cultures (Fig. IV-1) showed active transcription of many of these genes (Supplementary Information).

Based on these observations, we defined four key functional genes associated with denitrification and fermentation: *nirK* and *nirS*, encoding copper and cytochrome cd_1-type nitrite reductases, *nosZ*, encoding nitrous oxide reductase and *pflB*, encoding the catalytic subunit of pyruvate-formate-lyase. These genes were detected with high sensitivity with Hidden Markov Models on six-frame translations of all contigs. This way, the problems with the prediction of open reading frames could be overcome. All DNA regions for which one of these genes was predicted to be present were subsequently investigated with BLASTx against a database with general reference sequences, to be absolutely certain that the prediction of these genes was correct. The phylogenetic affiliation of the best BLAST hits was compared to the taxonomic assignments of the bins, to make sure that misbinning of short contigs did not lead to false conclusions. Table IV-2 shows the genes detected this way, their transcriptional activities, binning and phylogenetic affiliation. The *Alpha-* and *Epsilonproteobacteria* (bins A-D and bins K-N) all performed denitrification. Among *Gammaproteobacteria*, the three populations affiliated with *Vibrionales/Photobacteria* were fermentative, but two of them also reduced nitrous oxide to nitrogen. The other *Gammaproteobacteria* only performed denitrification. The population affiliated with *Acholeplasmatales* was strictly fermentative.

Table IV-2 I Key functional genes involved in denitrification (*NirK, NirS, NosZ,* encoding Copper-type nitrite reductase, cytochrome *cd1* nitrite reductase and nitrous oxide reductase, respectively) and fermentation (*pflB*, encoding pyruvate-formate-lyase) detected on binned contigs. Gene activity was inferred from transcriptomic analyses, a value of 1.0 indicates the average gene activity for the bin as a whole. Transcriptomes were only performed for the 2012 culture. If no transcripts were detected for populations present in the 2011 cultures, this is indicated with '-'.

Contig	Gene	Contig length	Gene position	Strand	Affiliation	Activity	Bin
25-B2/contig00017	*nirK*	71,778	45,446-46,495	-	*Maritalea*	1.5	A
25-B1/contig00217	*nirK*	9,174	2,768-3,662	-	*Maritalea*	1.7	C
25-A/contig00033	*nirS*	30,072	25,343-26,816	-	*Thalassospira*	0.1	D
25-A/contig00158	*nirS*	12,152	6,680-8,231	-	*Thioalkalivibrio*	3.2	D
10-B2/contig00974	*nirS*	2,242	904-2,232	-	*Colwellia*	2.6	E
25-B2/contig00523	*nirS*	2,510	980-2,228	-	*Sedimenticola*	0.0	J
25-B2/contig01160	*nirS*	1,675	4-1,195	-	*Sedimenticola*	3.8	J
10-A/contig00157	*nirS*	10,634	2,400-3,807	+	*Nitratiruptor*	3.9	K
25-A/contig00002	*nirS*	107,659	45,626-47,102	-	*Methylomonas*	-	L
25-A/contig00024	*nirS*	33,188	22,075-24,286	-	*Sulfurimonas*	-	L
10-B1/contig19936	*nirS*	313	1-313	+	*Sulfurovum*	-	M
25-B2/contig01207	*nosZ*	1,639	2-1,634	+	*Maritalea*	31	A
25-B2/contig03862	*nosZ*	794	6-792	-	*Maritalea*	7.4	A
25-B2/contig05570	*nosZ*	586	14-577	+	*Alcaligenes*	25	A
25-B1/contig00005	*nosZ*	50,905	24,280-26,089	-	*Ruegeria*	1.5	B
25-A/contig00045	*nosZ*	25,838	2,465-4,382	-	*Azospira*	1.9	D
10-B2/contig01391	*nosZ*	1,717	554-1,714	-	*Colwellia*	4.9	E
10-B2/contig00170	*nosZ*	12,996	5-1,668	-	*Photobacterium*	4.4	F
10-A/contig00784	*nosZ*	4,544	272-2,114	-	*Photobacterium*	0.8	H
10-B3/contig01180	*nosZ*	1,384	30-1,341	+	*Pseudomonas*	1.4	I
10-B3-contig01396	*nosZ*	1,256	25-385	+	*Pseudomonas*	2.7	I
25-B2/contig00886	*nosZ*	1,931	919-1,921	-	*Sedimenticola*	3.8	J
25-B2/contig00727	*nosZ*	2,135	8-889	-	*Sedimenticola*	3.7	J
10-A/contig00156	*nosZ*	10,642	5,801-7,757	-	*Campylobacter*	3.4	K
25-A/contig00195	*nosZ*	10,710	8,678-10,643	-	*Campylobacter*	0.6	L
10-A/contig04078	*nosZ*	1,065	7-1,063	-	*Wolinella*	-	N
10-B2/contig00028	*pflB*	33,743	4,060-6,813	+	*Vibrio*	0.04	F
10-B2/contig00038	*pflB*	31,309	8,080-9,932	-	*Photobacterium*	1.4	F
10-B2/contig00238	*pflB*	9,510	8,098-9,499	+	*Vibrio*	0.1	F
25-A/contig00439	*pflB*	5,996	54-1,652	+	*Enterobacter*	-	G
25-A/contig01759	*pflB*	1,841	46-1,834	-	*Vibrio*	1.2	G
25-A/contig02479	*pflB*	1,235	6-1,185	-	*Vibrio*	-	G
10-A/contig00058	*pflB*	15,024	9,499-11,539	+	*Vibrio*	1.8	H
10-A/contig00738	*pflB*	4,717	1,138-3,426	+	*Tolumonas*	-	H
10-A/contig01764	*pflB*	2,564	427-2,552	+	*Vibrio*	-	H
25-A/contig01805	*pflB*	1,800	9-1,793	-	*Acholeplasma*	-	O

Potential fermentation and denitrification rates were determined experimentally between days 195 and 235 for the 2012 cultures at both temperatures. During this period, denitrification in the 10 °C culture collapsed completely. Consistent with

metagenomic inferences, acetate and formate were the main fermentation products. Fermentation rates were not correlated with denitrification rates but remained high after denitrification collapsed (Fig. IV-6). Figure IV-4b confirmatively shows that in three out of four samples fermentative populations were more abundant in the 10 °C cultures than in the 25 °C cultures.

10°C   25°C
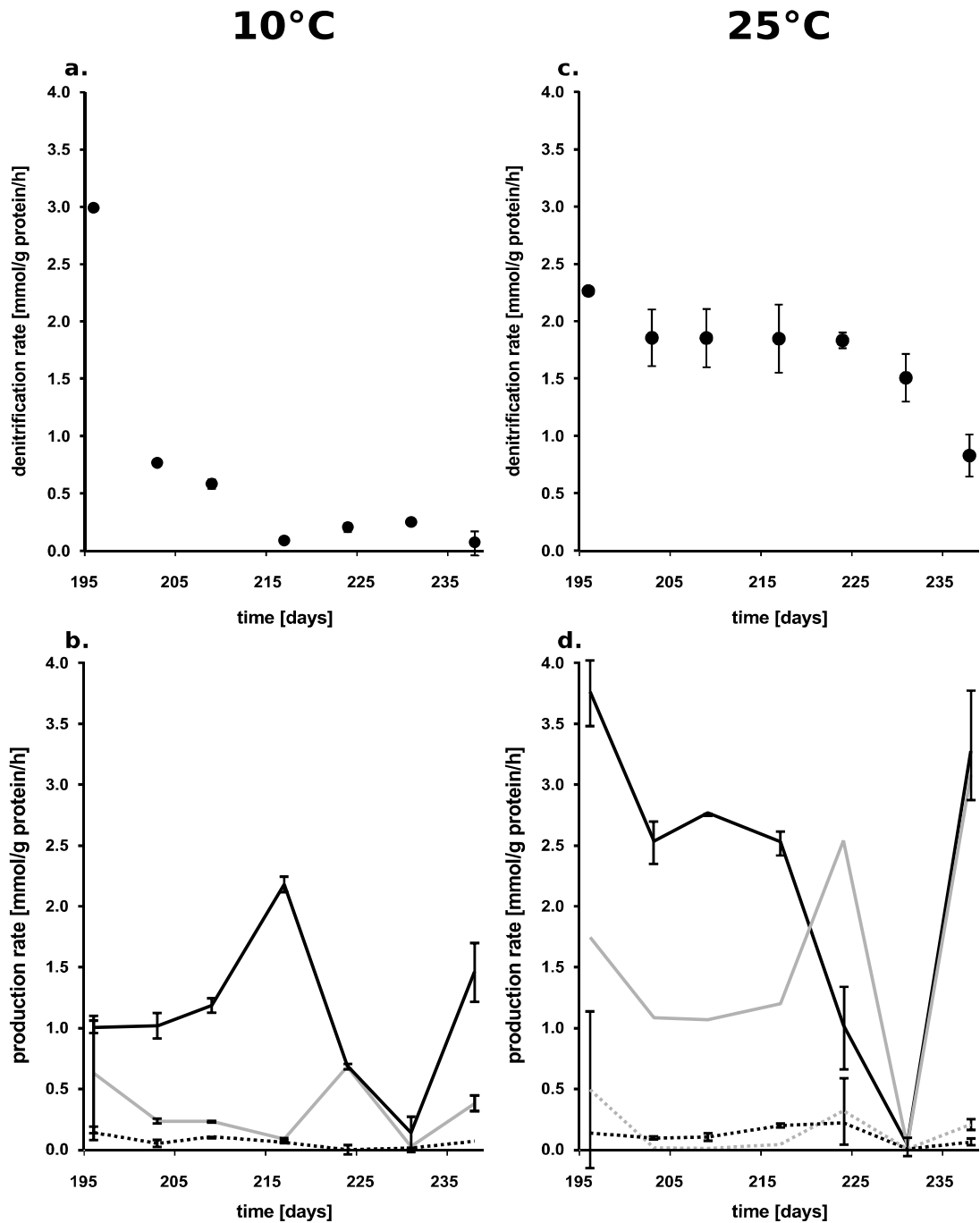
Figure IV-6 I Trends in potential denitrification rates (circles) and potential fermentation rates in the 10 °C (a,b) and 25 °C (c,d) cultures. Fermentation product formation rates are shown for formate (black solid line), acetate (grey solid line), succinate (black dashed line) and butyrate (grey dashed line).

170

This was to be expected, because as shown in Figure IV-1, the carbon to nitrogen ratio in the medium supplied to the 10 °C cultures was higher. Previous studies of continuous cultures performed under similar conditions and inoculated with bacteria from the same sediments showed that a consortium of fermentative and denitrifying bacteria was selected (Hanke *et al.* 2014, Kraft *et al.*, in press) and this was also observed in the present study. However, in the present case, the consortium was apparently unable to sustain denitrification at 10 °C.

It is possible that this was caused by the selection of denitrifying populations (such as the population related to *Colwellia psychrerythraea*) at 10 °C that did not use the fermentation products but competed directly with the fermentative populations for the supplied glucose and aminoacids. If fermentative populations have a higher affinity for these substrates, as previously suggested (Kraft *et al.,* in press), denitrifiers would be outcompeted for carbon substrates, leading to a collapse of denitrification. In this scenario, supply of additional carbon would provide temporal relief to the denitrifiers, until the fermentative populations caught up, as observed experimentally. Although the denitrifying populations did not encode pyruvate-formate-lyase, many species of the genus *Colwellia* and the family *Rhodobacteraceae* are facultatively fermentative. Further, half of the selected fermentative populations also contributed to denitrification (activity of *nosZ*). That means that the spontaneous assembly of a consortium with an effective division of labor may not be trivial and, apparently, this did not happen in two independent experiments inoculated with sediments sampled in two different years and was related to low temperature.

Interestingly, the population affiliated with *Terasakiella* (Bin D) encoded a putative nitric oxide dismutase in its genome (Fig. IV-7). Nitric oxide dismutase was previously proposed to catalyze the dismutation of two molecules of nitric oxide (NO) into oxygen (O_2) and nitrogen (N_2). This reaction enables the intra-aerobic oxidation of methane coupled to denitrification (Ettwig *et al.* 2010). The reaction was proposed to be facilitated by an enzyme similar to quinol dependent nitric oxide reductase encoded by the gene norZ (Ettwig *et al.* 2012). The active site of nitric oxide dismutase was shown to have several characteristic amino acid modifications that were also encoded in the gene detected in bin D.
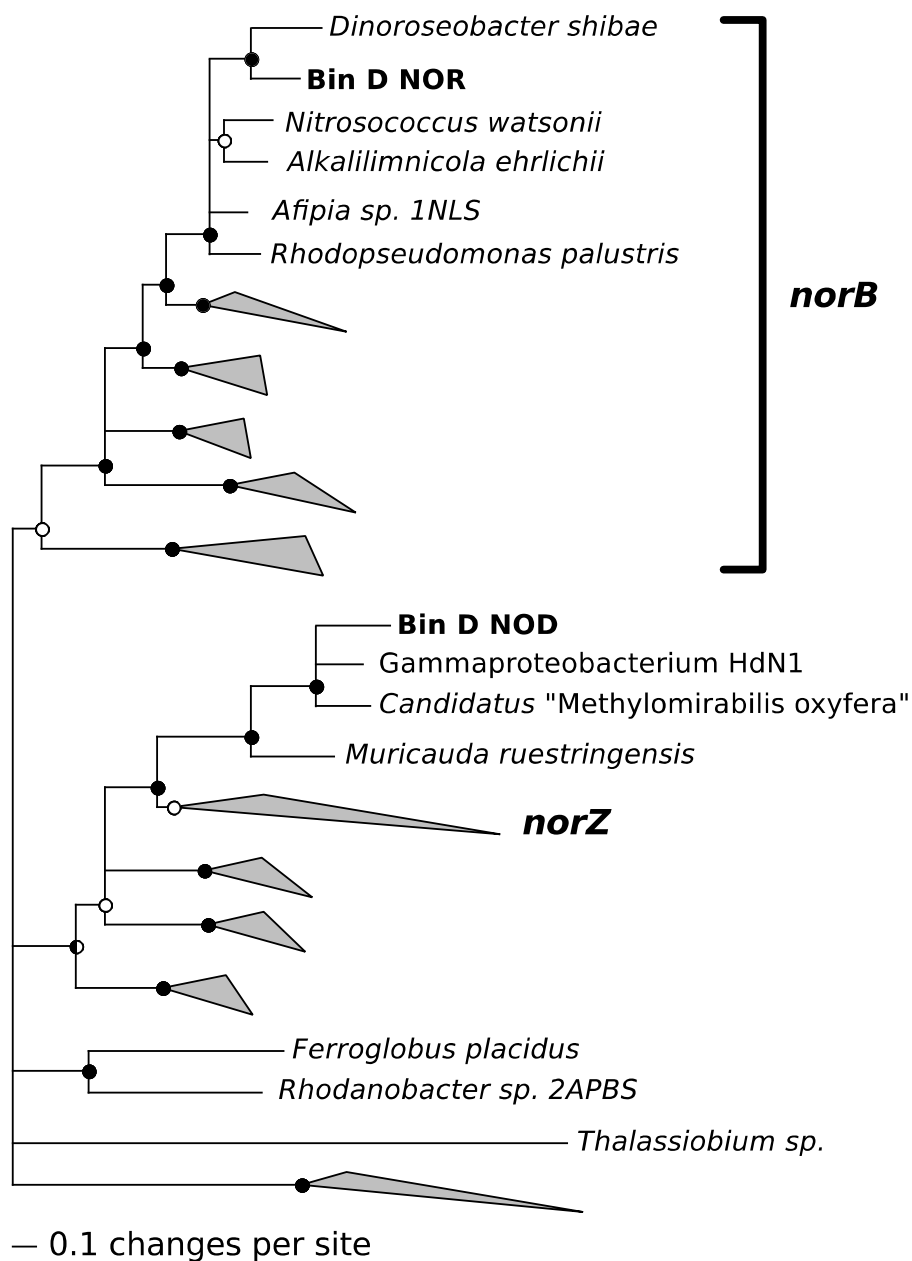
Figure IV-7 I Phylogenetic analysis of the nitric oxide reductase and putative nitric oxide dismutase genes of bin D.

The physiological role of the enzyme in the population enriched in the present study is unknown, as no methane or alkanes were provided in the medium and transcriptomics analysis showed that the gene was inactive in the culture. However, based on its 16S rRNA sequence, a nearly identical bacterium was isolated from an oil well in the North Sea (Bødtker *et al.* 2009), where both alkanes and nitrate were available, indicating that alkane oxidation coupled to denitrification might be an essential aspect of the physiology of these organisms.

References

Allen, A. P., J. F. Gillooly, and J. H. Brown. (2005) „Linking the global carbon cycle to individual metabolism." *Functional Ecology* 19:202-213.

Byrnes, B. H. (1990) „Environmental effects of N fertilizer use - An overview." *Fertilizer Research* 26:209-215.

Bendschneider, Kenneth and Rex J. Robinson. (1952) „A new spectrophotometric method for the determination of nitrite in sea water." *Journal of Marine Research* 11:87-96.

Bødtker, Gunhild, Christine L. Lysnes, Terje Torsvik, Eva Ø. Bjørnestad, and Egil Sunde. (2009) „Microbial analysis of backflowed injection water from a nitrate-treated North Sea oil reservoir." *Journal of Industrial Microbiology* 36:439-450.

Bondarev, Vladimir, Michael Richter, Stefano Romano, Jörn Piel, Anne Schwedt, and Heide N. Schulz-Vogt. (2013) „The genus *Pseudovibrio* contains metabolically versatile bacteria adapted for symbiosis." *Environmental Microbiology* 15(7):2095-2113.

Cubasch, U., *et al*. „Introduction." In *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*, by T. F. Stocker, *et al*. Cambridge, UK and New York, USA: Cambridge University Press, 2013.

Campbell, James H., *et al*. (2013) „UGA is an additional glycine codon in uncultured SR1 bacteria from the human microbiota." *Proceedings of the National Academy of Sciences* 110(14):5540-5545.

Carpenter, Lucy J., Stephen D. Archer, and Rachael Beale. (2012) „Ocean-atmosphere trace gas exchange." *Chemical Society Reviews* 41:6473-6506.

Crutzen, P. J. (1970) „The influence of nitrogen oxides on the atmospheric ozone content." *Quarterly Journal of the Royal Meteorological Society* 96:320-325.

Eddy, S. R. (2011) „Accelerated profile HMM searches." *PLOS Computational Biology* 7(10):e1002195.

Ettwig, Katharina F., *et al.* (2010) „Nitrite-driven anaerobic methane oxidation by oxygenic bacteria." *Nature* 464: 543-548.

Ettwig, Katharina F., Daan R. Speth, Joachim Reimann, Ming L. Wu, Mike S. M. Jetten, and Jan T. Keltjens. (2012) „Bacterial oxygen production in the dark." *Frontiers in Microbiology* 3:article 237.

Fuhrman, Jed A., *et al.* (2008) „A latitudinal diversity gradient in planktonic marine bacteria." *Proceedings of the National Academy of Science* 105(22):7774-7778.

Gruber, Nicolas and James N. Galloway. (2008) „An Earth-system perspective of the global nitrogen cycle." *Nature* 451:293-296.

Hanke, Anna & Marc Strous. (2010) „Climate, Fertilization, and the Nitrogen Cycle." *Journal of Cosmology* 8:1838-1845.

Hanke, Anna, *et al.* (2014) „Recoding of the stop codon UGA to glycine by a BD1-5/SN-2 bacterium and niche partitioning between *Alpha-* and *Gammaproteobacteria* in a tidal sediment microbial community naturally selected in a laboratory chemostat." *Frontiers in Microbiology* 5:Article 231

Katoh, Kazutaka, Kazuharu Misawa, Kei-ichi Kuma, and Takashi Miyata. (2002) „MAFFT: a novel method for rapid multiple sequence alignment based on fast fourier transform." *Nucleic Acid Research* 30(14):3059-3066.

Kraft, B., *et al.* „The environmental controls that govern the end product of bacterial nitrate respiration." *Science*, - in press.

Langmead, Ben and Steven L. Salzberg. (2012) „Fast gapped-read alignment with bowtie 2." *Nature Methods* 9(4):357-360.

Laslett, Dean and Bjorn Canback. (2004) „ARAGORN, a program to detect tRNA genes and tmRNA genes in nucleotide sequences." *Nucleic Acids Research* 32(1):11-16.

Lowry, Oliver H., Nira J. Rosebrough, A. Lewis Farr, and Rose J. Randall. (1951) „Protein measurement with the folin phenol reagent." *The Journal of Biological Chemistry* 193:265-275.

Miller, Christopher S., Brett J. Baker, Brian C. Thomas, Steven W. Singer, and Jillian F. Banfield. (2011) „EMIRGE: reconstruction of full-length ribosomal genes from microbial community short read sequencing data." *Genome Biology* 12:R44.

Osaka, Toshifumi, Kosuke Shirotani, Sachiko Yoshie, and Satoshi Tsuneda. (2008) „Effects of carbon source on denitrification efficiency and microbial community structure in a saline wastewater treatment process." *Water Research* 42:3709-3718.

Quast, Christian, *et al.* (2013) „The SILVA ribosomal RNA gene database project: improved data processing and web-based tools." *Nucleic Acids Research* 41:(D1):D590-D596.

Price, Morgan N., Paramvir S. Dehal, and Adam P. Arkin. (2010) „FastTree 2 – Approximately Maximum-Likelihood Trees for Large Alignments." *PLOS one* 5(3):e9490.

Seemann, Torsten. (2014) „Prokka: Rapid Prokaryotic Genome Annotation." *Bioinformatics* 30(14): 2068-2069.

Seiser, A. and L. Walz. (1925) „Stickstoffumsatz bei der Denitrifikation." *Archiv für Hygiene* 95:189-208.

Sharp, Christine E., Allyson L. Brady, Glen H. Sharp, Steven E. Grasby, Matthew B. Stott, and Peter F. Dunfield. (2014) „Humboldt's spa: microbial diversity is controlled by temperature in geothermal environments." *The ISME Journal* 8:1166-1174.

Smith, Keith A. (1997) „The potential for feedback effects induced by global warming on emissions of nitrous oxide by soils." *Global Change Biology* 3:327-338.

Solorzano, Lucia. (1969) „Determination of Ammonia in Natural Waters by the Phenolhypochlorite Method." *Limnology and Oceanography* 14(15):799-801.

Strous, M., B. Kraft, R. Bisdorf, and H. E. Tegetmeyer. (2012) „The binning of metagenomic contigs for microbial physiology of mixed cultures." *Frontiers in Microbiology* 3:Article 410.

Tallec, Gaelle, Josette Garnier, and Michel Gousailles. (2006) „Nitrogen removal in a wastewater treatment plant through biofilters: nitrous oxide emissions during nitrification and denitrification." *Bioprocess and Biosystems Engineering* 29:323-333.

Thomann, R. V. and J. A. Mueller. *Principles of surface water quality modeling and control.* New York: Harper & Row, 1987.

Veraart, Annelies J., Jeroen J. M. de Klein, and Marten Scheffer. (2011) „Warming Can Boost Denitrification Disproportionately Due to Altered Oxygen Dynamics." *PLOS one* 6(3):e18508.

Vitousek, Peter M., *et al*. (1997) „Human Alteration of the Global Nitrogen Cycle: Sources and Consequences." *Ecological Applications* 7(3):737-750.

Zhou, Jizhong, Mary Ann Bruns, and James M. Tiedje. (1996) „DNA Recovery from Soils of Diverse Composition." *Applied and Environmental Microbiology* 62(2):316-322.

Supplementary Material

Due to the large sizes of the supplementary tables, this data is not presented in the context of this thesis.

Supplementary Information for this manuscript is available online using the following link: http://www.mpi-bremen.de/Addendum.html

General discussion and conclusions

Importance of the nitrogen cycle

Carbon dioxide is the most important greenhouse gas in the atmosphere and contributes most to global warming. However, the importance of two other "gases of most concern" (Cubasch *et al.* 2013), methane (CH_4) and nitrous oxide (N_2O), are also significant and, because of their longer atmospheric lifetime, will become even more important in the future. From pre-industrial times (around 1750) until 2011 the atmospheric abundances of CO_2, N_2O and CH_4 have increased with 40, 20, and 150 %, respectively (Hartmann *et al.* 2013). Although efforts have been made to reduce the emission of greenhouse gases (GHG) for several years, the average annual increases in N_2O and CO_2 abundance between 2005 and 2011 are still in the same range as the ones determined between 1996 and 2005 (Hartmann *et al.* 2013). N_2O has a global warming potential approx. 300 times that of CO_2 over a 100-year horizon (Manne & Richels 2001) and a budget lifetime of about 120 years (Manne & Richels 2001, Prather & Hsu 2010). N_2O emissions to the atmosphere lead to ozone depletion in the mid to upper stratosphere (Prather & Hsu 2010). Currently, N_2O is the most important stratospheric ozone-depleting substance (Ravishankara *et al.* 2009) by serving as the major source for stratospheric nitric oxide (NO) and nitrogen dioxide (NO_2), which catalytically destroy ozone (Crutzen 1970). Given these facts, one can easily conclude that the nitrogen cycle has a considerable influence on the global climate. Taking into account that human activities have an even stronger influence on the nitrogen cycle (>100 % of the natural background (Hanke & Strous 2010)) than on the carbon cycle (5 % of the natural background (Hanke & Strous 2010)) the need for extensive research focusing on the nitrogen cycle as well as the development of strategies to control N_2O production becomes obvious.

N_2O is either produced by lightning, fossil fuel burning (Mackenzie *et al.* 2002, Gruber & Galloway 2008) or by microbial activity involved in nitrification and denitrification (reviewed by Carpenter *et al.* (2012)). Microbial activity is stimulated by human activities: the use of fertilizers and ammonia deposition from fossil fuel burning are the main processes that contribute to the increased abundance of

bioavailable nitrogen (esp. nitrate and ammonia) in soils, rivers, and coastal regions (Colijn *et al.* 2002, Duce *et al.* 2008, Wolff *et al.* 2010, Jørgensen *et al.* 2014) feeding nitrifying and/or denitrifying microbes which produce N_2O either as a by-product (nitrification) or as an intermediate (denitrification). Under certain circumstances such as high oxygen concentrations, as e.g. shown by the present work (Chen *et al.*, submitted), these N_2O fluxes are increased. Oxygen favors nitrification as an aerobic process, causing the release of N_2O as a by-product as well as NO_3^-, which subsequently feeds denitrification. Denitrification, although previously assumed to be anaerobic (see introduction), is active in the presence of oxygen as well (Robertson & Kuenen 1984, Gao *et al.* 2010, Chen *et al.*, submitted). However, aerobic denitrification is often incomplete as nitrous oxide reductase, the enzyme responsible for the reduction of N_2O to N_2, is inactivated by oxygen (Otte *et al.* 1996, Chen *et al.,* submitted). This eventually leads to the release of N_2O rather than N_2.

Sandy sediments in intertidal zones are flushed with oxygen during high tide twice a day. The presence of oxygen in combination with eutrophication of coastal regions (De Jonge *et al.* 2002, Colijn *et al.* 2002, Wolff *et al.* 2010), make these regions potential hotspots for the emission of N_2O (Gao *et al.* 2010). In this context, it is important to unravel the ecology of the microbial nitrogen cycle (Hanke & Strous 2010). Recent discovery of previously unknown biogeochemical processes such as nitrite-driven anaerobic methane oxidation (Ettwig *et al.* 2010) justify speculations that there is still potential for discovering whole new pathways involved in the nitrogen cycle.

Implementation of a custom chemostat for the study of mixed cultures

The work presented in the course of this thesis clearly illustrates the possibility to enrich for environmentally important bacterial populations from a natural sample in the course of a chemostat experiment (Hanke *et al.* 2014). The eco-physiological characterization of mixed microbial communities is important for several reasons such as the fundamental understanding of the conditions that cause the selection of certain microbes in biotechnological processes, as for example wastewater

treatment facilities (Ginige *et al.* 2007). To effectively and efficiently study the organisms, their interactions and their reactions to environmental influences by -omics approaches, FISH, and stable-isotope labeling techniques, a simplified community representing the most important species of the natural habitat is needed. With the presented setup such a community can indeed be enriched and theoretically cultivated for an indefinite amount of time. Techniques applied in course of this work comprised continuous nutrient determinations, off-gas analyses, batch incubations, stable-isotope labeling, FISH, and -omics approaches. The cultivation setup combined with these techniques enables the thorough study of *in situ* relevant organisms under conditions sufficiently close to conditions prevalent in the natural habitat to study processes that are also important *in situ*. The community can be studied with respect to its composition, the identity and function of each member, and the interactions of and relationships among the individual organisms (Hanke *et al.* 2014, Chen *et al.,* submitted, Hanke *et al.*, in prep.). Outcomes of repeated culturing approaches could be proven comparable, which is why we can conclude that this setup allows for reproducible investigations (Chen *et al.,* submitted, Hanke *et al.*, in prep.).

Conditions created by a cultivation system can, of course, never perfectly mirror *in situ* conditions. However, this is not necessary as long as the conditions are close enough to mimic the natural habitat in a way that specific aspects are reproduced (Dytczak *et al.* 2008, Hanke *et al.* 2014). As we were able to reproduce *in situ* phenomena such as aerobic denitrification we can conclude that the applied conditions were sufficiently close to natural conditions. This is a large step towards the more detailed investigation of *in situ* relevant processes and interactions than is possible by *in situ* studies. The latter are restricted by the complex situation in marine sediments created by a high diversity of metabolically closely interacting species, physical disturbances (tides, waves, currents, macrobenthos activity), and finally by transient chemical changes due to human activity (fertilized land runoff, wastewater disposal, motorsport, shipping traffic). Of course, these influences are part of the natural system the bacterial community inhabits. But taking these influences into account all at once would render the reliable discussion of the outcome of such a study impossible. Therefore, reducing the number of influences

with help of a laboratory setup, which creates conditions sufficiently close to nature, is a welcome complement to *in situ* studies.

The presented chemostat setup system was shown to generate stable physical culture conditions over a long period of time. Internal system variations detected (transient changes of community composition (Chen *et al.,* submitted, Hanke *et al.,* in prep.) and fluctuations in metabolic activity (Hanke *et al.,* in prep.) did not arise from varying external conditions as these were shown to be stable (Hanke *et al.* 2014). Instead, the observed variations might have resulted from the undetected presence and influence of bacteriophages on the community. Another possible scenario is the influence of adaptation and evolution/mutation events leading to competition, which were shown to affect continuous cultures (Ferenci 2007). An environment set up in a chemostat is, even if constant, both a selection condition for fitter mutants and a stimulus for changes in gene expression (Ferenci 2007). The resulting changes could have caused the observed shifts in metabolic activity and community composition. Mutations causing the development of better adapted (fitter) mutants might have resulted for the outcompetition of previously successful community members. Due to the observed strong metabolic interactions in the cultures, slight metabolic changes (for example the production of a new resource) resulting from minor mutation events could have finally had an overall effect on the entire community structure.

Our studies show that the presented setup is a promising way to successfully enrich K-strategists. K-strategists have lower growth and substrate removal rates but higher substrate affinities (and accordingly lower K_m-values) than r-strategist populations (Blackburne *et al.* 2007, Dytczak *et al.* 2008). Controlling the operational strategies in continuous cultures favor the enrichment of either K- or r-strategists (Ginige *et al.* 2007, Dytczak *et al.* 2008). Intended periods of starvation were shown to be beneficial for the enrichment of K-strategists (Martin-Hernandez *et al.* 2009). Our setup allowed the enrichment of K-strategists under low but continuous medium supply.

Compared to traditional culturing approaches with pure cultures, the presented continuous cultivation approach enables insight in the interplay of different organisms. Common pure culture approaches are a good way to study single

bacterial species under restricted conditions such as the lack of contact to other species. This is a condition rarely met in nature as most microbes depend on interactions with other microbes or higher organisms (Morris *et al.* 2013). Such interactions comprise, among others, intra- and inter-species quorum sensing (Atkinson & Williams 2009), commensalism (Boucher 1988), syntrophy/crossfeeding (Morris *et al.* 2013) and parasitism (Guerrero *et al.* 1986, Davidov *et al.* 2006).

A scientific understanding of microbial (metabolic) interactions is essential for biotechnological approaches such as wastewater treatment or the realization of the proposed recovery of methane gas from its petroliferous resources. It will furthermore enable us to understand how nature copes with energetic constraints *via* unique biochemical mechanisms.

Restrictions and possible improvements of the custom chemostat

To reflect potential for improvement of the culturing device, this section addresses drawbacks and possible strategies to overcome those.

As mentioned before, the culture conditions created by the setup are similar but not identical to *in situ* conditions. The creation of *in situ* conditions of a habitat as complex as tidally influenced marine sediment is not possible but would also not be a step forward compared to studying the habitat *in situ*. One drawback of our system in this respect is the suspended growth of the microbes, which do normally mostly live attached to sand grains. However, suspended growth is essential to achieve defined environmental conditions. With biofilms on sand, substrate gradients will form and it is no longer possible to relate metagenomic results to environmental conditions. In many of our cultures (Chen *et al.,* submitted, Hanke *et al.,* in prep.) we identified *Vibrionales* as the main fermentative populations while in the habitat that served as the source for the inoculum, *Flavobacteria* were shown to be the dominant fermenting bacteria (Llobet-Brossa *et al.* 1998). Flavobacteria are capable of the degradation of carbohydrate polymers and proteins (Kirchman 2002). However, our system was amended with monomeric rather than polymeric substrates, which caused the development of a *Vibrionales* rather than a *Flavobacteria* population as the main fermenting bacterial group. In

so far unpublished pilot experiments, supply of bovine serum albumin (BSA) as the carbon source led to the enrichment of *Flavobacteria* instead of *Vibrionales*.

With our finding that denitrification and fermentation occurred in parallel in single cultures (Chen *et al.,* submitted, Hanke *et al.,* in prep.) we were faced with a higher degree of metabolic interaction between the community members than anticipated. Moreover, these two metabolic pathways were most likely even performed by every population enriched at low temperature, that is, single cells of all enriched populations in that culture were presumably capable of both metabolisms in parallel. Resulting from this finding is the obvious need for metabolomic approaches to study environmentally based continuous cultures to clearly unravel the interacting processes in such enriched communities.

A risk for the cause of failing of a chemostat experiment is the possibility of culture contaminations, for example by handling procedures or by unperceived supply of contaminated medium. There are different ways to prevent contaminations such as constant sterile operation and setups that hinder microbes from entering the culture once the medium is contaminated (e.g. the "back growth buffer" mentioned in Ferenci (2007) and Hanke *et al.* (2014)). Most contaminations caused by human operation will probably not influence an environmental bacterial community substantially as conditions optimized for an environmental community will in many cases not suit human derived microbes. This might eventually lead to an outcompetition of the "invaders" by the microbes adapted to the applied conditions. However, this is just an assumption and the key for preventing contaminations of a chemostat is that the user is aware of the risk and continuously abides all procedures avoiding contaminations.

Technical setups are generally prone to failures. This cannot be prevented; its risk can just be minimized to a certain degree. Minimizing the risk for failure of a long-term culture experiment lasting for several months or even years is essential for meeting time schedules and deadlines. Strategies to reduce technical failures of a chemostat system as the one described here are:

1. Regular control of tubes and -connections related to bending, clogging, brittleness

2. Regular replacement and/or rinsing of tubes prone to clogging

3. Regular cleaning, checking and/or replacement of sensors

4. Regular control, reference measurements with respect to balances and pumps

5. Early development of alternative strategies for the case of a power-shutdown

A long-term continuous culture experiment should be thoroughly planned. Delays of culture related experiments should be avoided as the communities in continuous cultures vary with time (Ferenci 2007, Hanke *et al.,* in prep*.,* Chen *et al.,* submitted). If batch incubations with culture liquid are conducted, all samples necessary to characterize the status quo of the culture have to be taken at the same time. Optimally, these comprise the chemical status of the culture and the headspace as well as the status of the community itself. Under certain circumstances it can be necessary to conduct further incubations after identifying the members of the community by metagenome sequencing. To produce fundamental evidence for the existence of certain species derived from genome analyses and to prove their proposed metabolism and their function within the community, labeling experiments or comparable investigations might be necessary. If the time span between the sampling for –omics analyses and obtaining its results is too long, the culture might for several reasons (evolution, system failures) no longer allow for an experimental verification of the results. An example is the enrichment of a *Terasakiella*-affiliated organism encoding a putative nitric oxide dismutase in one of our cultures. This was an interesting finding as this enzyme could be shown to facilitate intra-aerobic methane oxidation (Ettwig *et al.* 2012). However, in our case it took up to several months to obtain the results of the metagenomic analyses of our experiments. When we finally got aware of the existence of this organism and the mentioned gene by metagenomic results the organism had disappeared in course of culture-dynamics and could hence not be studied in more detail. Accordingly, the realization of a much more speedy metagenomic analysis of the cultures than applied in the course of this thesis is highly recommended. Another point is the overall time that is needed to obtain publishable results, which can constitute a challenge. We had to face the problem that our finding about the use of the alternate genetic code by the BD1-5/SN-2-population had just been published shortly before we were able submit the manuscript.

Therefore, we highly recommend to speed up data analyses in the course of continuous cultures by conducting such experiments in tight cooperation with different team-members being responsible for different experiment related tasks, such as the following:

1. *Bioreactor related (lab)work*: daily care and checking of cultures, regular culture liquid sampling, measurement of headspace gas composition, measurement of nutrient concentrations in liquid samples, data evaluation
2. *Extra (lab)work*: planning and conduction of batch incubations and labeling experiments, data evaluation
3. *–omics analyses*: sample preparation for –omics approaches, realization of sequence analyses (genomics, transcriptomics, and/or proteomics), computational data evaluation

Another advantageous modification of the system with respect to labor-intensity would be a reduction in system-complexity. This would offer the chance to conduct more than a single study at once. The described setup allowed for the care of about two cultures at the same time by a single person. The number of replicates was restricted by the maintenance requirements, the space required for large-sized equipment such as pumps and culture vessels, and finally the time necessary for maintenance of the cultures. However, the parallel conduction of more than two experiments at the same time is highly favorable in many cases. Therefore, for studies which do not rely on the full set of control and automation the described system offers, we recommend to simplify the system according to individual needs. A simplification of the system can for example be achieved by replacing the pH-control based on the automated supply of acid and base solutions and the permanent pH control with a well buffered medium and random manual inspections. The size of the system can be reduced by using smaller vessels with a simpler mixing setup, for example with help of a magnetic stirrer below the vessel. Using more than one of these systems in parallel would allow to supply all vessels with medium and/or gas with only one gas and one liquid pipe. The costs for maintenance could accordingly be lowered. On the other hand it should be kept in mind that a simplified system might be less well controlled and therefore more prone to failures, especially if several systems are connected in series. For example, in case of medium contamination all vessels supplied by the

same source would be affected at once. Conclusively, the operator should carefully balance the assets and drawbacks of complex and simple systems and finally decide according to his needs.

Discussion of the most important experimental outcomes

The work behind this thesis led to different interesting results such as the microscopic visualization of a member the BD1-5/SN2 clade (Hanke *et al.* 2014). Members of this candidate *phylum* had so far only been investigated metagenomically (Johnson *et al.* 2009 (acc. no. FJ959979), Wrighton *et al.* 2012) because cultivated representatives were lacking. Moreover, we could demonstrate that the enriched representative of this bacterial division makes use of an alternate genetic code, using the opal codon UGA to encode for the amino acid glycine. The use of an alternate genetic code by members of the BD1-5/SN-2 candidate *phylum* had been inferred before from metagenomic data of environmental samples (Wrighton *et al.* 2012). The BD1-5/SN-2 organism enriched in our studies is moreover interesting, as we could only detect sparse genomic hints for metabolic activity (Hanke *et al.* 2014). This is similar to findings by (Wrighton *et al.* 2012) who assigned members of this candidate *phylum* a fermentation-based lifestyle resulting from the presence of a few genes encoding for enzymes involved in fermentation but could not detect any capacity for respiration. Based on the absence of genes for lipid biosynthesis and the tricarboxylic acid cycle, one may speculate that the selected BD1-5/SN-2 organism has a predatory lifestyle, which allows it to survive with a rather restricted set of enzymes. A predatory existence of this organism could not be shown, however, the association of its cells with other bacteria as shown with FISH is consistent with this idea (Hanke *et al.* 2014). Predatory or parasitic bacteria are found in nearly all habitats such as rivers, groundwater, estuaries, the open ocean, soil, and sewage (Jurkevitch 2007). However, further research is necessary to verify the lifestyle of members of the enigmatic BD1-5/SN-2 candidate *phylum*.

Additional to the finding that the selected BD1-5/SN-2 bacterium uses an alternate genetic code we found evidence for low levels of mistranslations in this organism, leading to the occasional translation of the opal stop codon to tryptophan, alanine,

lysine, and aspartate. We hypothesize that coincidentally occurring mistranslations may be the basis for evolutionary events leading to the development of alternate genetic codes like the ones known so far, such as UGA/UAA Stop→Gln: reviewed in (Knight *et al.* 2001), AUA Ile→Met: reviewed in (Knight *et al.* 2001), UGA Stop→Trp: (McCutcheon *et al.* 2009), UGA Stop→Gly: (Hanke *et al.* 2014).

The finding that aerobic and anaerobic processes co-occur in single cells causing a loss of overall microbial productivity (Chen *et al.,* submitted) is another striking result obtained in the course of this thesis. It challenges the theory that thermodynamic sorting of redox processes, known as the "microbial redox tower" (Fenchel & Jørgensen 1977, Madigan 2006), optimizes microbial productivity. It is also supported by findings that fermentative bacteria outcompete denitrifying organisms for carbon at relatively low temperatures or that fermentative metabolism may outcompete denitrification within the same cell (Hanke *et al.,* in prep.). This is counter-intuitive with respect to the low energy yield derived from fermentation compared to denitrification. The results suggested that the thermodynamic disadvantage of fermentation is compensated for by its kinetic advantage. Fermentative enzymes are located in the cytosol, whereas denitrification is associated with the cytoplasmic membrane and the periplasm. Because the cytosol has more space available, more enzymes can be produced sustaining higher rates. Substrate affinity is proportional to the consumption rate. If fermentation and denitrification are performed by different populations, uptake rates of carbon compounds of fermentative bacteria may be higher than for denitrifying bacteria, because fermentative bacteria have more membrane space available for transporters, whereas denitrifying bacteria need to allocate membrane space to their respiratory enzymes. At lower temperatures the results suggested that denitrification might be outcompeted by fermentation. Whereas at higher temperatures effective division of labor was observed between specialized denitrifying and fermentative cells, at low temperatures cells of almost all enriched populations appeared to perform denitrification and fermentation in parallel. In that case, denitrification was outcompeted by fermentation, leading to a loss of the denitrification function for the ecosystem. Future research should address the question whether this scenario may also occur in natural ecosystems.

The theory that microbial consumption of electron donors and acceptors follows thermodynamic rules that ensure highest possible energy yield and biomass production has already been challenged by the investigation of metabolic phenomena such as aerobic denitrification and the Crabtree and Warburg Effects (see introduction). The theory that natural selection leads to an optimized microbial productivity in natural ecosystems was falsified in this thesis: natural selection leads to competition for optimized substrate uptake rates rather than for an optimized productivity. Our experimental and mathematical modeling results show that this phenomenon explained 50 % of the productivity losses in a microbial community evolved by natural selection (Chen *et al.,* submitted). Our findings were supported by analyzes of genome and transcriptome data from natural habitats, according to which parallel aerobic and anaerobic metabolic processes may also occur in nature, at low oxygen concentrations. The *in situ* relevance of "cryptic fermentation" might be addressed in future studies by metabolomic approaches. The finding is also relevant to climate change, because the productivity of microbial ecosystems is an important factor in global primary productivity: marine micoorganisms make up for a substantial part of biomass production on Earth and therefore act as a sink for atmospheric CO_2. Accordingly, a loss of microbial productivity leads to a reduced negative feedback on the atmospheric CO_2 concentration.

The finding that anaerobic and aerobic processes can proceed in parallel was explained by two microbial strategies that together enable the occurrence of anaerobic processes such as fermentation, denitrification, and sulfate reduction under aerobic conditions. These strategies are:

1. The formation of micro-aggregates, which create micro-niches locally providing conditions supporting the occurrence of anaerobic processes by enabling complete oxygen consumption in combination with mass transport limitation effectively depleting the oxygen.
2. Active oxygen consumption by *bd*-type terminal oxidases to protect oxygen-sensitive enzymes, such as N_2O-reductase and pyruvate-formate-lyase. *bd*-type terminal oxidases conserve less energy than heme-copper terminal oxidases leading to dissipation of energy as heat and lower productivity

Using these strategies, bacteria formerly assumed to depend on anoxia were shown to be able to actively metabolize their substrates in the presence of oxygen. One example is a fermenting *Vibrionales* population detected in studies by (Chen *et al.,* submitted), which effectively protected its pyruvate-formate-lyase by the efficient reduction of oxygen with help of a *bd*-type terminal oxidase. This population potentially also reduced nitrous oxide to nitrogen and nitrate to nitrite. With these reduction reactions it disposed of excess electrons resulting from fermentation, such as glycolytic conversion of glucose to pyruvate. This strategy allowed *Vibrionales* to effectively ferment in the presence of oxygen, although, according to the microbial redox tower this metabolic activity is restricted to anoxia. This led to the unexpected aerobic production of formate, one of the main fermentation products of this population. Aerobic accumulation of formate production could only be possible if the *Vibrionales* had a higher affinity for oxygen than the formate-consuming *Arcobacter* population.

References

Abdo, Zaid, Ursel M. E. Schuette, Steven J. Bent, Christopher J. Williams, Larry J. Forney, and Paul Joyce. (2006) „Statistical methods for characterizing diversity of microbial communities by analysis of terminal restriction fragment length polymorphisms of 16S rRNA genes." *Environmental Microbiology* 8(5): 929-938.

Abraham, W.-R. (2014) „Applications and impacts of stable isotope probing for analysis of microbial interactions." *Appl Microbiol Biotechnol* 98: 4817-4828.

Allen, A. P., J. F. Gillooly, and J. H. Brown. (2005) „Linking the global carbon cycle to individual metabolism." *Functional Ecology* 19: 202-213.

Aller, Robert C. „The effects of macrobenthos on chemical properties of marine sediment and overlying water." In: *Animal-Sediment Relations*, by P. L. McCall (ed.) and M. J. S. Tevesz, 53-96. Plenum Press, 1982.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. (1990) „Basic Local Alignment Search Tool." *Journal of Molecular Biology* 215: 403-410.

Amann, Rudolf I., Wolfgang Ludwig, and Karl-Heinz Schleifer. (1995) „Phylogenetic Identification and *In Situ* Detection of Individual Microbial Cells without Cultivation." *Microbiological Reviews* 59(1): 143-169.

Andrews, John H., and Robin F. Harris. (1986) „r- and K-Selection and Microbial Ecology." *Advances in Microbial Ecology* 9: 99-147.

Atherton, Rachel J., Andrew J. Baird, and Giles F. S. Wiggs. (2001) „Inter-tidal Dynamics of Surface Moisture Content on a Meso-tidal Beach." *Journal of Coastal Research* 17(2): 482-489.

Atkinson, Steve, and Paul Williams. (2009) „Quorum sensing and social networking in the microbial world." *Journal of the Poyal Society Interface* 6: 959-978.

Barer, Michael R. and Colin R. Harwood. (1999) „Bacterial Viability and Culturability." *Advances in Microbial Physiology* 41: 93-137.

Baumann, B., M. Snozzi, A. J. Zehnder, and J. R. Van Der Meer. (1996) „Dynamics of denitrification activity of *Paracoccus denitrificans* in continuous culture during aerobic-anaerobic changes." *Journal of Bacteriology* 178(15): 4367-4374.

Bazylinski, Dennis A. and R. P. Blakemore. (1983) „Denitrification and Assimilatory Nitrate Reduction in *Aquaspirillum magnetotacticum*." *Applied and Environmental Microbiology* 46(5): 1118-1124.

Beck, Christoph and H. Kaspar von Meyenburg. (1968) „Enzyme Pattern and Aerobic Growth of *Saccharomyces cerevisiae* Under Various Degrees of Glucose Limitation." *Journal of Bacteriology* 96(2): 479-468.

Beck, M., M. Grunwald, C. Kohlmeier, O. Dellwig, and H.-J. Brumsack. „Observatory in the Wadden Sea: Nutrient cycling and export to the North Sea." *OCEANS 2009 - Europe.* Bremen: IEEE, 2009. 1-3.

Beck, Melanie, Olaf Dellwig, Gerd Liebezeit, Bernhard Schnetger, and Hans-Jürgen Brumsack. (2008) „Spatial and seasonal variations of sulphate, dissolved organic carbon, and nutrients in deep pore waters of intertidal flat sediments." *Estuarine, Coastal and Shelf Science* 79: 307-316.

Beck, Melanie and Hans-Jürgen Brumsack. (2012) „Biogeochemical cycles in sediment and water column of the Wadden Sea: The example Spiekeroog Island in a regional context." *Ocean & Coastal Management* 68: 102-113.

Behrendt, A., D. de Beer, and P. Stief. (2013) „Vertical activity distribution of dissimilatory nitrate reduction in coastal marine sediments." *Biogeosciences* 10: 7509-7523.

Bendschneider, Kenneth and Rex J. Robinson. (1952) „A new spectrophotometric method for the determination of nitrite in sea water." *Journal of Marine Research* 11: 87-96

Bietz, Hauke. (2004) "*Die Entwicklung der deutschen Wattforschung: Ein wissenschaftshistorischer Beitrag zur Bedeutung der Pionierarbeiten.*" (PhD Thesis) Wiefelstede

Bijkerk, A. H. E. and R. J. Hall. (1977) „A Mechanistic Model of the Aerobic Growth of *Saccharomyces cerevisiae*.“ *Biotechnology and Bioengineering* 19: 267-296.

Billerbeck, Markus, Ursula Werner, Lubos Polerecky, Eva Walpersdorf, Dirk de Beer, and Markus Huettel. (2006) „Surficial and deep pore water circulation governs spatial and temporal scales of nutrient recycling in intertidal sand flat sediment.“ *Marine Ecology Progress Series* 326: 61-76.

Blackburne, Richard, Vel M. Vadivelu, Zhiguo Yuan, and Jürg Keller. (2007) „Kinetic characterization of an enriched *Nitrospira* culture with comparison to *Nitrobacter*.“ *Water Research* 41: 3033-3042.

Blainey, Paul C. (2013) „The future is now: single-cell genomics of bacteria and archaea.“ *FEMS Microbiology Reviews* 37: 407-427.

Bødtker, Gunhild, Christine L. Lysnes, Terje Torsvik, Eva Ø. Bjørnestad, and Egil Sunde. (2009) „Microbial analysis of backflowed injection water from a nitrate-treated North Sea oil reservoir.“ *Journal of Industrial Microbiology* 36: 439-450.

Boetius, Antje, *et al*. (2000) „A marine microbial consortium apparently mediating anaerobic oxidation of methane.“ *Nature* 407: 623-626.

Bondarev, Vladimir, Michael Richter, Stefano Romano, Jörn Piel, Anne Schwedt, and Heide N. Schulz-Vogt. (2013) „The genus *Pseudovibrio* contains metabolically versatile bacteria adapted for symbiosis.“ *Environmental Microbiology* 15(7): 2095-2113.

Boucher, Douglas H. *The Biology of Mutualism: Ecology and Evolution.* Oxford University Press, 1988.

Boudreau, Bernard P., *et al*. (2001) „Permeable Marine Sediments: Overturning an Old Paradigm .“ *EOS* 82(11): 133-140.

Britten, R. J. and D. E. Kohne. (1968) „Repeated Sequences in DNA. Hundreds of thousands of copies of DNA sequences have been incorporated into the genomes of higher organisms.“ *Science* 161(3841): 529-540.

Brockamp, Olaf and Michael Zuther. (2004) „Changes in clay mineral content of tidal flat sediments resulting from dike construction along the Lower Saxony coast of the North Sea, Germany." *Sedimentology* 51: 591-600.

Broda, E. (1977) „Two kinds of lithotrophs missing in nature." *Zeitschrift für Allg. Mikrobiologie* 17(6): 491-493.

Byrnes, B. H. (1990) „Environmental effects of N fertilizer use - An overview." *Fertilizer Research* 26: 209-215.

Camacho, C., *et al.* (2009) „BLAST+: architecture and applications." *BMC Bioinformatics* 10: Article 421.

Campbell, James H., *et al.* (2013) „UGA is an additional glycine codon in uncultured SR1 bacteria from the human microbiota." *Proceedings of the National Academy of Sciences* 110, Nr. 14: 5540-5545.

Canfield, Don E., *et al.* (2010) „A Cryptic Sulfur Cycle in Oxygen-Minimum–Zone Waters off the Chilean Coast." *Science* 330: 1375-1378.

Carpenter, Lucy J., Stephen D. Archer, and Rachael Beale. (2012) „Ocean-atmosphere trace gas exchange." *Chemical Society Reviews* 41: 6473-6506.

Castelle, Cindy J., *et al.* (2013) „Extraordinary phylogenetic diversity and metabolic versatility in aquifer sediment." *Nature Communications* 4:2120.

Chen, Jianwei, *et al.* (submitted) „Competition between thermodynamically unsorted redox processes limits productivity of microbial ecosystems."

Cline, J. (1969) „Spectrophotometric determination of hydrogen sulfide in natural waters." *Limnology and Oceanography* 14: 454-458.

Codispoti, L. A., *et al.* (2001) „The oceanic fixed nitrogen and nitrous oxide budgets: Moving target as we enter the anthropocene?" *Scientia Marina* 65(2): 85-102.

Colijn, Franciscus, Karl-Jürgen Hesse, Norbert Ladwig, and Urban Tillmann. (2002) „Effects of the large-scale uncontrolled fertilisation process along the continental coastal North Sea." *Hydrobiologia* 484: 133–148.

Colijn, Franciscus, and Gerhard C. Cadée. (2003) „Is phytoplankton growth in the Wadden Sea light or nitrogen limited?" *Journal of Sea Research* 49: 83-93.

Crutzen, P. J. (1970) „The influence of nitrogen oxides on the atmospheric ozone content." *Quarterly Journal Of The Royal Meteorological Society* 96: 320-325.

Cubasch, U., *et al.* „Introduction." In: *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*, by T. F. Stocker, et al. Cambridge, UK and New York, USA: Cambridge University Press, 2013.

CWSS (2008), Common Wadden Sea Secretariat. „Nomination of the Dutch-German Wadden Sea as World Heritage Site - Volume one." Wilhelmshaven, 2008.

CWSS (2012), Common Wadden Sea Secretariat. „The Wadden Sea, Germany and Netherlands (N1314) - Extension Denmark and Germany - Volume one." Wilhelmshaven, 2012.

D'Andrea, Anthony F., Robert C. Aller, and Glenn R. Lopez. (2002) „Organic matter flux and reactivity on a South Carolina sandflat: The impacts of porewater advection and macrobiological structure." *Limnology and Oceanography* 47(4): 1056-1070.

Daehnke, Kirstin, Kay Emeis, Astrid Johannsen, and Birgit Nagel. (2010) „Stable isotope composition and turnover of nitrate in the German Bight." *Marine Ecology Progress Series* 408: 7-18.

Daramola, M. O. and L. Zampraka. (2008) „Experimental study of the production of biomass by *Sacharomyces cerevisiae* in a fed batch fermentor." *African Journal of Biotechnology* 7(8): 1107-1114.

Davidov, Yaacov, Dorothee Huchon, Susan F. Koval, and Edouard Jurkevitch. (2006) „A new a-proteobacterial clade of *Bdellovibrio*-like predators: implications for the mitochondrial endosymbiotic theory." *Environmental Microbiology* 8(12): 2179-2188.

De Beer, Dirk, Frank Wenzhöfer, Timothy G. Ferdelman, Susan E. Boehme, and Markus Huettel. (2005) „Transport and mineralization rates in North Sea sandy intertidal sediments, Sylt-Rom Basin, Wadden Sea." *Limnology and Oceanography* 50(1): 113-127.

De Deken, R. H. (1966) „The Crabtree Effect: A Regulatory System in Yeast." *Journal of General Microbiology* 44: 149-156.

De Jonge, Victor N., M. Elliott, and E. Orive. (2002) „Causes, historical development, effects and future challenges of a common environmental problem: eutrophication." *Hydrobiologia* 475/476: 1-19.

Dendooven, Al. and J. M. Anderson. (1994) „Dynamics of Reduction Enzymes Involved in the Denitrification Process in Pasture Soil." *Soil Biology & Biochemistry* 26(11): 1501-1506.

Diaz-Ruiz, R., S. Uribe-Carvajal, A. Devin, and M. Rigoulet. (2009) „Tumor cell energy metabolism and its common features with yeast metabolism." *Biochimica et Biophysica Acta* 1796: 252-265.

Diaz-Ruiz, Rodrigo, Michel Rigoulet, and Anne Devin. (2011) „The Warburg and Crabtree effects: On the origin of cancer cell energy metabolism and of yeast glucose repression." *Biochimica et Biophysica Acta* 1807: 568-576.

Dittmann, Sabine (ed.). *The Wadden Sea Ecosystem: stability properties and mechanisrns .* Berlin, Heidelberg: Springer Verlag, 1999.

Duce, R. A., *et al.* (2008) „Impacts of Atmospheric Anthropogenic Nitrogen on the Open Ocean." *Science* 320: 893-897.

Duerr, Hans Peter. *„Rungholt - Die Suche nach einer versunkenen Stadt".* Frankfurt/Main, Leipzig: Insel Verlag, 2005.

Dytczak, Magdalena A., Kathleen L. Londry, and Jan A. Oleszkiewicz. (2008) „Activated sludge operational regime has significant impact on the type of nitrifying community and its nitrification rates." *Water Research* 42: 2320-2328.

Eddy, S. R. (2011) „Accelerated profile HMM searches." *PLOS Computational Biology* 7(10): e1002195.

Engström, Pia, Tage Dalsgaard, Stefan Hulth, and Robert C. Aller. (2005) „Anaerobic ammonium oxidation by nitrite (anammox): Implications for N_2 production in coastal marine sediments." *Geochimica et Cosmochimica Acta* 69(8): 2057-2065.

Eriksson, Peder G., and Stefan E. B. Weisner. (1999) „An Experimental Study on Effects of Submersed Macrophytes on Nitrification and Denitrification in Ammonium-Rich Aquatic Systems." *Limnology and Oceanography* 44(8): 1993-1999.

Ettwig, Katharina F., *et al.* (2010) „Nitrite-driven anaerobic methane oxidation by oxygenic bacteria." *Nature* 464: 543-548.

Ettwig, Katharina F., Daan R. Speth, Joachim Reimann, Ming L. Wu, Mike S. M. Jetten, and Jan T. Keltjens. (2012) „Bacterial oxygen production in the dark." *Frontiers in Microbiology* 3: Article 237.

Fenchel, T. M. and B. Barker Jørgensen. „Detritus Food Chains of Aquatic Ecosystems: The Role of Bacteria." Vol. 1. In: *Advances in Microbial Ecology*, by M. Alexander. New York: Springer Verlag, 1977.

Ferenci, Thomas. „Bacterial Physiology, Regulation and Mutational Adaptation in a Chemostat Environment." Vol. 53. In: *Advances in Microbial Physiology*, by Robert K. Poole, 169-229. Academic Press, 2007.

Ferguson, Stuart J. (1994) „Denitrification and its control." *Antonie van Leeuwenhoek* 66: 89-110.

Ferreira, Leonardo M. R. (2010) „Cancer metabolism: The Warburg effect today." *Experimental and Molecular Pathology* 89: 372-380.

Flemming, Burg and Karen Ziegler. (1995) „High-Resolution Grain Size Distribution Patterns and Textural Trends in the Backbarrier Environment of Spiekeroog Island (Southern North Sea)." *Senckenbergiana Maritima* 26(1/2): 1-24.

Flemming, Burghard W., and Richard A. Jr. Davis. (1994) „Holocene Evolution, Morphodynamics and Sedimentology of the Spiekeroog Barrier Island System (Southern North Sea)." *Senckenbergiana Maritima* 24: 117-155.

Fock, Heino O. (2003) „Changes in the seasonal cycles of inorganic nutrients in the coastal zone of the southeastern North Sea from 1960 to 1997: effects of eutrophication and sensitivity to meteoclimatic factors." *Marine Pollution Bulletin* 46: 1434-1449.

Forster, Stefan, Markus Huettel, and Wiebke Ziebis. (1996) „Impact of boundary layer flow velocity on oxygen utilisation in coastal sediments." *Marine Ecology Progress Series* 143: 173-185.

Froelich, P. N., *et al.* (1979) „Early oxidation of organic matter in pelagic sediments of the eastern equatorial Atlantic: suboxic diagenesis." *Geochimica et Cosmochimica Acta* 43: 1075-1090.

Fuhrman, Jed A., *et al.* (2008) „A latitudinal diversity gradient in planktonic marine bacteria." *Proceedings of the National Academy of Science* 105(22): 7774-7778.

Gao, Hang, *et al.* (2010) „Aerobic denitrification in permeable Wadden Sea sediments." *The ISME Journal* 4: 417-426.

Gao, Hang, *et al.* (2012) „Intensive and extensive nitrogen loss from intertidal permeable sediments of the Wadden Sea." *Limnology and Oceanography* 57(1): 185-198.

Gatenby, Rober A. and Robert J. Gillies. (2004) „Why do cancers have high aerobic glycolysis?" *Nature Reviews Cancer* 4: 891-899.

Gatt, Shimon and E. Racker. (1959) „Regulatory Mechanisms in Carbohydrate Metabolism: I. Crabtree Effect in Reconstructed Systems." *The Journal of Biological Chemistry* 234: 1015-1023.

Gayon, V., and G. Dupetit. (1886) „Recherches sur la réducion des nitrate spar les infiniment petits." *Société des Sciences Physiques et Natuelle de Bordeaux* 3(2): 201-307.

Ginige, M. P., G. Carvalho, J. Keller, and L. L. Blackall. (2007) „Eco-physiological characterization of fluorescence *in situ* hybridization probe-targeted denitrifiers in activated sludge using culture-independent methods." *Letters in Applied Microbiology* 44(4): 399-405.

Gogvadze, Vladimir, Boris Zhivotovsky, and Sten Orrenius. (2010) „The Warburg effect and mitochondrial stability in cancer cells." *Molecular Aspects of Medicine* 31: 60-74.

Gray, James P. and Russell P. Herwig. (1996) „Phylogenetic Analysis of the Bacterial Communities in Marine Sediments." *Applied and Environmental Microbiology* 62(11): 4049-4059.

Grossart, Hans-Peter, Andrea Schlingloff, Michael Bernhard, Meinhard Simon, Brunkhoff, and Thorsten Brinkhoff. (2004) „Antagonistic activity of bacteria isolated from organic aggregates of the German Wadden Sea." *FEMS Microbiology Ecology* 47: 387-396.

Gruber, Nicolas and James N. Galloway. (2008) „An Earth-system perspective of the global nitrogen cycle." *Nature* 451: 293-296.

Grunwald, Maik, *et al*. (2010) „Nutrient dynamics in a back barrier tidal basin of the Southern North Sea: Time-series, model simulations, and budget estimates." *Journal of Sea Research* 64: 199-212.

Guerrero, Ricardo, Carlos Pedrós-Alió, Isabel Esteve, Jordi Mas, David Chase, and Lynn Margulis. (1986) „Predatory prokaryotes: Predation and primary consumption evolved in bacteria." *Proceedings of the National Academy of Sciences* 83: 2138-2142.

Hanke, Anna and Marc Strous. (2010) „Climate, Fertilization, and the Nitrogen Cycle." *Journal of Cosmology* 8: 1838-1845.

Hanke, Anna, *et al.* (2014) „Recoding of the stop codon UGA to glycine by a BD1-5/SN-2 bacterium and niche partitioning between *Alpha-* and *Gammaproteobacteria* in a tidal sediment microbial community naturally selected in a laboratory chemostat." *Frontiers in Microbiology* 5: Article 231.

Hanke, Anna, Jasmine Berg, Theresa Hargesheimer, Halina E. Tegetmeyer, Christin E. Sharp, and Marc Strous. (in prep.) „Temperature as a factor for natural selection on marine denitrifying bacterial communities in continuous cultures."

Hartmann, D. L., *et al.* „Observations: Atmosphere and Surface." In: *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*, by T. F. Stocker, *et al.* Cambridge, New York: Cambridge University Press, 2013.

Hernandez, Dennis and John J. Rowe. (1988) „Oxygen Inhibition of Nitrate Uptake Is a General Regulatory Mechanism in Nitrate Respiration." *The Journal of Biological Chemistry* 263(17): 7937-7939.

Huettel, M. and G. Gust. (1992) „Impact of bioroughness on interfacial solute exchange in permeable sediments." *Marine Ecology Progress Series* 89: 253-267.

Huettel, M., W. Ziebis, and S. Forster. (1996) „Flow-induced uptake of particulate matter in permeable sediments." *Limnology and Oceanography* 41(2): 309-322.

Huettel, M., W. Ziebis, S. Forster, and G. W. Luther III. (1998) „Advective transport affecting metal and nutrient distributions and interfacial fluxes in permeable sediments ." *Geochimica et Cosmochimica Acta* 62(4): 613-631.

Huettel, Markus and Ian T. Webster. „Porewater Flow in Permeable Sediments." In: *The benthic boundary layer*, by B. P. Boudreau and B. B. Jørgensen [eds], 144-179. Oxford University Press, 2001.

Hulth, Stefan, *et al.* (2005) „Nitrogen removal in marine environments: recent findings and future research challenges." *Marine Chemistry* 94: 125-145.

Hyatt, D., G.-L. Chen, P. F. LoCascio, M. L. Land, F. W. Larimer, and L. J. Hauser. (2010) „Prodigal: prokaryotic gene recognition and translation initiation site identification." *BMC Bioinformatics* 11: 119.

Jansen, Stefan, Eva Walpersdorf, Ursula Werner, Markus Billerbeck, Michael E. Böttcher, and Dirk de Beer. (2009) „Functioning of intertidal flats inferred from temporal and spatial dynamics of O_2, H_2S and pH in their surface sediment." *Ocean Dynamics* 59: 317-332.

Janssen, F., M. Huettel, and U. Witte. (2005) „Pore-water advection and solute fluxes in permeable marine sediments (II): Benthic respiration at three sandy sites with different permeabilities (German Bight, North Sea)." *Limnology and Oceanography* 50(3): 779-792.

Johnson, Wesley R., Manolito Torralba, Particia A. Fair, Gregory D. Bossart, Karen E. Nelson, and Pamela J. Morris. (2009) „Novel diversity of bacterial communities associated with bottlenose dolphin upper respiratory tracts." *Environmental Microbiology Reports* 1(6): 555-562.

Jørgensen, Bo Barker. „Bacteria and marine biogeochemistry." In: *Marine Geochemistry*, by H. D. Schulz and M. Zabel (eds), 169-206. Berlin Heidelberg: Springer Verlag, 2006.

Jørgensen, Linda, Stiig Markager, and Marie Maar. (2014) „On the importance of quantifying bioavailable nitrogen instead of total nitrogen." *Biogeochemistry* 117: 455-472.

Jurkevitch, Edouard. (2007) „Predatory Behaviors in Bacteria - Diversity and Transitions." *Microbe Magazine* 2(2): 67-73.

Kartal, Boran and Marc Strous. „Methods to study consortia and mixed cultures." In: *Accessing Uncultivated Microorganisms: from the Environment to Organisms and Genomes and Back*, by Karsten Zengler (ed.), 205-219. Washington, DC: ASM Press, 2008.

Katoh, Kazutaka, Kazuharu Misawa, Kei-ichi Kuma, and Takashi Miyata. (2002) „MAFFT: a novel method for rapid multiple sequence alignment based on fast fourier transform." *Nucleic Acid Research* 30(14): 3059-3066.

Kiel Reese, Brandi, Angela D. Witmer, Stacey Moller, John W. Morse, and Heath J. Mills. (2014) „Molecular assays advance understanding of sulfate reduction despite cryptic cycles." *Biogeochemistry* 118: 307-319.

Kiel Reese, Brandi, Heath J. Mills, and Scot E. Dowd. (2013) „Linking Molecular Microbial Ecology to Geochemistry in a Coastal Hypoxic Zone." *Geomicrobiology Journal* 30: 160-172.

Kieskamp, Willem Michel, Lutz Lohse, Eric Epping, and Willem Helder. (1991) „Seasonal variation in denitrification rates and nitrous oxide fluxes in intertidal sediments of the western Wadden Sea." *Marine Ecology Progress Series* 72: 145-151.

Kirchman, David L. (2002) „The ecology of *Cytophaga-Flavobacteria* in aquatic environments." *FEMS Microbiology Ecology* 39: 91-100.

Knight, Robin D., Stephen J. Freeland, and Laura F. Landweber.(2001) „Rewiring the Keyboard: Evolvability of the Genetic Code." *Nature Reviews Genetics* 2(1): 49-58.

Körner, Heinz and Walter G. Zumft. (1989) „Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of *Pseudomonas stutzeri*." *Applied and Environmental Microbiology* 55(7): 1670-1676.

Kraft, B., *et al.* (in press.) „The environmental controls that govern the end product of bacterial nitrate respiration." *Science*.

Krul, J. M. and R. Veeningen. (1977) „The synthesis of the dissimilatory nitrate reductase under aerobic conditions in a number of denitrifying bacteria, isolated from activated sludge and drinking water." *Water Research* 11: 39-43.

Langmead, Ben and Steven L. Salzberg. (2012) „Fast gapped-read alignment with bowtie 2." *Nature Methods* 9(4): 357-360.

Laslett, Dean and Bjorn Canback. (2004) „ARAGORN, a program to detect tRNA genes and tmRNA genes in nucleotide sequences." *Nucleic Acids Research* 32(1): 11-16.

Lenk, Sabine, Julia Arnds, Katrice Zerjatke, Niculina Musat, Rudolf Amann, and Marc Mußmann. (2011) „Novel groups of *Gammaproteobacteria* catalyse sulfur oxidation and carbon fixation in a coastal, intertidal sediment." *Environmental Microbiology* 13(3): 758-774.

Liu, W.-T., T. L. Marsh, H. Cheng, and L. J. Forney. (1997) „Characterization of Microbial Diversity by Determining Terminal Restriction Fragment Length Polymorphisms of Genes Encoding 16S rRNA." *Applied and Environmental Microbiology* 63(11): 4516-4522.

Llobet-Brossa, Enric, Ramon Rosseló-Mora, and Rudolf Amann. (1998) „Microbial Community Composition of Wadden Sea Sediments as Revealed by Fluorescence *In Situ* Hybridization." *Applied and Environmental Microbiology* 64(7): 2691–2696.

Llobet-Brossa, Enrique, *et al.* (2002) „Community structure and activity of sulfate-reducing bacteria in an intertidal surface sediment: a multi-method approach." *Aquatic Microbial Ecology* 29: 211-226.

Lo, Ian, *et al.* (2007) „Strain-resolved community proteomics reveals recombining genomes of acidophilic bacteria." *Nature* 446: 537-541.

Lowry, Oliver H., Nira J. Rosebrough, A. Lewis Farr, and Rose J. Randall. (1951) „Protein measurement with the folin phenol reagent." *The Journal of Biological Chemistry* 193: 265-275.

Lüders, K. (1953) „Die Entstehung der ostfriesischen Inseln und der Einfluß der Dünenbildung auf den geologischen Aufbau der ostfriesischen Küste." *Probleme der Küstenforschung* 5: 5-15.

Mackenzie, Fred T., Leah May Ver, and Abraham Lerman. (2002) „Century-scale nitrogen and phosphorus controls of the carbon cycle."*Chemical Geology* 190: 13-32.

Madigan, Michael T. and John M. Martinko. *Brock Mikrobiologie.* Vol. 11. Addison-Wesley, 2006.

Mandel, M. and J. Marmur. "Use of ultraviolet absorbance-temperature profile for determining the guanine plus cytosine content of DNA*."* Vol. 12B, In. *Methods in Enzymology*, by Grossman L. and Moldave K. (eds), 195-206. New York: Academic Press, 1968.

Manne, Alan S. and Richard G. Richels. (2001) „An alternative approach to establishing trade-offs among greenhouse gases." *Nature* 410: 675-677.

Martín-Hernández, Mariángel, Julián Carrera, Julio Perez, and María Eugenia Suárez-Ojeda. (2009) „Enrichment of a K-strategist microbial population able to biodegrade p-nitrophenol in a sequencing batch reactor." *Water Research* 43: 3871-3883.

McCutcheon, John P., Bradon R. McDonald, and Nancy A. Moran. (2009) „Origin of an Alternative Genetic Code in the Extremely Small and GC–Rich Genome of a Bacterial Symbiont." *PLoS Genetics* 5(7): e1000565.

Meiberg, J. B. M., P. M. Bruinenberg, and W. Harder. (1980) „Effect of Dissolved Oxygen Tension on the Metabolism of Methylated Amines in Hyphomicrobium X in the Absence and Presence of Nitrate: Evidence for 'Aerobic' Denitrification." *Journal of General Microbiology* 120: 453-463.

Meiklejohn, Jane. (1940) „Aerobic denitrification." *Annals of Applied Biology* 27(4): 558-573.

Miller, Christopher S., Brett J. Baker, Brian C. Thomas, Steven W. Singer, and Jillian F. Banfield. (2011) „EMIRGE: reconstruction of full-length ribosomal genes from microbial community short read sequencing data." *Genome Biology* 12: R44.

Miller, C. S., K. M. Handley, K. C. Wrighton, K. R. Frischkorn, B. C. Thomas, and J. F. Banfield. (2013) „Short-Read Assembly of Full-Length 16S Amplicons Reveals Bacterial Diversity in Subsurface Sediments." *PLOS one* 8(2): e56018.

Möbius, Karl August. *Die Auster und die Austernwirthschaft.* Berlin: von Wiegandt, Hemple & Parey, 1877.

Morris, Brandon E. L., Ruth Henneberger, Harald Huber, and Christine Moissl-Eichinger. (2013) „Microbial syntrophy: interaction for the common good." *FEMS Microbiology Reviews* 37: 384-406.

Moyer, C. L., F. C. Dobbs, and D. M. Karl. (1994) „Estimation of Diversity and Community Structure through Restriction Fragment Length Polymorphism Distribution Analysis of Bacterial 16S rRNA Genes from a Microbial Mat at an Active, Hydrothermal Vent System, Loihi Seamount, Hawaii." *Applied and Environmental Microbiology* 60(3): 871-879.

Mußmann, Marc, Kousuke Ishii, Ralf Rabus, and Rudolf Amann. (2005) „Diversity and vertical distribution of cultured and uncultured *Deltaproteobacteria* in an intertidal mud flat of the Wadden Sea." *Environmental Microbiology* 7(3): 405-418.

Novick, Aaron, and Leo Szilard. (1950) „Description of the Chemostat." *Science* 112: 715-716.

Osaka, Toshifumi, Kosuke Shirotani, Sachiko Yoshie, and Satoshi Tsuneda. (2008) „Effects of carbon source on denitrification efficiency and microbial community structure in a saline wastewater treatment process." *Water Research* 42: 3709-3718.

Otte, Sandra, Nicole G. Grobben, Lesley A. Robertson, Mike S. M. Jetten, and J. Gijs Kuenen. (1996) „Nitrous oxide production by *Alcaligenes faecalis* under transient and dynamic aerobic and anaerobic conditions." *Applied and Environmental Microbiology* 62(7): 2421-2426.

Overmann, Jörg. "Principles of Enrichment, Isolation, Cultivation and Preservation of Prokaryotes*."* Vol. 1, In: *The Prokaryotes - A Handbook on the Biology of Bacteria*, by Martin Dworkin, Stanley Falkow, Eugene Rosenberg, Karl-Heinz Schleifer and Erko Stackebrandt (eds), 80-136. Berlin Heidelberg: Springer Verlag, 2006.

Palsson-McDermott, Eva M. and Luke A. J. O'Neill. (2013) „The Warburg effect then and now: From cancer to inflammatory diseases." *Bioassays* 35: 965-973.

Parkin, Timothy B. and James M. Tiedje. (1984) „Application of a soil core method to investigate the effect of oxygen concentration on denitrification." *Soil Biology & Biochemistry* 16(4): 331-334.

Pohorelic, Brant K. J., Johanna K. Voordouw, Elisabeth Lojou, Alain Dolla, Jens Harder, and Gerrit Voordouw. (2002) „Effects of Deletion of Genes Encoding Fe-Only Hydrogenase of *Desulfovibrio vulgaris* Hildenborough on Hydrogen and Lactate Metabolism." *Journal of Bacteriology* 184(3): 679-686.

Prather, Michael J. and Juno Hsu. (2010) „Coupling of Nitrous Oxide and Methane by Global Atmospheric Chemistry." *Science* 330: 952-954.

Precht, Elimar and Markus Huettel. (2003) „Advective pore-water exchange driven by surface gravity waves and its ecological implications." *Limnology and Oceanography* 48(4): 1674-1684.

Precht, Elimar, Ulrich Franke, Lubos Polerecky, and Markus Huettel. (2004) „Oxygen dynamics in permeable sediments with wave-driven pore water exchange." *Limnology and Oceanography* 49(3): 694-705.

Price, Morgan N., Paramvir S. Dehal, and Adam P. Arkin. (2010) „FastTree 2 – Approximately Maximum-Likelihood Trees for Large Alignments." *PLOS one* 5(3):9490.

Pruesse, E., *et al*. (2007) „SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB." *Nucleic Acids Research* 35(21): 7188-7196.

Quast, Christian, *et al*. (2013) „The SILVA ribosomal RNA gene database project: improved data processing and web-based tools." *Nucleic Acids Research* 41: (D1):D590-D596.

Rao, Alexandra M. F., Mark J. McCarthy, Wayne S. Gardner, and Richard A. Jahnke. (2007) „Respiration and denitrification in permeable continental shelf deposits on the South Atlantic Bight: Rates of carbon and nitrogen cycling from sediment column experiments." *Continental Shelf Research* 27: 1801-1819.

Ravishankara, A. R., John S. Daniel, and Robert W. Portman. (2009) „Nitrous Oxide (N_2O): The Dominant Ozone-Depleting Substance Emitted in the 21st Century." *Science* 326: 123-125.

Reichenbach, Hans. "The Order Cytophagales*."* Vol. 3. In: *The Prokaryotes - A Handbook on the Biology of Bacteria*, by Stanley Falkow, Eugene Rosenberg, Karl-Heinz Schleifer, Erko Stackebrandt and Martin Dworkin (ed.), 549-590. Berlin: Springer-Verlag, 2006.

Reise, Karsten. (2002) „Sediment mediated species interactions in coastal waters ." *Journal of Sea Research* 48: 127-141.

Rinke, Christian, *et al.* (2013) „Insights into the phylogeny and coding potential of microbial dark matter." *Nature* 499: 431-437.

Risgaard-Petersen, Nils, *et al.* (2004) „Anaerobic ammonium oxidation in an estuarine sediment." *Aquatic Microbial Ecology* 36: 293-304.

Risgaard-Petersen, Nils, Søren Rysgaard, Lars Peter Nielsen, and Niels Peter Revsbech. (1994) „Diurnal variation of denitrification and nitrification in sediments colonized by benthic microphytes." *Limnology and Oceanography* 39(3): 573-579.

Robertson, Lesley A., Ed W. J. van Niel, Rob A. M. Torremans, and Gijs Kuenen. (1988) „Simultaneous Nitrification and Denitrification in Aerobic Chemostat Cultures of *Thiosphaera pantotropha*." *Applied and Environmental Microbiology* 54(11): 2812-2818.

Robertson, Lesley A., and Gijs Kuenen. (1984) „Aerobic denitrification: a controversy revived." *Archives of Microbiology* 139: 351-354.

Rolland, Filip, Joris Winderickx, and Johan M. Thevelein. „Glucose-sensing and -signalling mechanisms in yeast." *FEMS Yeast Research* 2: 183-201.

Rossignol, Rodrigue, Robert Gilkerson, Robert Aggeler, Kunihiro Yamagata, S. James Remington, and Roderick A. Capaldi. (2004) „Energy Substrate Modulates Mitochondrial Structure and Oxidative Capacity in Cancer Cells." *Cancer Research* 64: 985-993.

Rusch, Antje, Markus Huettel, Clare E. Reimers, Gary L. Taghon, and Charlotte M. Fuller. (2003) „Activity and distribution of bacterial populations in Middle Atlantic Bight shelf sands ." *FEMS Microbiology Ecology* 44: 89-100.

Rusch, Antje and Markus Huettel. (2000) „Advective particle transport into permeable sediments - evidence from experiments in an intertidal sandflat." *Limnology and Oceanography* 45(3): 525-533.

Sacks, L. E. and H. A. Barker. (1949) „The Influence of Oxygen on Nitrate and Nitrite Reduction." *Journal of Bacteriology* 58(1): 11-22.

Schlisio, Susanne. (2009) „Neuronal apoptosis by prolyl hydroxylation: implication in nervous system tumours and the Warburg conundrum." *Journal of Molecular Medicine* 13(10): 4104-4112.

Seemann, Torsten. (2014) „Prokka: Rapid Prokaryotic Genome Annotation." *Bioinformatics* 30(14): 2068-2069.

Seiser, A. and L. Walz. (1925) „Stickstoffumsatz bei der Denitrifikation." *Archiv für Hygiene* 95: 189-208.

Seitzinger, Sybil P. (1988) „Denitrification in freshwater and coastal marine ecosystems: Ecological and geochemical significance." *Limnology and Oceanography* 33(4) part 2: 702-724.

Sharp, Christine E., Allyson L. Brady, Glen H. Sharp, Steven E. Grasby, Matthew B. Stott, and Peter F. Dunfield. (2014) „Humboldt's spa: microbial diversity is controlled by temperature in geothermal environments." *The ISME Journal* 8: 1166-1174.

Smith, Keith A. (1997) „The potential for feedback effects induced by global warming on emissions of nitrous oxide by soils." *Global Change Biology* 3: 327-338.

Solorzano, Lucia. (1969) „Determination of Ammonia in Natural Waters by the Phenolhypochlorite Method." *Limnology and Oceanography* 14(15): 799-801.

Strous, Marc, John A. Fuerst, Evelien H. M. Kramer, Susanna Logemann, Gerard Muyzer, Katinka T. Van de Paas-Schoonen, Richard Webb, J. Gijs Kuenen and Mike S. M. Jetten (1999) „Missing lithotroph identified as new planctomycete." *Nature* 400: 446-449.

Strous, M., B. Kraft, R. Bisdorf, and H. E. Tegetmeyer. (2012) „The binning of metagenomic contigs for microbial physiology of mixed cultures." *Frontiers in Microbiology* 3: Article 410.

Sussmann, Ilene, Maria Erecinska, and David F. Wilson. (1980) „Regulation of Cellular Energy Metabolism - The Crabtree Effect." *Biochimica et Biophysica Acta* 591: 209-223.

Sutherland, Robert M. (1988) „Cell and Environment Interactions in Tumor Microregions: The Multicell Spheroid Model." *Science* 240: 177-184.

Takaya, Naoki, Maria Antonina Catalan-Sakairi, Yasushi Sakaguchi, Isao Kato, Zhemin Zhou, and Hirofumi Shoun. (2003) „Aerobic Denitrifying Bacteria That Produce Low Levels of Nitrous Oxide." *Applied and Environmental Microbiology* 69(6): 3152-3157.

Tallec, Gaelle, Josette Garnier, and Michel Gousailles. (2006) „Nitrogen removal in a wastewater treatment plant through biofilters: nitrous oxide emissions during nitrification and denitrification." *Bioprocess and Biosystems Engineering* 29: 323-333.

Teske, A., C. Wawer, G. Muyzer, and N. B. Ramsing. (1996) „Distribution of sulfate-reducing bacteria in a stratified fjord (Mariager Fjord, Denmark) as evaluated by most-probable-number counts and denaturing gradient gel electrophoresis of PCR-amplified ribosomal DNA fragments." *Applied and Environmental Microbiology* 62(4): 1405-1415.

Thamdrup, Bo, Henrik Fossing, and Bo Barker Jørgensen. (1994) „Manganese, iron, and sulfur cycling in a coastal marine sediment, Aarhus Bay, Denmark." *Geochimica et Cosmochimica Acta* 58(23): 5115-5129.

Thamdrup, Bo and Tage Dalsgaard. (2002) „Production of N$_2$ through Anaerobic Ammonium Oxidation Coupled to Nitrate Reduction in Marine Sediments .“ *Applied and Environmental Microbiology* 68(3): 1312-1318.

Thevelein, Johan M. (1994) „Signal Transduction in Yeast.“ *Yeast* 10: 1753-1790.

Thiele, S., B. M. Fuchs and R. I. Amann. "Identification of Microorganisms Using the Ribosomal RNA Approach and Fluorescence *In Situ* Hybridization." Vol. 3. In: *Treatise on Water Science*, by Peter Wilderer (ed.), 171-189. Oxford: Academic Press, 2011.

Thomann, R. V. and J. A. Mueller. *Principles of surface water quality modeling and control.* New York: Harper & Row, 1987.

Tomiyama, Arata, *et al*. (2006) „Critical Role for Mitochondrial Oxidative Phosphorylation in the Activation of Tumor Suppressors Bax and Bak.“ *Journal of the National Cancer Institute* 98(20): 1462-1473.

Torsvik, V., R. Sørheim, and J. Goksøyr. (1996) „Total bacterial diversity in soil and sediment communities a review.“ *Journal of Industrial Microbiology* 17: 170-178.

Tyson, Gene W., *et al*. (2004) „Community structure and metabolism through reconstruction of microbial genomes from the environment.“ *Nature* 428: 37-43.

Van de Leemput, Ingrid, Annelies J. Veraart, Vasilis Dakos, Jeroen J. M. de Klein, Marc Strous, and Marten Scheffer. (2011) „Predicting microbial nitrogen pathways from basic principles.“ *Environmental Microbiology* 13(6): 1477-1487.

Vance-Harris, Cynthia and Ellery Ingall. (2005) „Denitrification pathways and rates in the sandy sediments of the Georgia continental shelf, USA.“ *Geochemical Transactions* 6(1): 12-18.

Vander Heiden, Matthew G., Lewis C. Cantley, and Craig B. Thompson. (2009) „Understanding the Warburg Effect: The Metabolic Requirements of Cell Proliferation.“ *Science* 324: 1029-1033.

Veech, R. L., J. W. Lawson, N. W. Cornell, and H. A: Krebs. (1979) „Cytosolic phosphorylation potential." *The Journal of Biological Chemistry* 254: 6538-6547.

Veraart, Annelies J., Jeroen J. M. de Klein, and Marten Scheffer. (2011) „Warming Can Boost Denitrification Disproportionately Due to Altered Oxygen Dynamics." *PLOS one* 6(3): e18508.

Vitousek, Peter M., *et al.* (1997) „Human Alteration of the Global Nitrogen Cycle: Sources and Consequences." *Ecological Applications* 7(3): 737-750.

Wagner, Michael and Susanne Haider. (2012) „New trends in fluorescence *in situ* hybridization for identification and functional analyses of microbes." *Current Opinion in Biotechnology* 23: 96-102.

Warburg, O., K. Posener and E. Negelein. (1924) „Ueber den Stoffwechsel der Tumoren." *Biochemische Zeitschrift* 152: 319-344.

Warburg, Otto, Franz Wind, and Erwin Negelein. (1927) „The metabolism of tumors in the body." *Journal of General Physiology* 8(6): 519-530.

Warburg, Otto. (1956) „On the Origin of Cancer Cells." *Science* 123(3191): 309-314.

Weinhouse, Sidney. (1956) „On Respiratory Impairment in Cancer Cells." *Science* 124: 267-268.

Weinhouse, Sidney. (1972) „Glycolysis, Respiration, and Anomalous Gene Expression in Experimental Hepatomas: G. H. A. Clowes Memorial Lecture." *Cancer Research* 32(10): 2007-2016.

Wethey, David S., Sarah A. Woodin, Nils Volkenborn, and Karsten Reise. (2008) „Porewater advection by hydraulic activities of lugworms, *Arenicola marina*: A field, laboratory and modeling study." *Journal of Marine Research* 66: 255-273.

Widdel, Friedrich, Sylvia Schnell, Silke Heising, Armin Ehrenreich, Bernhard Assmus, and Bernhard Schink. (1993) „Ferrous iron oxidation by anoxygenic phototrophic bacteria." *Nature* 362: 834-836.

Wilms, Reinhard, Henrik Sass, Beate Köpke, Jürgen Köster, Heribert Cypionka, and Bert Engelen. (2006a) „Specific Bacterial, Archaeal, and Eukaryotic Communities in Tidal-Flat Sediments along a Vertical Profile of Several Meters." *Applied and Environmental Microbiology* 72(4): 2756-2764.

Wilms, Reinhard, Beate Köpke, Henrik Sass, Tae Soo Chang, Heribert Cypionka, and Bert Engelen. (2006b) „Deep biosphere-related bacteria within the subsurface of tidal flat sediments." *Environmental Microbiology* 8(4): 709-719.

Wolff, W. J., J. P. Bakker, K. Laursen, and K. Reise. (2010) „The Wadden Sea quality status report - synthesis report 2010." *Wadden Sea Ecosystem* 29: 25-74.

Wrighton, Kelly C., *et al.* (2012) „Fermentation, Hydrogen, and Sulfur Metabolism in Multiple Uncultivated Bacterial *Phyla*." *Science* 337: 1661-1665.

Zhou, Jizhong, Mary Ann Bruns, and James M. Tiedje. (1996) „DNA Recovery from Soils of Diverse Composition." *Applied and Environmental Microbiology* 62(2): 316-322.

Ziebis, Wiebke, Markus Huettel, and Stefan Forster. (1996) „Impact of biogenic sediment topography on oxygen fluxes in permeable seabeds." *Marine Ecology Progress Series* 140: 227-237.

Zumft, Walter G. (1997) „Cell Biology and Molecular Basis of Denitrification." *Microbiology and Molecular Biology Reviews* 61(4): 533-616.

Acknowledgements

First of all, I want to especially thank Prof. Marc Strous for being the competent, always enthusiastic, friendly and helpful supervisor he was. Without your constantly optimistic and motivating mentoring I would not be at this point now!

Also special thanks go to my Defense Committee members Prof. Friedrich Widdel, Prof. Marc Strous, Prof. Wilhelm Hagen, Dr. Gaute Lavik, Dr. Ute Meyer and Christian Arend.

Moreover, I thank my Thesis Committee members Prof. Marc Strous, Dr. Jeanine Geelhoed, Prof. Juliane Filser, Prof. Friedrich Widdel, Dr. Bernhard Fuchs, Prof. Wilhelm Hagen, and Dr. Gaute Lavik, who participated during the regularly held meetings and supported my work with valuable ideas and helpful criticism.

I thank Theresa for being the nicest and best-tempered technician I could have wished for. Without your great lab organization and your cooperativeness in terms of issues with experiments, the past 5 years could have ended differently.

Not to forget, I want to thank my lab rotations Jasmine and Emmo for their participation in two of my experiments. Your almost infinite willingness to do hundreds of measurements without any need for external pushing is exceptional! In particular, thank you, Emmo, for creating the probably most remarkable name for a continuous culture: "Kluut"!

Special thanks also go to Jianwei for his cooperation in terms of our common labwork and his at all times patient dealing with my questions. The latter is also true for Jeanine who stayed calm and patient even when I asked her the same question a second time because I had forgotten the answer…

I furthermore thank Halina for conducting the sequencing analyses of my culture samples and her even-tempered way in dealing with all my emails regarding the sequencing results. Additionally, I thank Regina and Ines for their "background activity" in dealing with the genome and transcriptome samples.

I thank Christiane for -always cheerful- guiding me through the mar*mic* world!

I thank my office mates Zainab, Beate, and Stefanie for their patient listening and encouragements during times in which my cultures tried to tear my nerves apart.

For their kind help with any kind of lab related questions and measurements I thank Tim, Ramona, Gaby, Sergio, Zwiebel, Anna, Jessika, Christin, and Stefano.

As being the "basis" for all the work I have done during the last years in the MPI I first of all thank Bernd for providing me with literally every document I couldn't retrieve on my own. Moreover, I thank the workshop people Harald, Paul, Volker, and Georg, the "technical heads" Michael and Heiner, the "IT helpers" Olaf, Oliver, Carsten and Christine, and all the ones supporting my work many different ways: Stefan, Anja and Jörg, Manfred, Tomas, Algrid, Ulrike, Sandra, Ralf, Birgit, Bettina, and Heike. It was always nice having a chat with you in the hallway!

Without my family and their constant interest in my wellbeing I would not be at this point. Mama, Papa, ich kann mich immer uneingeschränkt auf Euch und Eure Unterstützung in absolut jeder Lebenslage verlassen. Dafür kann ich Euch gar nicht genug danken!! Oma, danke für Dein konsequentes Verständnis für meine (zu oft zeitlich eingeschränkte) Situation – und für Deine liebenswerte Fähigkeit, mich auf andere Gedanken zu bringen wann immer ich mit Dir spreche!

Thanks to all my friends who were available whenever I needed somebody from the "real world" to chat, to talk rubbish, or to just enjoy normal life outside science. You encouraged me and made me laugh whenever I rather thought of crying! In particular I want to thank Sean for his patient willingness to go through page after page of my thesis.

Thanks to my "new" OLS-colleagues who never got tired of asking for the recent status of my PhD work, of keeping their fingers crossed and motivating and supporting me in every way they were able to. I thank Joschi in particular for his highly qualified advice with respect to all my texts he read and all help he offered.

Last but not least - and full of deep gratefulness - I thank Peter for being at my side as solid as a rock through all the years of excitement, frustration and doubts. You were always there and buffered my emotions in a way nobody else would have tolerated. When frustration seemed to overwhelm me you constantly showed me what is so much more important than a PhD: Love and a home!

Erklärung

Erklärung gemäß §6 Abs. 5 der Promotionsordnung der Universität Bremen für die mathematischen, natur- und ingenieurwissenschaft-lichen Fachbereiche

Hiermit versichere ich, dass ich die vorliegende Dissertation

**Continuous Cultivation
of Janssand Microbial Communities**
Response to Varying
Oxygen Concentrations and Temperatures

- ohne unerlaubte fremde Hilfe angefertigt,

- keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt

- und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

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

Bremen, 30. Juli 2014

_____

(Anna Hanke)